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7. IMMUNE SYSTEM AND MICROFLORA

EDITORS:

PETER J. HEIDT
VOLKER RUSCH
DIRK VAN DER WAAIJ

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EDITORS:

Peter J. Heidt, Ph.D., B.M.
Department of Animal Science
Biomedical Primate Research Centre (BPRC)
Lange Kleiweg 139
2288 GJ - Rijswijk
The Netherlands

Volker Rusch, Dr. rer. nat.
Institute for Integrative Biology
Kornmarkt 2
D-35745 Herborn-Dill
Germany

Dirk van der Waaij, M.D., Ph.D.
Professor emeritus, University of Groningen
Hoge Hereweg 50
9756 TJ - Glimmen
The Netherlands

Verlag wissenschaftlicher
Schriften und Bücher
Am Kornmarkt 2
Postfach 1664
D-35745 Herborn-Dill
Germany
Telephone: +49 - 2772 - 921100
Telefax: +49 - 2772 - 921101

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Participating authors

Herma Z. Apperloo-Renkema, M.D., Ph.D., Laboratory for Medical Microbiology, University Hospital Groningen, Oostersingel 59, 9713-EZ Groningen, The Netherlands.

Josef Beuth, M.D., Ph.D., Institute for Scientific Evaluation of Naturopathy, University of Cologne, Robert-Koch-Straße 10, D-50931 Cologne, Germany.

John J. Cebra, Ph.D., Department of Biology, University of Pennsylvania, 415 South University Avenue, Philadelphia, PA, 19104-6018, USA.

John O. Hunter, M.D., Ph.D., Department of Gastroenterology, Addenbrooke's Hospital, Cambridge CB2 2QQ, England.

Gijsbert J. Jansen, M.Sc., Laboratory for Medical Microbiology, University of Groningen, Oostersingel 59, 9713-EZ Groningen, The Netherlands.

Bart C. Meijer, M.D., Ph.D., Laboratory for Medical Microbiology, University Hospital Groningen, Oostersingel 59, 9713-EZ Groningen, The Netherlands.

Tore Midtvedt, M.D., Ph.D., Department of Medical Microbial Ecology, Karolinska Institute, Box 60 400, S-17177 Stockholm, Sweden.

Dirk van der Waaij, M.D., Ph.D., Laboratory for Medical Microbiology, University Hospital Groningen, Oostersingel 59, 9713-EZ Groningen, The Netherlands.

Laurens van der Waaij, M.D., Laboratory for Medical Microbiology, University Hospital Groningen, Oostersingel 59, 9713-EZ Groningen, The Netherlands.

Carol Wells, Ph.D., Departments of Laboratory Medicine & Pathology and Surgery, Box 198 UMHC; University of Minnesota; Minneapolis, MN 55455, USA.

Michael H.F. Wilkinson, M.Sc., Laboratory for Medical Microbiology, University of Groningen, Oostersingel 59, 9713-EZ Groningen, The Netherlands.

Kurt Zimmermann, Dr. rer. nat., Symbio Herborn Group, Auf den Lüppen 8, D-35745 Herborn-Hörbach, Germany.

Dirk van der Waaij, M.D., Ph.D., Hoge Hereweg 50, 9756 TJ - Glimmen, The Netherlands

COLONISATION RESISTANCE AND ANTIMICROBIAL DEFENCE OF THE DIGESTIVE TRACT; TWO POTENT PHYSIOLOGICAL DEFENCE MECHANISMS TO INFECTIONS

DIRK VAN DER WAAIJ

Laboratory for Medical Microbiology, University of Groningen, Groningen, The Netherlands

SUMMARY

It is to be expected that the successful use of antibiotics for treatment of infections by opportunistic bacteria will soon (in several decades) come to an end. As was recently reported at the Third Western Pacific Congress on Chemotherapy and Infectious Diseases, in some countries antibiotics are already useless. Not only for the treatment of infections by potentially pathogenic microorganisms but also for treatment of infections by pathogens. Therefore, we must look for other solutions for the treatment - if possible prevention - of infectious diseases. An obvious approach could be to (artificially) maintain the physiologic antimicrobial defence capacity.

The normal physiologic clearance mechanism is given the name 'antimicrobial defence'. Hence, a deficiency of this mechanism could be indicated with 'defence deficiency'. If the immune system plays a role in the sequence of opsonisation, phagocytosis and destruction of penetrating opportunistic microorganisms, they may - one stronger than another - be suppressive to the immune system; i.e. they may induce a status of specific and perhaps even a general suppression of inflammation. This contrasts what occurs in infections by pathogenic microorganisms.

An experimental model in mice presents evidence that bacteraemias may occur clinically unnoticed. These bacteraemias could possibly be regarded as evidence of an overflow of the normal physiologic clearance capacity of the antimicrobial defence. Because opportunistic bacteria may occasionally reach along this route the central (systemic) immune system, a need was felt to be able to measure the interaction between the immune system and the intestinal flora in greater detail. Therefore, we have recently developed techniques in our laboratory. This enables us to study in man and animals the specific (and the aspecific?) influence of opportunistic bacteria on the immune system in a direct way. These techniques also permit measurement of the effect of the immune system on bacteria in the intestines of the subject.

The *Enterococcus faecalis* preparation Symbioflor 1® provided by Symbiofarm, was selected as a next best to *Enterococcus faecalis* strains isolated from the endogenous flora of each subject (healthy volunteer).

Regarding the possibility of successful application of autovaccins and/or selected pure cultures of potentially pathogenic strains, we con

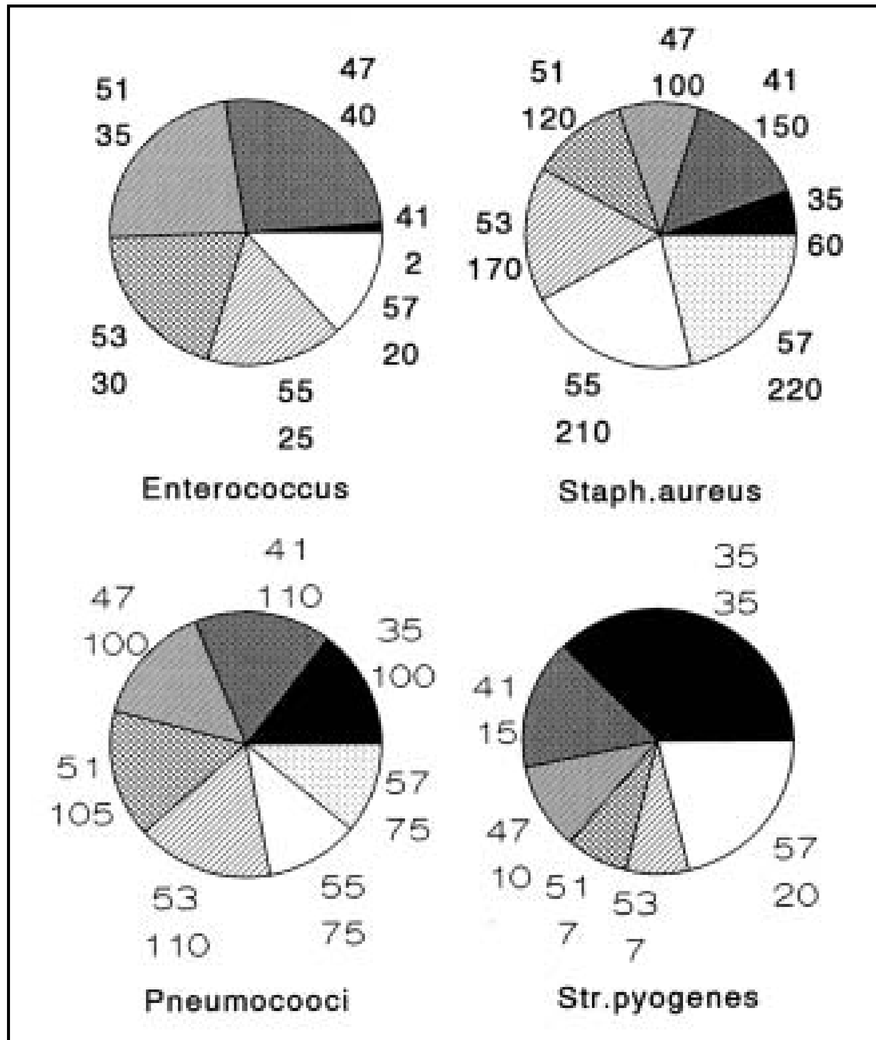

Figure 1: Bacteraemias by Gram-positive bacteria in the years 1935, 1941, 1947, 1951, 1953, 1955, and 1957. The numbers around the pie-diagrams represent these years of study (top numbers) as well as the number of cases involved (undermost numbers) (*Finland* et al., 1959).

clude from evidence in the literature and from our own recent observations, that oral preparations like Symbioflor 1® may really work in patients; i.e. may either stimulate or suppress the immune system. Preparations of this kind applied in electively hospital admitted subjects may enhance the clearance of translocating bacteria and/or prevent inflammatory responses to these microorganisms in patients with some degree of 'defence deficiency'. This implies that preparations of this kind, made for oral use, urgently require further detailed evaluation.

The results obtained by objective measurements in ten healthy volunteers indicate clearly the development of a broad-spectrum of immune suppression during and for some time after Symbioflor 1® treatment.

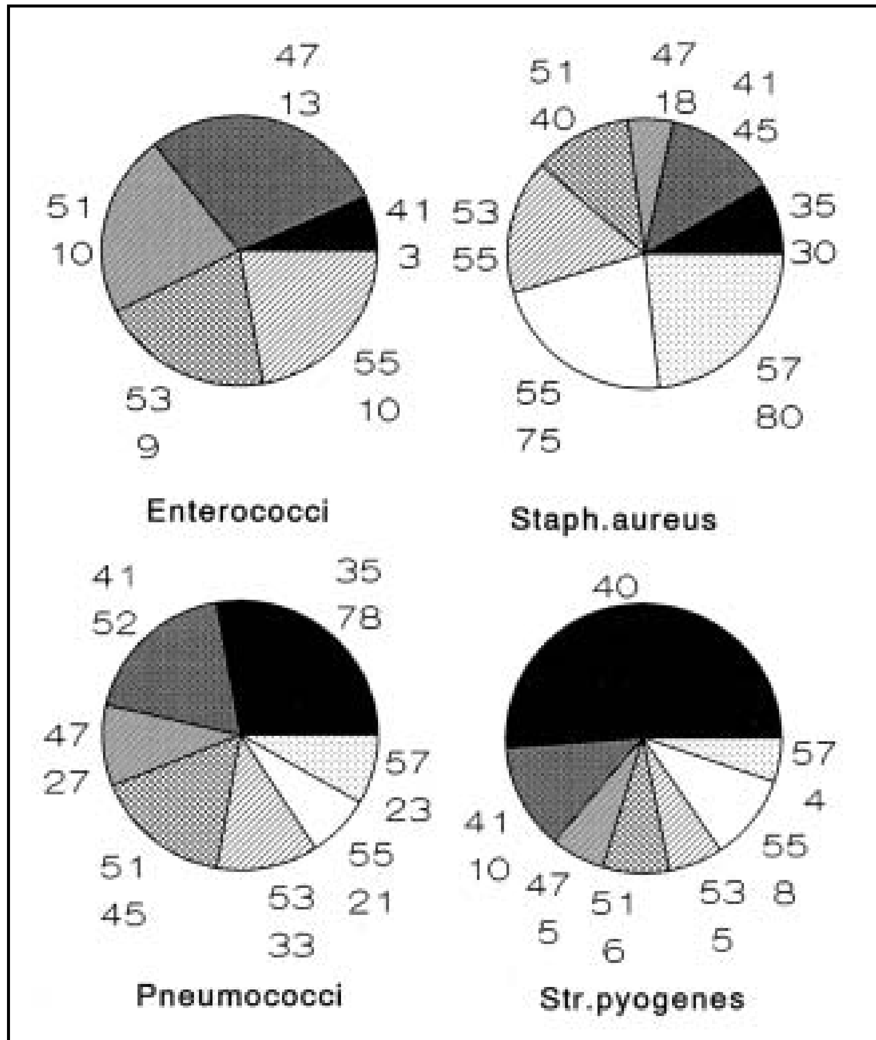

Figure 2: Deaths in patients associated with Gram-positive bacteria. The same years of study and the same way of presentation of the data is used as in Figure 1 (*Finland* et al., 1959).

INTRODUCTION

Change of the spectrum of bacteria associated with hospital infections

Since the introduction of antimicrobial drugs such as sulpha-preparations and later antibiotics, the pattern of 'hospital infections' and the bacteria involved, have changed dramatically. Before 1935 when sulpha-drugs were for the first time taken in use, the majority of infections in hospitalised patients concerned primary pathogens such as *Salmonella* spp., *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *Pertussis* spp., pneumococci and the like (*Finland* et al., 1959; *Julianelle* and *Siegel*, 1945). However, as Finland reported and the end of the fifties, after 1935 and after 1941 in particular, when penicillin and streptomycin be-

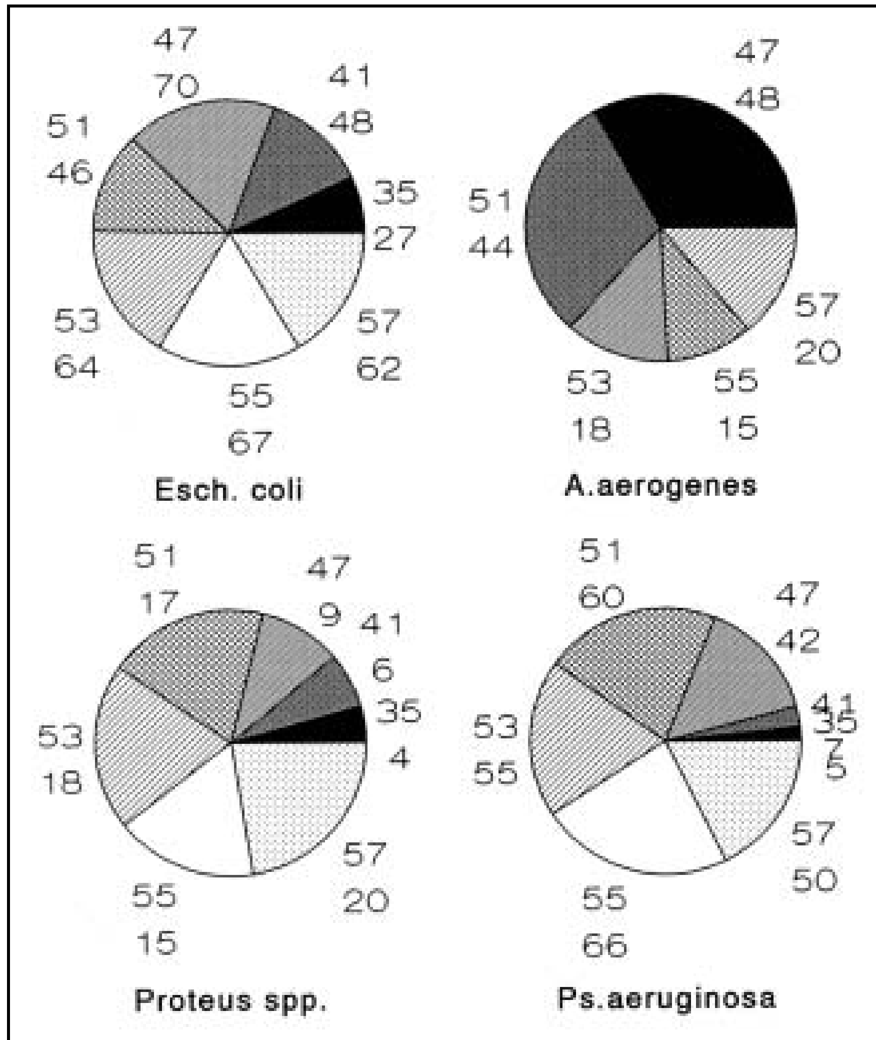

Figure 3: Bacteraemias by Gram-negative bacteria in the years 1935, 1941, 1947, 1951, 1953, 1955 and 1957. The numbers around the pie-diagram represent these years of study (top numbers) as well as the number of cases involved (undermost numbers) (*Finland* et al., 1959).

came available, the pattern of infections in hospitals changed rapidly into infections caused by potentially pathogenic (opportunistic) bacteria such as *Staphylococcus aureus* (Figures 1 and 2). When penicillin and streptomycin were taken in use, opportunistic Gram-negative enterobacteria and *Pseudomonas* spp. became predominant in hospital infections (Figures 3 and 4). With the introduction of broad-spectrum antibiotics such as tetracyclines and chloramphenicol, also fungi - such as *Candida* species - became more common as infection causative microorganisms (*Rogers*, 1959; *McGovern* et al., 1953).

Development of antibiotic resistance

The excitement about the therapeutic possibilities provided by antibiotics, did

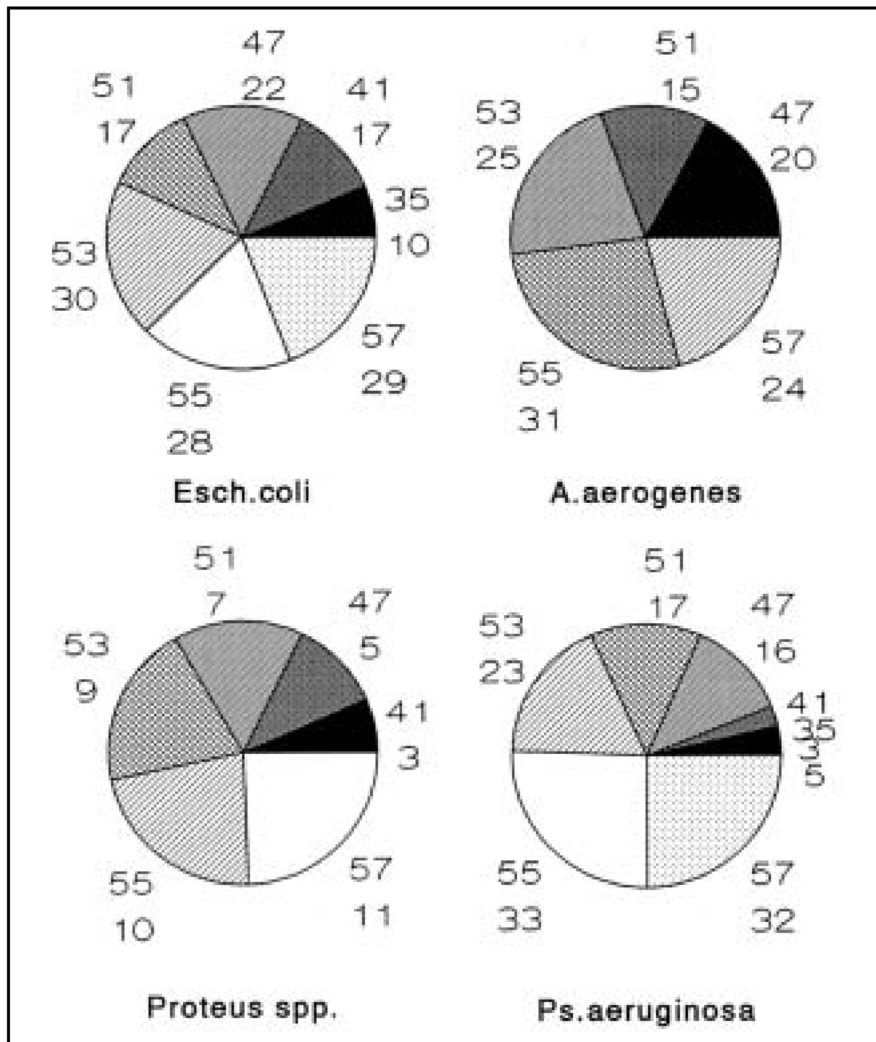

Figure 4: Deaths in patients associated with Gram-negative bacteria. The same years of study and the same way of presentation of the data is used as in Figure 3 (*Finland* et al., 1959).

unfortunately stop further studies on endogenous and exogenous bacteria (vaccines) and their specific and aspecific stimulation/suppression of the immune system. Although in comparison with our present facilities under relative primitive conditions, such studies were in progress in the forties. The fact that development of resistance to antimicrobial agents was observed soon when penicillin and streptomycin were taken in use, was generally regarded as a minor problem. It was believed that the problem of resistance development could be overcome quite easily, as it would be a matter of further technical improvement only.

The influence of antimicrobials on the endogenous microflora

Already in the forties, it was recognised that antimicrobial drugs may adversely influence the oropharyngeal and intestinal microflora (*Julianelle* and *Siegel*, 1945). It was found that resistant potentially pathogenic bacteria and

fungi got growth preference during treatment (*Mangiaracine*, 1951). Later in the late sixties, this mechanism was studied in greater detail by myself and was named colonisation resistance of the digestive tract (*van der Waaij* et al., 1971).

LESSONS BY THE EVOLUTION OF LIFE

Ecosystems with a static defence

On the basis of what has occurred during the evolution, it is conceivable that resistance to antimicrobial drugs would develop. Not only antibiotics would evoke resistance but also to primary manmade antimicrobials like sulpha preparations, trimethoprim and quinolones. This is plausible on the basis of the course of developments in the evolution. The first bacteria that developed on earth were autotrophic. Later on, bacteria developed with a more complex metabolism. At different places, different ecosystems may have developed from these bacterial combinations (*Gould*, 1989). Cross-contamination between ecosystems may have occurred by an airborne route; i.e. bacteria may have been transported by wind. This may have forced some bacteria in each ecosystem to produce an antibiotic substance to which co-colonisers of the endogenous ecosystem were obviously essentially resistant. Newly arriving bacteria on the other hand may mostly have become killed by these antibiotic substances. Only if the newcomers came in sufficiently high numbers and if they could make use of locally available nutrients, newcoming bacteria may have been in the position to adapt, i.e. to mutate in time and develop resistance. Then these bacteria could perhaps settle and multiply in the niche in which they had landed.

After bacteria, first monocellular eucaryotes and later multicellular organisms may have developed from these microorganisms (*Margulis*, 1993). When much later in the history of life on earth, multicellular organisms (animals) developed and when these animals became more complex, they got a digestive tract with a bacterial ecosystem. This ecosystem of the digestive tract may have developed - just like in the open outside world - on the basis of the principle that these communities should defend themselves - and therewith their host - to foreign bacteria. This is at least what the intestinal flora appears to do these days (*van der Waaij*, 1990).

On the basis of the foregoing hypothesis, adaptation to antimicrobial substances present in foreign ecosystems by mutation and selection may therefore be as old as the gradually developed more complex bacterial communities exist on our planet since the beginning of autotrophic life.

The need of a dynamic (rapidly adjustable) and specific defence in animals

Because the higher organisms developed means to move themselves by feet, wings or by swimming in the water they could actively and quite rapidly move from one place (environmental bacteria) to another (other bacteria in the environment). This caused contamination of their digestive tract with many different bacteria which were so far foreign and which might be dangerously invasive. Therefore, a more complex defence system - the successor of the monocellular organism that may have protected itself by phagocytosis - was required than just phagocytosis and the static anti-microbial substances (antibiotics) produced by the intestinal ecosystem. The defence of higher animals

to 'antibiotic' resistant (the antibiotic-like of the ecosystem) foreign bacteria, had to become fast and readily adjustable. In addition, in higher animals the defence had to act specific to invasive newcomers.

In the digestive tract, a static defence provided by a bacterial ecosystem (among else by antibiotic-like substances) may have been responsible for the prevention of colonisation by these newcomers as still is the case. Because of the protective function of the intestinal ecosystem to the host organism regarding pathogenic (foreign) microorganisms, the bacteria indigenous to the digestive tract in fact form an essential part of the host organism. However, this protection may have been insufficient to cope with many foreign bacteria. A dynamic defence system of the tissues of the host organism was required for survival of the species. The defence system however, had to be specific, as it should leave the indigenous ecosystem unaffected. To this end, a specific defence system may have developed in animals. Would bacteria, which colonise the digestive tract, get across the epithelial lining of the intestines, they had to be cleared without notice (without ensuing inflammation) to the host. Foreign, rapidly multiplying and invasive bacteria on the other hand, had to be rapidly killed and cleared. The 'specific part of the defence system' that developed in animals is presently known as the immune system.

Regarding the evolution of the immune system, the defence to pathogenic microbes had to be rapidly readjustable, because otherwise 'immune-resistance' might develop. Strains of bacteria, fungi and viruses may change rapidly their major antigenic composition (antigenic mimicry) and thus (try to) escape from the immune activity by developing this type of resistance.

Practical consequences of the course of the development of life on earth

The practical value of these 'lessons of the evolution' for the clinical use of antibiotics, have unfortunately not been taken in consideration in the fifties. The usefulness of antibiotics therefore became unfortunately overestimated. At a much earlier stage, it should have been realised that: Firstly, antibiotics represent a static defence. The only flexibility in the system is provided by the pharmaceutical industry; i.e. when it introduces a new modified antibiotic for practically no resistance exists. Secondly, the opportunistic microorganisms involved in hospital infections could only become massively involved in infections because of the introduction of antimicrobial drugs:

- In the first place, antibiotics have strongly (positively) influenced the progress in development of almost every specialism of medicine. This, however, has resulted in a strong increase of the percentage of compromised patients in our hospitals.
- Secondly, antibiotics with their unspecific activity to a wide range of different bacteria, often affected the intestinal ecosystem of the patients treated and thus permitted colonisation by resistant strains.

GROUPING OF BACTERIA ACCORDING TO THEIR 'DEGREE OF PATHOGENICITY'

For practical (medical) purposes, all bacteria presently known could be grouped in three major groups with increasing 'pathogenicity' for man and

animals. The first two of these groups may differ in particular between animal species:

1. A huge group, which is by far the largest, is not pathogenic at all. Bacteria belonging to this group can be found in the digestive tract and on the skin of all healthy human subjects as well as on the skin of animals and plants. They live in one way or another, in peaceful co-existence with the immune system.
2. A much smaller group, which is well-maintained under control by the immune system of the digestive tract (the so-called gut associated lymphoid tissue). This control occurs - as we all daily experience - without causing any sign or symptom of disease. Representatives of this group, which is called potentially pathogenic or opportunistic, can be found in practically every healthy human subject, in every animal and on plants as well.
3. A (fortunately) small group which is pathogenic; e.g. can cause disease upon contamination with sufficient numbers. These bacteria are often not readily controlled by the immune system of man and animals. This group therefore causes mostly disease upon contamination of a susceptible subject and, depending on its pathogenicity in relation to the condition of the host, even death. In contrast to representatives of group one and two, bacteria of group three are normally not found in healthy subjects. If, however, they are isolated from excreta of healthy subjects, the individual should be regarded as a potential transmitter and a source of infection.

As mentioned in the introduction, microbes which cause hospital infections are in general potentially pathogenic (opportunistic) and differ of primary pathogenic microorganisms by their capacity to normally colonise healthy subjects (man as well as animals) for extended periods of time. Yet, albeit in low numbers, opportunistic bacteria may translocate from time to time from the intestines into the gut associated lymphoid tissues (GALT). This occurs without evoking signs or symptoms of infection (*van der Waaij* et al., 1972; *Wells* et al., 1988), which contrasts to what occurs normally upon invasion of primary pathogenic bacteria and viruses. Macrophages - both tissue macrophages and monocytes in the circulation - may play a key role in the clearance of translocating microorganisms as well as of necrotic tissues and cells (*Border*, 1988). Macrophages could be regarded as the successors of the most primitive defence system that developed during evolution in the most primitive multicellular organisms.

A commonly known representative of a serious primary pathogen in this third group is the bacterium that causes Plague. Several other examples of pathogens, which are nowadays still causing disease, are bacteria that cause tuberculosis, typhoid fever, diphtheria or pertussis. These bacteria are pathogenic because of different properties:

- They may fool the immune system or the may produce an agent which is toxic to cells of the defence (immune?) system. The defence system may for these reasons not respond sufficiently fast therewith giving free way to the pathogen for some time. If this period of 'retarded response' is too long, the patient may not survive.
- Another reason for grouping a bacterium pathogenic to man, is based on adverse (tissue destructive) effects of the immune response itself may have. The host individual becomes largely ill due to tissue destruction like in tuberculosis or typhoid fever. Tissue destruction in these cases is partly the result of intracellular bacterial multipli-

cation, but also the result of complement binding and agents released by cells of the immune system; i.e. a consequence of the immune response to the infecting microorganism.

HOSPITAL INFECTIONS IN RELATION TO THE FOREGOING

Patients, who suffer of infections during their stay in a hospital by potentially pathogenic microorganisms, clearly have a deficit in their defence capacity. They may less rapidly and less efficiently clear translocating bacteria because they all have to some degree a 'defence deficiency'. Healthy persons with normal defence capacity on the other hand, being exposed to same extend to these bacteria because they work in the hospital, may become colonised by several different opportunistic species (*Chambers* et al., 1987) without signs or symptoms. They therefore, do not need to take any precautions to protect themselves to contamination by potentially pathogenic bacteria excreted by patients. However, in the Intensive Treatment (IT) wards, most patients have a serious defence deficiency. Therefore, if isolation precautions are taken in IT-units, they are taken because of hospital hygienic considerations. The precautions are necessary to prevent spread of resistant opportunistic microorganisms among these (often antibiotic treated) patients. Nowadays, the deficit in our knowledge concerning the pathogenesis of infections by opportunistic bacteria indeed is most experienced in IT-wards. In these high care stations, the infection rate (practically 100% caused by opportunistic microorganisms) may be as high as 31% (*Craven* et al., 1988).

URGENTLY REQUIRED RESEARCH

General

The basic questions in infectious diseases by opportunistic microorganisms have unfortunately not been studied in greater detail since the forties. The central issue in infectious diseases in hospitals should have been a study of factors involved in antimicrobial defence. By now, it should be known which defence factors are affected in severely ill patients with increased risk of infections.

Regardless our detailed knowledge of the functioning of the immune system (*Kagnoff*, 1987; *Lee*, 1985), it is still an open question how the immune system interacts with the bacteria of the ecosystem of the digestive tract in the first place. Secondly, research is necessary to elucidate all factors which play a role in the defence mechanism that enable healthy individuals to live unaffected in daily contact with residing as well as with newly ingested opportunistic microorganisms (potential pathogens).

If we had undertaken studies of this kind in the previous decades, we might at the present be able to prevent many infections. We would never have reached the frightening situation of the present with often multiply bacterial resistance to antibiotics. Instead we would perhaps be able to monitor the condition of the defence capacity of our patients at risk during their hospital stay. If indicated, we would be able to either boost 'antimicrobial defence' (possibly with so-called 'auto-vaccines' or with selected pure cultures such as for exam-

ple Symbioflor 1® (*Rusch*, 1985). This treatment would be given in advance to (elective) admission of patients to the hospital. In acute (non-elective) patients, we might be able to supply them with those factors required to prevent 'defence deficiency'. In compromised patients therefore, we may become able to prevent along these lines most infections by opportunistic microbes in the future.

Personal view

In Groningen in the Netherlands we are of opinion, that we should perform as many investigations in a systematic way as we can, in order to fill the gap of our knowledge concerning normal interactions between the immune system and intestinal microflora as soon as possible. It is concluded that in the initial studies, we could best start with monitoring the normal function and responses of the 'antimicrobial defence' to microbial stimuli from the digestive tract. This means studies in healthy volunteers on the basis of a model of opportunistic infections in mice. Such studies may elucidate how we should make practical use of measurements concerning the clearance of potentially pathogenic bacteria after translocation.

RESEARCH DEVELOPMENT IN GRONINGEN SO FAR

On the basis of the foregoing arguments, we have realised that we should try to develop an animal model in which we could study the antimicrobial defence capacity. In addition a need was felt to be able to measure - and thereby to monitor - the interaction(s) between the gastro-intestinal microflora and the immune system (both the 'gut associated' and the 'systemic') as well.

Experiments in animals to study antimicrobial defence capacity

In patients, so far the culturing of blood is the only way to determine - albeit at a late stage - evidence of translocation of bacteria. A bacteraemia in a healthy subject could perhaps be regarded as an 'overflow' of the system. The system would normally clear all translocating bacteria. Therefore, we have made use of the classical method of experimentally evoking translocation in mice, namely by oral contamination with an opportunistic bacterium (*van der Waaij* et al., 1971). In this system, we have collected 10 ml of tail blood at regular intervals for culturing, to investigate whether mice have a 'measurable overflow' of their antimicrobial defence system for bacteria following oral contamination with high numbers of opportunistic bacteria. In our study, a bacterial strain was used of which we knew by experience that translocation would occur to a considerable degree.

Animal model to study the 'normal' defence capacity in mice

Eight weeks old female C3H mice were orally contaminated in groups of five with various doses of a streptomycin and neomycin resistant (SMR) strain of *Escherichia coli*. The endogenous *E. coli* strains were all sensitive to streptomycin and neomycin. In total five groups of otherwise untreated animals were contaminated with respectively 10^2, 10^4, 10^6, 10^8 and 10^{10} SMR *E. coli*. At six hours, 24 hours and thereafter daily for five days, ten ml of tail blood was collected with a calibrated heparinised capillary under aseptical conditions. To this end, the very end of the tail was cut. Quantitative culturing on MacConkey agar (OXOID) made SR-*E. coli* selective with streptomycin (50 mg/l). After incubation overnight,

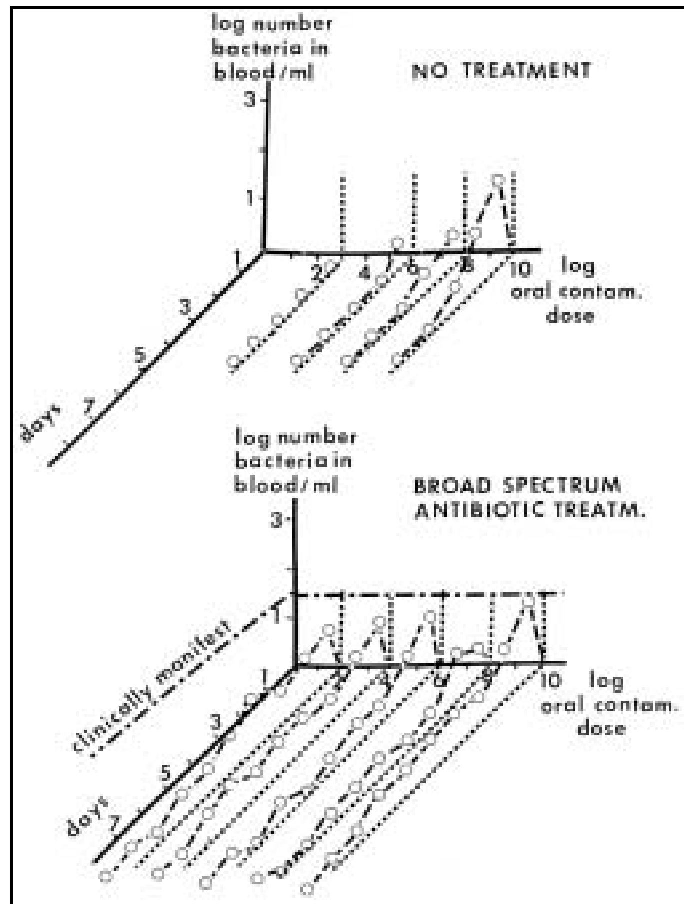

Figure 5: Translocation of SMR-*E. coli* in conventional and in antibiotic treated mice (5 mice per contamination dose).

the number of colonies was counted and the number of bacteria per ml of blood was thus estimated. Log median values are presented in Figure 5.

This experiment was repeated with mice which had been treated orally with streptomycin and neomycin in the drinking water (*van der Waaij* et al., 1971) for seven to ten days before oral contamination. Antibiotic treatment was continued during the eight days of the experiment. During the experimental period, tail blood was sampled for culturing twice daily.

The results of this study are depicted as mean log values per day in Figure 5. Firstly, it is important to read from this figure, that indeed healthy mice may experience a bacteraemia for some time after oral contamination. However, this only occurred following oral contamination with very high numbers of opportunistic bacteria. This may have caused abnormally high numbers of these bacteria in the intestines (*van der Waaij* and *Berghuis*, 1974). Clinically, the animals remained healthy: They did not show evidence of diarrhoea, and they showed normal activity. Secondly, the results also clearly show the influence of antibiotic treatment. The antibiotics suppressed the autochthonous microflora (decreased colonisation resistance). Yet, these mice did also not

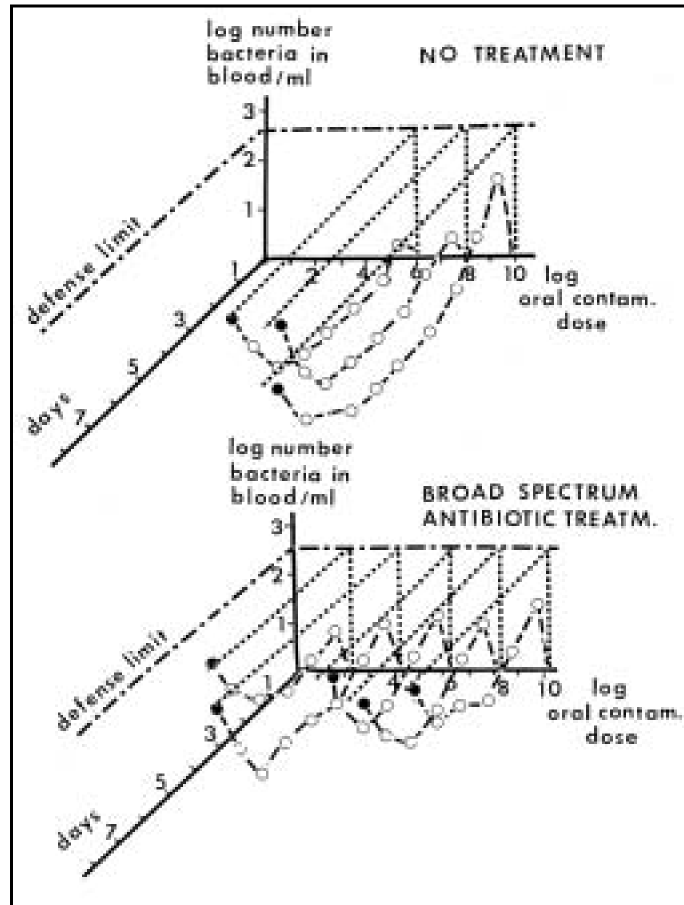

Figure 6: Translocation of SMR-*E. coli* in lethally irradiated (7 Gy) conventional and in irradiated antibiotic treated mice (5 mice per contamination dose).

show clinical signs of infection. However, they obviously had soft faeces since the onset of antibiotic treatment.

Animal model to study the effect of decreased defence capacity in antibiotic treated subjects

Two groups of 25 mice were irradiated with 7 Gy four days before oral contamination. One group had been antibiotic decontaminated with streptomycin and neomycin like in the previous experiment since seven to ten days before irradiation. Antibiotic treatment was continued after SMR-*E. coli* contamination in the antibiotic decontaminated mice. The animals in both experimental groups were contaminated in groups of five, again with respectively 10^2, 10^4, 10^6, 10^8 and 10^{10} SMR-*E. coli*. Tail blood was collected and cultured at the same intervals as in the previous two experiments. However, in the antibiotic treated mice after day three following contamination and after day four in the untreated mice, collection of tail blood was not always successful. Means of the number of bacteria per ml of blood are therefore at days three respectively day four calculated of less than four mice. Moribund animals were killed and their hearts blood was taken for quantitative culturing.

The results of these experiments are

depicted in Figure 6. The data of the two lowest contamination doses are omitted in the not antibiotic treated group for the sake of simplicity. These results did not differ from what was seen in the unirradiated mice, their curves might only confuse the presentation.

Conclusion

The first conclusion is that in mice with a strongly suppressed defence capacity, low contamination doses may - like in untreated mice - also not result in intestinal concentrations adequate to cause bacteraemia in the present model. However, from oral contamination dosages of 10^6 on, following an early peak the contaminant may reach for the second time an intestinal concentration associated with a quite strong translocation or decreased clearance as evidenced by bacteraemia.

Secondly, the results depicted in Figures 5 and 6 clearly show that the higher the numbers which the SMR-*E. coli* may have reached in the intestines, the shorter was the interval for which bacteraemia disappeared following the initial peak. All animals died with a rather equal number of approximately 5.10^2 SMR-*E. coli* per ml of blood.

From the results of this experimental study it is tentatively concluded that lethal irradiation may cause destruction of the defence system and therewith a rapid exhaustion of the clearance capacity. If under such circumstances opportunistic bacteria colonise the intestines in high numbers, they may (still) translocate and - not being normally cleared - they may soon reach lethal numbers in the blood stream and perhaps other organ systems.

Mice which had the opportunistic bacterium in very high (overgrowth) numbers in their digestive tract because of broadspectrum antibiotic treatment, survived significantly shorter than comparable animals in the untreated but lethally irradiated control group. Because of the decreased colonisation resistance, in the antibiotic treated subjects, the size of the contamination dose did not longer play a role. Oral doses of 10^2 SMR-*E. coli* had the same (rapidly lethal) effect as the highest doses of 10^{10}. These experiments make likely, that the defence capacity is formed by two mechanisms:

1. a mechanism based on bacterial interactivity, and
2. a radiosensitive mechanism typical for organisms of eucaryotic origin. The latter being primarily designed to clear the tissues of invading microorganisms (foreign as well as indigenous).

Studies aiming to obtain insight in the interaction between the autochthonous microflora and the immune system in man

Because it is very likely that the immune system is involved in the complex clearance mechanism for translocating bacteria, we decided to study the physiologic interactions between the immune system and the intestinal microflora as a first step. To this end, it was realised that we should develop techniques, which would permit us to study humoral and cellular reactivity to indigenous intestinal bacteria in greater detail. Furthermore, it was realised that in these studies washed (uncultured) intestinal bacteria had to be used in stead of pure cultures, since the bacteria might change their antigenic composition because of culturing, isolation and typing. It is known that bacteria may change outer-membrane antigens during pure culturing steps.

As will be discussed by three coworkers in greater detail during this Old Herborn University Seminar, two techniques have been set up to study humoral responses to intestinal bacteria

and to bacteria-like particles in washed faeces:

- The first technique involves 'fluorescence immuno-micromorphometry'. Briefly, this involves a microscope equipped with UV-light and phase-contrast optics. A high-resolution video camera reads the microscopic fields sequentially with normal light and with UV. Computer software especially developed for this purpose, sorts these bacteria on the basis of shape and seize, counts them and measures the titres of isotypes of antibodies which may 'coat' the bacteria. Evaluation of a sample may take a day; an experienced technician can study five samples per day. Antibody coating may take place in the gut *in vivo*. However, *in vitro* incubation of bacteria with either autologous or allogenous sera is also being performed to study possible differences in response of the systemic immune system to autologous indigenous bacteria and those of other (allogenous) persons (*Apperloo-Renkema* et al., 1992; *Jansen* et al., 1993a).
- The second approach investigated makes use of FAX-techniques. The FAX-analysis of faecal suspensions permits rapid evaluation of ten times as many bacterial particles (events) per sample. However, the FAX gives less specific information than the fluorescence immuno-morphometry does. An experienced technician can study forty samples per day (*van der Waaij*, 1994).
- Finally, techniques have been developed to study cellular responses to (washed) indigenous and allogenous intestinal microflora. This technique is now operational. However, these cellular studies are much more laborious and may in fact require experimental pre-studies in animals to clarify their outcome.

ONGOING STUDIES ON HUMORAL (IMMUNE) INTERACTION WITH INTESTINAL BACTERIA IN MAN

The results of these studies are presented by my co-workers during this Old Herborn University Seminar. Briefly: the first opportunistic bacterium we have studied in human volunteers, was *Enterococcus faecalis*. We selected this bacterium because two to three days after admission of patients to IT-wards, it usually becomes predominant in the patient's oropharyngeal microflora. This occurs in a high percentage of multi-trauma patients. It was assumed therefore, that the *Enterococcus* might be - post or propter - be associated with the defence deficiency seen in these patients. In addition, in several IT-units, *Enterococcus* is being used as an indicator organism to 'monitor' the 'defence capacity' by culturing throat washings sequentially.

A study of the influence of enterocci on the humoral immune response(s) following oral application should obviously start in healthy subjects because healthy persons have a normal 'defence capacity'. They therefore do not run a risk of infection by orally given strains of opportunistic microbes.

For a study in healthy volunteers the best approach would have been the use of an auto-vaccine containing endogenous *Enterococcus faecalis* strains in each subject. However, we had no experience in preparing auto-vaccines. A standard pure culture was therefore considered a next best. This assumption is based on the fact that many different foreign *Enterococcus faecalis* strains are being ingested frequently and thus may provide multiply antigenic information

about the species to the gut associated immune system. We were therefore very pleased by the offer of Symbiopharm to use their strains of *Enterococcus faecalis* called Symbioflor 1®.

Jansen and co-authors (1993b) recently reported in a letter to the medical journal Infection about our first findings with Symbioflor 1®. Nine persons of a group of ten volunteers who took Symbioflor 1® daily for three subsequent weeks showed a significant decrease of their antibody titre to *Enterococcus faecalis*. This titre continued to decrease furthermore in the subsequent three weeks. The one volunteer in who the titre did not decrease significantly had an abnormal low titre already in the five study weeks previous to Symbioflor 1® treatment. This could possibly be ascribed to the spastic colon this volunteer was known to suffer of.

Because not only the IgG antibody titre against *Enterococcus faecalis* appeared to decrease significantly, but also most IgG titres to other indigenous bacteria, it is tentatively concluded that *Enterococcus faecalis* treatment may also suppress the immune response to other potentially pathogenic microbes and therewith may suppress inflammatory response would they translocate (*Jansen* et al., 1993b).

Conclusion

These data indicate that there is a suppressive effect of oral treatment with Symbioflor 1® on the humoral immune response to not only *Enterococcus faecalis*, but evidently also to a wide range of other bacterial antigens. An autovaccine may have a similar or even a stronger effect than selected pure cultures of this kind, because the immune system may have more experience with bacteria in autovaccines than with orally applied immunologically foreign strains.

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MICROFLORA ASSOCIATED CHARACTERISTICS: CHARACTERISATION OF THE COMPOSITION OF THE MICROFLORA BY VARIOUS BIOCHEMICAL TESTS CONCERNING BACTERIAL PRODUCTS AND CHEMICAL MODIFICATION OF BILE ACIDS

TORE MIDTVEDT

Department of Medical Microbial Ecology, Karolinska Institute, Stockholm, Sweden

SUMMARY

The interplay between the host and his microbes can be followed by studying a set-up of biochemical reactions in intestinal content and faeces. In this review, the interest has been focused on two topics, i. e. presence/absence of β-aspartylglycine and microbial transformation of bile acids.

INTRODUCTION

Normally, humans harbour an intestinal microflora that numbers 10^{13}-10^{14} microbes, which is a population equalling or exceeding the total number of cells comprising the whole human body. From man's cradle to his grave, this population forms dynamic ecosystems, governed by a wide variety of host- and microflora-derived physiochemical conditions, including pH, redox potential, nutrient availability, peristalsis, and transit time.

Several approaches can be made to the task of evaluating the gastrointestinal microflora. In the past, many reports have dealt with the composition of the human gastrointestinal flora, and great efforts have been expended in isolation, identification and enumeration of the hundreds of species constituting this flora. Additionally, nearly as much efforts have been made to follow alterations in the composition and balance of the flora after various external factors, such as changes in dietary habits and oral or parenteral administration of antimicrobial agents. In earlier studies, coliform bacteria most often were used as indication organisms. However, as skills and techniques have improved, the trend has been to study alterations in the numbers of both aerobic and anaerobic microorganisms. And - as underlined by other speakers at this symposium - a qualitative and quantitative evaluation of the gastrointestinal microflora in man is extremely time-consuming and difficult to perform and, to the best of my knowledge, a full-scale evaluation has so far never been carried out.

Another approach is to study the metabolic capacity of the microbial flora ("what can the microbes do"). It goes without saying that most often, these studies are carried out *in vitro*. A long series of biochemical transformations have been studied. Some *in vivo* metabolic capacity tests have been worked out, and some of them are well established in clinical medicine, as the bile acid deconjugation test (see later) and the lactulose test.

A third approach is more directly to

Table 1: Some intestinal structures and functions influenced by the microflora

Parameter	MAC	GAC	Microbes involved
Intestinal wall	Thick lamina propria	Thin lamina propria	Unknown
	Irregular villi	Regular villi	Unknown
	High cell turnover	Low cell turnover	Unknown
Caecum size	Normal	Enlarged	Unknown
Intestinal smooth muscle activity	Vivid spontaneous contractions	Markedly reduced contractions	Unknown
Sensitivity toward biogenic amines	Normal	Markedly reduced	Unknown
Amounts of biogenic amines	MAC=GAC	GAC=MAC	
Bile acid metabolism	Deconjugation	No deconjugation	Several species
	Dehydrogenation	No dehydrogenation	Several species
	Dehydroxylation	No dehydroxylation	Few species, mostly anaerobic, non-spore-forming, Gram + rods
Bilirubin metabolism	Deconjugation	Little deconjugation	Several species
	Urobilinogen formation	No urobilinogen formation	*Clostridium ramosum*
Cholesterol	Mainly coprostanol	Only cholesterol	An *Eubacterium* species
Dipeptidases (such as β-aspartylglycine)	Absent	High amounts	Unknown
Intestinal gases	H_2, CH_4, CO_2	No H_2 or CH_4, reduced CO_2	Unknown
Mucus	Absent in faeces	High amount	Several species, such as*Peptostreptococcus micros*, *Bacteroides ruminococcus*, and *Bifidobacterium*
SCFAs	High amounts, several acids	Small amounts, few acids	Probably several species
Tryptic activity in faeces	Little or no activity	High activity	Unknown

study the functional status of the gastrointestinal flora ("what have the microbes done"). In order to do so, it is necessary to clarify which mechanisms and reactions are related to the host and which to the microflora itself, respectively. With a slight travesty of the well-known terminology introduced by Claude Bernhard, the host himself can be characterised as the milieu interieur, the microflora as the milieu exterieur, and the host and his microflora as the milieu total.

Studies on adult mammals, birds, fishes, insects and reptiles with no microbial flora, i. e. germfree individuals, have established long series of values with regard to anatomical structures, physiological and biochemical functions in a milieu interieur, i.e. the macroorganism itself.

When such baselines are first estab-

Table 2: Microflora-associated characteristics investigated

Parameter	Microbial interaction	Method	Function
Bile acids	Deconjugation 7α-dehydroxylation	Gas chromatography	Entero-hepatic circulation
Bilirubin Urobillogen formation	Deconjugation Spectrophotometry	Spectrophotometry circulation	Entero-hepatic
Cholesterol	Coprostanol formation	Gas chromatography	Entero-hepatic circulation
Mucin	Breakdown	Gel electrophoresis	Mucosal
Tryptic activity	Inactivation	Spectrophotometry	Pancreatic
Short Chain Fatty Acids	Presence	Gas chromatography	Dietary
β-aspartylglycine	Absence	High voltage electrophoresis	Dietary

lished, the normal function(s) of the flora as well as alterations in these functions can be worked out. In such functional studies, two new terms - MAC and GAC - have been shown to be of considerable value. A MAC (i.e. a Microflora-Associated Characteristic) can be defined as the recording of any anatomical structure, or physiological or biochemical function in a macroorganism, which has been influenced by the microflora. When microbes actually influencing the parameter under study are absent - as in germfree animals, newborns, and/or in relation to ingestion of antibiotics - the recording of a MAC can be defined as a GAC (i.e. Germfree Animal Characteristic). Consequently, a set-up of GACs describes the milieu total under germfree conditions whereas a similar set-up of MACs describes the milieu total. A simple equation: Milieu total minus milieu interieur gives milieu exterieur ("what have the microbes done") as an answer. Below, some of the most well known set-ups of MACs/GACs are summarised and some data concerning the most active part (species when known) of the microbial flora is quoted (Table 1).

SOME ACTUAL GAC/MAC PARAMETERS FOR ROUTINE ANALYSIS

The list shown above covers just some few of the GAC/MAC pairs, which exist within the gastrointestinal tract. However, it goes without saying that the list is too "ambitious" to be used in a routine setting. In Table 2 the tests which we have found to be of great value when evaluating the functional part of the intestinal microflora are listed. Most of the tests have been the subject of a Ph.D. thesis (*Høverstad*, 1985; *Norin*, 1985; *Carlstedt-Duke*,

1987; *Saxerholt*, 1990), and are very well standardised. In the following part, the interest will be related to two areas, i.e. colonisation resistance and microbial transformation of bile acids.

PARAMETERS RELATED TO COLONISATION RESISTANCE

Presence/absence of β-aspartylglycine

As underlined and summarised by *van der Waaij* in this volume, the presence in faeces from conventional mammals, including man of some dipeptides, especially β-aspartylglycine, indicates that the normal microbial intestinal ecosystems are seriously altered. In short, the biochemical background for presence of β-aspartylglycine may be as follows. Dietary proteins are the main targets of intestinal proteolytic enzymes. Biochemically, β-aspartylglycine is a member of a group of β-carboxyl dipeptides, formed in the intestinal tract when dietary proteins are broken down by host derived proteolytic enzymes (*Welling* et al., 1985). The β-carboxyl dipeptide bindings are suggested to be broken down only by microbial derived proteolytic enzymes. This is substantiated by findings in germfree rats and mice (*Welling* and *Groen*, 1978; *Norin* and *Midtvedt*, l987). Adult germfree rats and mice always excrete β-aspartylglycine in their faeces, whereas their conventional counterparts never do. Thus, presence/absence of β-aspartylglycine represents a GAC/MAC system. However, the microbe(s) capable of switching this particular GAC to a MAC is (are) now known.

Thus, presence of this particular GAC, i. e. presence of β-aspartylglycine, is depending on (i), the presence of dietary precursor(s); (ii), the presence of host derived proteolytic enzymes, and (iii), the absence of microbial derived proteolytic enzymes.

In some past and on-going studies on GAC/MAC parameters, we have followed the presence/absence of β-aspartylglycine in experimental animals as well as in humans, and some of our results will be reported upon hereunder.

Beta-aspartylglycine/antibiotics/human

The following antibiotics were given for 6 days to groups of healthy volunteers: Ampicillin 500 mg q.i.d., bacitracin 25,000 IU q.i.d., clindamycin 150 mg q.i.d., co-trimoxazole 150/800 mg b.i.d., doxycycline 200 mg day 1, followed by 100 mg daily, erythromycin 250 mg q.i.d., metronidazole 400 mg t.i.d., nalidixic acid 500 mg q.i.d., ofloxacin 200 mg b.i.d., and vancomycin 240 mg q.i.d. Faecal sampling was done before, during and after medications. Drug concentrations were measured in faeces and blood on day 6. All faecal samples were investigated for alterations in the following MAC parameters: Presence of β-aspartylglycine, conversion of cholesterol to coprostanol, bilirubin deconjugation and formation of urobilinogen, 7-alpha-dehydroxylation of bile acids, breakdown of mucin, inactivation of mucin and production of short chain fatty acids. The results are published elsewhere (*Steinbakk*, 1992), and here will be commented upon alterations in the β-aspartylglycine parameter only.

Beta-aspartylglycine was absent in all individuals prior to administration of antibiotics, and present in one individual during the administration. This individual received ampicillin and was the only individual not having detectable amounts of β-lactamases in her faeces on day 6. In that particular sample, high

amount of β-aspartylglycine was found to be present, together with 480 mg/kg of ampicillin. From these series of experiments, we concluded that presence of β-aspartylglycine was a rare event following oral ingestion of antibiotics.

In another study, clindamycin was given to conventional rats (4 mg/kg for 5 days). Additionally, some rats received clindamycin together with 2 strains of lactobacilli (*Carlstedt-Duke*, 1987). Faeces were analysed for variations in some MAC patterns. However, β-aspartylglycine could not be detected in any sample taken prior to, during or after ingestion of clindamycin.

Establishment of a β-aspartyl-degrading flora in ex-germfree rats

A time-course study for the establishment of some biochemical microbial intestinal functions was undertaken in ex-germfree rats conventionalised, i.e. colonised with conventional flora, in three different ways: Untreated (Group 1); contact with visitor rats (Group 2); inoculated with intestinal contents from conventional rats (Group 3). The biochemical parameters studied were the same as mentioned above. The results, which are described in detail elsewhere (*Midtvedt* et al., 1987), showed that the way in which the microbes ware introduced and the biochemical functions themselves were of importance. Concerning the β-aspartylglycine parameter, a significant difference was found between group 1 and group 3. On day 3 after being taken out of their germfree isolators, all the rats in Group 1 showed presence of β-aspartylglycine whereas it was absent in all the rats in Group 3. Fourteen and 21 days after they have been taken out of their isolators, all the rats in Groups 2 and 3 had switched from a GAC to a MAC pattern. Although the specific microbial species involved in this GAC/MAC switch are virtually unknown, it might be reasonable to assume that the capability of performing this reaction is not rare among intestinal microorganisms.

Time-schedule for presence of β-aspartylglycine in young germfree rats

As mentioned, it has been shown that high amount of β-aspartylglycine always is present in faeces from adult germfree rats and mice. In a series of experiments, we intended to follow when β-aspartylglycine starts to be present in young germfree rats and also whether, and to what extent, weaning may influence upon the occurrence of β-aspartylglycine in faeces. The experiments, which are described in greater details elsewhere (*Norin* and *Midtvedt*, 1987), were performed as follows. A litter of germfree AGUS rats was raised together with their mother up to day 17, when the animals were randomly divided into following two groups.

Group I: Six rats, were weaned onto water and a commercially obtained, pelleted rat food *ad libitum* (R3, Ewos, Södertälje, Sweden)

Group II: Five rats, were receiving mother's milk only during the period 17-23 days of age (the mother was taken away from the young rats twice daily, when she was given full access to the diet R3)

From both groups of young rats, individual samples of faeces were obtained every morning by rectal stimulation. The samples were immediately frozen at -20°C and stored until analyses. The results, which are given in Table 3, show that β-aspartylglycine is absent up to day 17 after birth; then a quantitative rather than a qualitative difference is established between Group I and Group II. Several explanations to these findings may be possible. The initial diet, i.e. mother's milk may over the time

Table 3: β-Aspartylglycine in faeces from young germfree rats

β-Aspartylglycine*	Day	17	18	19	20	21	22	23
Group I (R3 diet)		-	+++	+++	+++	+++	+++	+++
Group II (Milk)		-	+	+	++	++	++	+++

*: no β-asp-gly
+ and ++: increasing concentration
+++: adult level of β-asp-gly

vary in its content of precursors for the formation of β-aspartylglycine, the host derived proteolytic activity; i.e. trypsin, may vary in its activity during the same time schedule, the intestinal mucosa in young animals may allow an absorption of β-aspartylglycine, etc. The data obtained in the older animals, i.e. the animals older than 17 days, may give support to an assumption that more than one mechanism is at work.

Presence of β-aspartylglycine in faeces from children 0-24 months of age

In a longitudinal study we have followed the establishment of some biochemical MACs in a cohort of Swedish children from 0-24 months of age. Meconium was collected by the staff at the maternity ward and the parents collected faeces from the children at 1, 3, 6, 9, 12, 15, 18, 21, and 24 months of age. All samples were stored in clean plastic vials at -20°C until analysed. Based on their diet regimens, the children were divided into groups in a manner similar to that of *Cooperstock* and *Zedd* (1983). The MACs listed above have been investigated, and some of the results have already been published (*Midtvedt* et al., 1988, *Midtvedt* and *Midtvedt*, 1992). Concerning the β-aspartylglycine parameter it can briefly be stated that none of the samples taken prior to 6 months and after 9 months of age contained any β-aspartylglycine. In some few samples taken at 6 and 9 months of age, small amounts of β-aspartylglycine could be detected.

General comments on β-aspartylglycine

Our data support the view that presence of β-aspartylglycine is a rare event in adult individuals receiving oral antibiotics.

Our data indicate that in young animals and in children, the β-aspartylglycine parameter might be more controversial than in adults. Obviously, further investigations have to be carried out in order to clarify the value of this parameter in children.

MICROBIAL BILE ACID TRANSFORMATION

Comparative work in germfree and conventional animals have substantiated that microbial bile acid metabolism creates several GAC/MAC systems. The main microbial interactions include deconjugation, oxidation/reduction, dehydroxylation and hydroxylation. In mammals, bile acid metabolism can briefly be described as follows.

The bile acid derives from cholesterol by hepatic transformation. In man, cholic acid and chenodeoxycholic acid are the two most commonly occurring primary bile acids. Within the liver, the primary bile acids are conjugated, mainly with the amino acid taurine or

glycine at C-24 and excreted into the bile. A minor part may be present as sulphate or glucuronide conjugates; the esterification takes place mainly at C-3. In the intestinal tract, the conjugated bile acids are attacked by microbial enzymes and converted to a variety of metabolites. The so-called secondary bile acids thus formed may either be excreted into the faeces, or absorbed and sometimes further metabolised by hepatic enzymes to tertiary bile acids before re-excretion by the bile into the intestine where they can be further attacked by microbial enzymes. The bile acids are undergoing enterohepatic circulation several times each day. Most of the absorption takes place by an active transport in the distal ileum. However, a passive transport over the mucosa of some bile acids may take place in the whole small intestine. In general, microbial converted bile acids have reduced capacity to participate in the normal absorption of fat, and some of the derivatives may have a reduced absorption rate. The bile acids, which are not absorbed, are excreted in the faeces.

To summarise: The final composition of biliary and faecal bile acids are an interplay between liver biosynthetic enzymes and intestinal microbial transformation, both factors may vary with age. The following will be focused upon some main pairs of bile acid GACs/MACs.

Deconjugation of bile acids

As mentioned, bile acids are excreted by the liver in the form of conjugates and the findings are similar in germfree and conventional animals. In conventional animals nearly all bile acids present in faeces are in their free forms, and this is contrary to the findings in germfree animals where all bile acids present in faeces are found as conjugates. The intestinal hydrolysis is, as far as we know, exclusively brought about by the action of microbial enzymes.

The first successful isolation of a bacterium capable of hydrolysing conjugated bile acids was made by *Frankel* (1936). Since then, many reports have been made and several reviews have been written. The capability to split bile acid conjugates, especially glycine and taurine conjugates, is commonly occurring among intestinal microorganisms. Under normal conditions, the deconjugation appears to be restricted to the large bowel and the terminal part of the ileum. However, under pathological conditions the numbers of deconjugating microbes may increase in the proximal part of the small intestine. Deconjugation of radiolabelled bile acids is a test commonly used for diagnosing microbial overgrowth in the small intestine.

Deconjugation may temporarily be reduced following intake of several antibiotics (*Gustafsson* and *Norin*, 1977), but normal levels of deconjugation are usually retained shortly after the intake has been stopped.

In an on-going study on the establishment of microbial function in newborns, we have found that deconjugation is one of the first GAC/MAC switches to be established in infants. One month after birth, nearly all faecal bile acids were present in their unconjugated forms (*Jönsson* et al., unpublished results).

Oxidation-reduction of hydroxyl groups

Bile acids may carry hydroxyl groups at C-3, C-6, C-7, C-12, C-16 and C-23 positions, respectively. The main primary bile acids in most mammalian species, including man, are cholic acid and chenodeoxycholic acid with hydroxyl groups at C-3, C-7, C-12 and C-3, C-7, respectively. Most of the work with microbial bile acid hydroxyl dehydrogenases has been made on these

bile acids. It has been shown that such dehydrogenases are produced by a very wide range of bacterial genera (*Midtvedt* and *Norman*, 1967; *Dickinson* et al., 1971, *Prevot*, 1961; *Midtvedt*, 1974).

In conventional rats, dehydrogenating microorganisms are present in high numbers in caecal contents and faeces, but present in low numbers in the small intestine (*Midtvedt* and *Norman*, 1968). Under pathological conditions, as after the establishment of a blind loop, dehydrogenating microorganisms are present in high numbers in the blind loop and throughout the small intestine (*Midtvedt* et al., 1969).

The reduction of a keto group leading to the formation of a β-hydroxyl group can partly be performed by microbial enzymes but can also partly be performed by the liver. Formation of 3β-derivatives seems to be a sole microbial capacity. Most of the strains so far studied are anaerobes. Unpublished results from my laboratory indicate that some aerobes - as some *Pseudomonas* strains - can perform this reaction. At least in conventional rats, the 3β-forming microbes are lacking or present in low numbers only in the small intestine, but are present in the large intestine.

As for deconjugation, dehydrogenation can also be influenced upon by intake of antibiotics. Similarly, microbial oxidation-reduction of the various hydroxyl groups is established soon after birth.

Dehydroxylation of bile acids

Here, the interest has been focused on the dehydroxylation of the hydroxyl group at C-7 in cholic and chenodeoxycholic acid, leading to the formation of deoxycholic and lithcholic acid, respectively. The first microbial strains capable of performing this reaction were described in 1966 by *Gustafsson* et al. Since then, there have been several reports and most of the authors have found this capability to be a rare one among intestinal microorganisms. Most reports identify Gram-positive, non-sporeforming, anaerobic rods, probably belonging within the *Eubacterium* group (*Gustafsson* et al, 1966; *Hirano* et al., 1981; *Hylemon* et al., 1980; *Midtvedt*, 1967), whereas strains of *Clostridium* (*Aries* and *Hill*, 1970; *Ferrari* and *Beretta*, 1977; *Hayakawa* and *Hattori*, 1970; *Hill*, 1985; *Hill* et al., 1971; *Hirano* et al., 1981; *Stellwag* and *Hylemon*, 1979), *Bacteroides* (*Aries* and *Hill*, 1970; *Bokkenheuser* et al., 1969; *Edenharder* and *Slemrova*, 1976; *Hill* et al., 1971) and *Bifidobacterium* (*Ferrari* et al., 1980; *Hill* et al., 1971) have been described.

As for deconjugating and dehydrogenating microbes, 7-alpha-dehydroxylating microbes are usually absent or present in low numbers only in the small intestine whereas they are present in high numbers in the large intestine.

A significant effect upon 7-alpha-dehyroxylation has been found after intake of several antibiotics, in man (*Canzi* et al., 1985, *Gustafsson* et al., 1977; *Andreasson* et al., 1988) as well as in animals (*Gustafsson* and *Norman*, 1977; *Gustafsson* et al., 1993). Obviously, this is a GAC/MAC parameter worth to be studied in greater detail.

In contrast to deconjugation and dehydrogenation, 7-alpha-dehydroxylation is a rare event to be established in infants. Unpublished results indicate that it may take months until this function is established (*Jönsson* et al., unpublished results).

Hydroxylation of bile acids

In vitro, several microbial strains can hydroxylate bile acids. However, no hydroxylation of bile acids seems to take place in the intestinal tract of mammals (*Midtvedt*, 1974).

To summarise: Deconjugation, dehydrogenation and dehydroxylation are

three main MAC/GAC switches of considerable interest in clinical medicine, Alterations may reflect that the microbes are outside their normal habit (as in the case of the conjugate-breath-test for bacterial overgrowth) or are eradicated (following intake of antibiotics). Whether, and to what extent these alterations reflect alterations in colonisation resistance remains to be settled.

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DETERMINATION OF COLONISATION RESISTANCE OF THE DIGESTIVE TRACT BY BIOTYPING OF *ENTEROBACTERIACEAE* ISOLATED FROM SUBSEQUENT FAECAL SAMPLES

HERMA Z. APPERLOO-RENKEMA and DIRK VAN DER WAAIJ

Laboratory for Medical Microbiology, University Hospital Groningen, Groningen, The Netherlands

SUMMARY

Nine healthy volunteers were studied during six weeks, in order to determine the natural variation in the number of different biotypes of *Enterobacteriaceae* per faecal sample. The numbers of biotypes ranged from 1-15 per faecal sample, the mean number of biotypes varied between 2.6 and 7.3 different biotypes per faecal sample per healthy volunteer. Inter-individual variations of five biotypes in the mean number of biotypes per faecal sample are normal.

We assessed the minimal number of faecal samples that should be taken for comprehensive biotyping so as to determine reliably the mean number of different biotypes representative for the Colonisation Resistance (CR) of an individual. It was found that minimally four faecal samples are required.

We validated measurement of the CR in 10 healthy individuals by determination of the mean number of different biotypes of *Enterobacteriaceae* isolated from four faecal samples per volunteer by comparison with the golden standard for CR. The golden standard for CR is oral contamination with a neomycin resistant *Escherichia coli* (NR-*E. coli*) strain and measurement of the faecal concentration of this strain during 14 days after the contamination. Both measures are significantly correlated ($p<0.05$). The NR-*E. coli* strain could be cultured from faecal samples of 4/10 volunteers as long as 300 days after contamination.

INTRODUCTION

Man and his microflora constitute an intriguing ecosystem. The intestinal bacteria even outnumber the eukaryotic host cells. Yet both man and his microflora succeed in peaceful symbiosis. Not much is known about this ecosystem because its numerous relations are difficult to study.

Now that the direct relations are difficult to approach, perhaps it is possible to deal with the results of the interactions. One such result of the myriad of interactions between the host and his intestinal microflora is the Colonisation Resistance (CR). CR is a property of the indigenous intestinal microflora that controls the growth and therewith the chance of translocation of potentially pathogenic bacteria across the gut wall. CR is accomplished by means of the action of antimicrobial metabolites (*Walker*, 1990). Other factors that influence colonisation of bacteria are ability to adhere to the gut wall and the

length of the lag phase of the bacteria (*Freter*, 1983, 1986). Competition for niches and nutrients e.g. has also been assigned a role in this prevention of colonisation by foreign bacteria. The role of the indigenous mainly anaerobic flora in the maintenance of the CR for potentially pathogenic bacteria was shown indirectly in studies in which antibiotics were administered. Antibiotics that killed many anaerobic bacteria decreased the CR, whereas antibiotics that left the anaerobic part of the microflora as it was, did not affect the CR (*Hentges* et al., 1984; *Hofstra* et al., 1988; *van der Waaij* et al., 1972a; *van der Waaij*, 1982).

The stronger the suppression of ingested bacteria by the anaerobic flora, i.e. the stronger the CR, the less different potentially pathogenic bacteria are apparently able to colonise the gut. *Enterobacteriaceae* species - particularly *E. coli* biotypes - in general form an important part of the facultatively anaerobic (aerobic) flora of the digestive tract. The quality of the CR and therewith its protective capacity may vary between individuals of the same species (*van der Waaij and Heidt*, 1990).

The digestive tracts of individuals with an impairment of their CR, e.g. due to destruction of large parts of the anaerobic flora by antibiotic treatment, are likely to become colonised in high concentrations by potentially pathogenic bacteria (*Hahn* et al., 1978; *van der Waaij* et al., 1977, 1978). Dominant clones of *Enterobacteriaceae* - those that grow out to high numbers - represent the strains which are most likely to translocate to lymphatic organs and the liver (*Tancrede*, 1985). In immunocompromised patients translocation of *Enterobacteriaceae* may result in a life-threatening septicaemia (*van der Waaij* et al., 1977, 1978; *Schimpff* et al., 1972). The concept of CR and translocation of potentially pathogenic bacteria in case of impairment of the CR form the rationale behind selective decontamination of the digestive tract (SDD) in immunocompromised patients (*van der Waaij*, 1992).

CR was first studied in the seventies in mice. In mice, CR is defined as the resistance of the digestive tract against colonisation by orally ingested potentially pathogenic bacteria (*van der Waaij* et al., 1971). In mice CR has been defined as the $\log^{10}$ of the oral bacterial dose followed by a persistent "take" in 50% of the contaminated animals (*van der Waaij* et al., 1971). For an individual mouse the CR can be directly expressed as the $\log^{10}$ concentration of a specific potentially pathogenic bacterial species found in the faeces two weeks after contamination. Measurement of CR can also be accomplished by quantitative biotyping of one or more *Enterobacteriaceae* strains isolated from faeces collected at three of four daily intervals. This was considered a useful method because of the finding that, after oral administration of a single dose of an *Escherichia coli* strain to mice, the population density of these bacteria in the intestinal tract (faeces) varied inversely with CR (*van der Waaij* and *Berghuis*, 1974).

We have analysed the data of a comprehensive biotyping study in healthy volunteers performed in the seventies. Biotyping of *Enterobacteriaceae* provide a valuable and reproducible method for differentiation below the species level. Some species can be subdivided in more than 50 different biotypes (*van der Waaij* et al., 1972b, 1975]. The object of our analysis was to assess the natural variation in the number of different biotypes of *Enterobacteriaceae* found in the faecal samples of healthy volunteers. For practical reasons we investigated how many faecal samples should be studied minimally in order to obtain a reliable value for the mean number of

different biotypes of *Enterobacteriaceae* as a measure for CR.

Furthermore, this method of determining the CR was validated in healthy human volunteers by comparison with a golden standard for CR: Oral contamination with an *E. coli* strain and measuring its faecal concentration two weeks after administration. Ten healthy volunteers were given orally a NR-*E. coli* strain. Thereafter the mean faecal concentration of this strain was assessed from day 1 to day 14. These concentrations were compared with the mean number of different biotypes of *Enterobacteriaceae* in the faeces in four faecal samples obtained from each volunteer within one week prior to the oral contamination (*Apperloo-Renkema* et al., 1990).

SUBJECTS, MATERIALS AND METHODS

Natural variation in biotypes of *Enterobacteriaceae* and minimal number of faecal samples to be studied

Subjects

Nine healthy volunteers, seven male and two female, aged 22-51 years, participated in the study. None of them had taken antibiotics for eight weeks or during the experiment nor had they suffered any infective illness during the period they were on study.

Sampling

Two faecal samples were obtained every week from each volunteer for six weeks.

Biotyping

Faeces were inoculated directly onto MacConkey agar (Merck) and were additionally suspended 1:9 (w/v) in Brain Heart Infusion (BHI) broth (Oxoid). These faecal suspensions were then diluted 1:9 (v/v) in BHI broth. After incubation the various suspensions were subcultured on MacConkey agar. *Enterobacteriaceae* species were identified and typed with 19 different fermentation reactions selected for the *Enterobacteriaceae*. For comprehensive biotyping a minimum of 20 colonies was cultured per sample. Details of the biotyping technique have been described previously (*van der Waaij* et al., 1972b).

Determination of the minimal number of faecal samples

We estimated the lowest number of faecal samples by applying the standard error of the mean (SEM) of stepwise adding the data derived from subsequent samples. If adding data of additional samples did not alter the standard error of the mean significantly, it was decided that the minimal number of samples had been reached (*Apperloo-Renkema* et al., 1990).

Statistical analysis

Statistical software has been developed for data storage and processing of the results of the biotyping experiment (*Apperloo-Renkema* et al., 1990).

Oral contamination and biotyping

Volunteers and sampling

Ten healthy volunteers, seven males and three females, aged 22-44, entered this study after having given written informed consent. The experiment was approved of by the Medical Ethical Committee of the University Hospital Groningen.

Before oral contamination, four faecal samples were collected per volunteer within one week. After oral contamination with an *E. coli* strain, faecal samples were collected daily, whenever faeces were produced. After three months

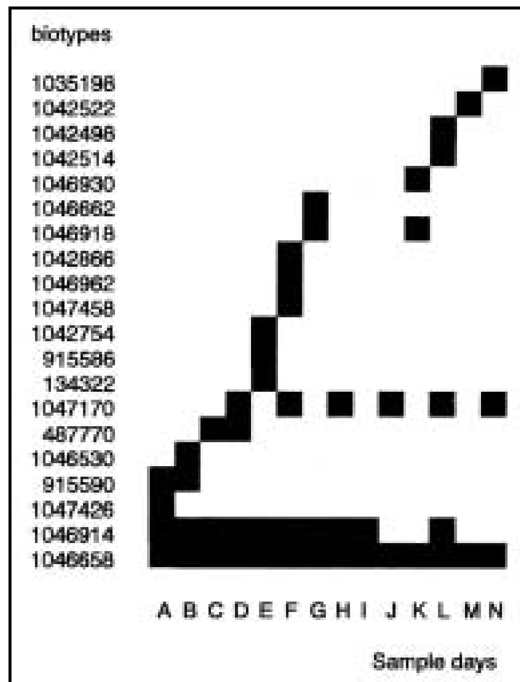

Figure 1: Biotyping diagram of a healthy individual. On the X-axis the sample days are shown, on the Y-axis different biotypes of *Enterobacteriaceae* are given.

the frequency of faecal sampling diminished to once per month. In the period of one month before until three months after the oral contamination, no volunteer had suffered or did suffer from a gastrointestinal disease or had taken antibiotics.

Oral contaminating strain

The *E. coli* strain used for oral contamination was identified with the API-20E system (Analytab Products Inc., Montalieu Vercieu, France). The biotype of the strain was 1144512. The strain was resistant to neomycin (minimal inhibitory concentration >250 mg/l). The strain will be referred to as NR-*E. coli*. For oral contamination the volunteers ingested 10 ml chocolate milk to which 1 ml of an overnight culture at 37°C (approximately 10^9 bacteria/ml) of the NR-*E. coli* strain had been added.

Determination of the faecal concentration of the NR-E. coli strain.

Faeces were suspended 1:9 (w/v) in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, UK) and subsequently diluted 1:9 (v/v) in BHI. After overnight incubation at 37°C the suspensions were subcultured on MacConkey agar (Merck, Darmstadt, FRG) to which neomycin had been added (250 mg/l). The concentrations were determined by standard dilution methods for both the NR-*E. coli* strain the endogenous strains of *Enterobacteriaceae*.

Measurement of CR by comprehensive biotyping of Enterobacteriaceae

CR was defined as the reciprocal value of the mean number of different biotypes of *Enterobacteriaceae* isolated from four faecal samples obtained within one to two weeks (*Apperloo-Renkema* et al., 1990). Details of the

Table 1: Results of the first study on the natural variation of the number of biotypes of *Enterobacteriaceae* found in faecal samples of nine healthy individuals

	healthy individuals								
	1	2	3	4	5	6	7	8	9
Number of faecal samples:	13	14	15	13	10	12	14	15	14
Mean number of different biotypes of *Enterobacteriaceae*:	5.0	4.9	6.1	3.0	2.6	5.8	3.6	7.3	6.7
Range of different biotypes of *Enterobacteriaceae*:	1-9	2-15	2-9	1-7	1-5	2-14	2-6	2-15	3-13

biotyping technique have been described previously (*van der Waaij* et al., 1977). The strains were stored at -20°C until use for specific antibody titration.

Measurement of CR by oral contamination

CR can be expressed directly as the $\log^{10}$ concentration of a specific potentially pathogenic bacterial species found in the faeces two weeks after contamination. Because of the normal fluctuations in the faecal concentration of *Enterobacteriaceae* we assessed the mean faecal concentration of this strain daily on day one until day 14 after oral contamination instead of the faecal concentration on day 14 only.

RESULTS

Natural variation in biotypes of *Enterobacteriaceae* and minimal number of faecal samples to be studied

In the first study the mean number of faecal samples investigated per volunteer was 13, range 10-15. The development of the pattern of the number of different biotypes of a volunteer with time is presented as an example in a diagram (Figure l). The variation in the number of different biotypes isolated from the faecal samples of these nine volunteers ranged between 1 and 15; the variation in the mean number of different biotypes per faecal sample was 2.6-7.3 (Table 1). In the mean number of biotypes inter-individual variations of five biotypes per faecal sample were found. There was a random variation of number of different biotypes with time.
We calculated the standard error of the mean (SEM) number of different biotypes per faecal sample for each volunteer separately, for increasing numbers of faecal samples. Including four faecal samples instead of one for determination of a reliable mean number of different biotypes per faecal sample yielded a considerable gain of 50% in accuracy of determination of that mean number of different biotypes. Including a fifth faecal sample yielded only an extra 5% in accuracy.

Oral contamination and biotyping

The strains of *Enterobacteriaceae* isolated from the faecal samples of the volunteers collected in the week before the oral contamination were sensitive to neomycin in contrast to the strain used for oral experimental contamination. The oral intake of the chocolate milk with the NR-*E. coli* strain was well tolerated as none of the volunteers suffered from any clinical symptoms. The

Table 2: Results of the study on oral contamination and biotyping in 10 healthy individuals

	healthy individuals									
	1	2	3	4	5	6	7	8	9	10
Mean number of different biotypes of faecal *Enterobacteriaceae* before oral contamination:	2.00	1.25	1.25	2.25	2.00	1.25	2.25	2.00	2.25	1.25
Mean faecal concentration of NR-*E. coli*, day 1-14 after oral contamination*:	4.4	1.3	3.3	4.3	4.0	2.2	4.4	3.8	4.8	3.7
Mean concentration of faecal Enterobacteriaceae before oral contamination:	3.4	3.0	4.4	4.1	4.6	4.5	3.5	3.0	3.5	2.4

*: ^{10}log/g faeces

strain was cleared from the intestines within 14 days in one case. After one year four volunteers were still colonised with the NR-*E. coli* strain.

The mean number of different biotypes isolated from the faecal samples collected prior to the oral contamination ranged between 1.25 and 2.25 different biotypes between the volunteers (Table 2). The mean concentration of the NR-*E. coli* strain measured from day 1 to 14 correlated significantly with the mean number of different biotypes of *Enterobacteriaceae* isolated from the four faecal samples of the volunteers collected before the oral contamination ($r = 0.80$, $p<0.05$).

The mean concentration of *Enterobacteriaceae* in the faecal samples before the oral contamination ranged between 2.4 and 4.6 (log^{10}/g faeces). No correlation was found between the mean concentration of *Enterobacteriaceae* in the faecal samples before the oral contamination and the mean concentration of the oral contaminating strain measured from day 1 to 14 ($r = -0.01$).

DISCUSSION

The results of our study show considerable differences in the mean number of biotypes of *Enterobacteriaceae* excreted by healthy volunteers per faecal sample. In addition, there are also clear differences in the number of new biotypes found in the faeces of these volunteers. These inter-individual differences appeared constant and could be expressed adequately in the mean number of biotypes per sample. These differences may bear a relationship to the quality of the individual CR of the digestive tract of the volunteers; this possible relationship was subject of the second study presented here.

Examination of data of the nine volunteers shows that the number of biotypes per faecal sample per individual does not show a pattern dependent on time. The error in calculating the mean number of biotypes obviously decreases the more samples are involved in the calculation. Reliable assessment of the mean number of biotypes as measure of CR can apparently be achieved by ex-

amination and biotyping of four faecal samples.

In 10 healthy volunteers CR was measured in two ways. The first, the "golden standard" worked out in mice, is the faecal concentration of an oral contaminating strain measured after 14 days, and the second was a measure applicable in hospitalised patients for studies of the effect of antibiotic treatment on the protective indigenous microflora (*van der Waaij* et al., 1977). Since the concentration of the oral contaminant in faeces was not constant but fluctuated, the mean concentration on days 1 to 14 was used instead of those on day 14. Both measures for CR were significantly correlated ($p<0.05$). Therefore, we decided to use biotyping of *Enterobacteriaceae* isolated from four faecal samples obtained within one week as a measure for CR instead of the ethically less acceptable method of oral contamination. In the first study, the mean number of different biotypes of *Enterobacteriaceae* in the healthy individuals was found to be higher than in the second study. This difference is mainly accounted for by the difference in biotyping techniques that were applied in the respective studies. The elder method focussed on as many differences in outcome of biochemical reactions as possible (*van der Waaij* et al., 1972b), whereas the latter (API system) targeted at as many different strains of *Enterobacteriaceae* as possible. The first approach yielded more different "biotypes" than the second.

In this study, we found no correlation between concentrations of endogenous *E. coli* strains and the concentration of the NR-*E. coli* strain on day 14 after contamination. Higher concentrations of endogenous *Enterobacteriaceae* are found sometimes after broad-spectrum antibiotic therapy and are then indicative for a disturbed intestinal ecosystem and a lowered CR. But in the normal situation the concentration of endogenous *Enterobacteriaceae* cannot be used as such an indicator.

One might hypothesise that the higher the CR, the sooner a newly ingested bacterium from the environment will be eliminated from the gastrointestinal tract. However, the speed of clearance depends not only on the strain but also on the number of bacteria ingested. Ingestion of 10^9 bacteria of a strain by the volunteers in our study may rarely occur in normal life. Indeed, in several subjects the elimination of the NR-*E. coli* strain lasted quite long. Clearance of laboratory strains (*Kaijser*, 1983) or *Pseudomonas aeruginosa* (*Buck*, 1969) occurs within a few weeks to a few months.

This measurement of CR provides us with a tool for the study of one aspect of the ecosystem constituted by man and his intestinal microflora. E.g. it is possible now to study differences in CR between different animal species (*van der Waaij* and *van der Waaij*, 1990). Moreover the relationship between the CR and development of wasting disease in mice was studied this way (*van der Waaij* and *Heidt*, 1990). A possible relation between CR and activity of Systemic Lupus Erythematosus (SLE) in man was also subject of study and revealed slight differences between patients with active and inactive SLE versus healthy individuals (*Apperloo-Renkema*, unpublished results).

To conclude: We assessed natural variation in biotypes of *Enterobacteriaceae* in faeces from healthy individuals. The minimal number of faecal samples was assessed that should be taken for comprehensive biotyping in order to determine reliably the mean number of different biotypes representative for the Colonisation Resistance (CR) of an individual. It was found that minimally four faecal samples are required.

We validated measurement of the CR

in 10 healthy individuals by determination of the mean number of different biotypes of *Enterobacteriaceae* isolated from four faecal samples per volunteer by comparison with the golden standard for CR. The golden standard for CR is oral contamination with a neomycin resistant *Escherichia coli* (NR-*E. coli*) strain and measurement of the faecal concentration of this strain during 14 days after the contamination. Both measures are significantly correlated ($p<0.05$). The NR-*E. coli* strain could be cultured from faecal samples of 4/10 volunteers as long as 300 days after contamination.

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ECOLOGICAL IMPACT OF ANTIMICROBIAL AGENTS ON HUMAN INTESTINAL MICROFLORA

CHARLOTTA EDLUND and CARL-ERIC NORD

Department of Microbiology, Huddinge University Hospital, Karolinska Institute, Stockholm, Sweden

INTRODUCTION

Administration of antimicrobial agents has been shown to induce disturbances in the ecological balance of the intestinal microflora. The role of the normal microflora is still poorly understood, but there is evidence that alterations in the microflora may have important clinical consequences. One of the major functions of the endogenous gastrointestinal microflora is protection of new colonisation by potentially pathogenic microorganisms as well as protection of overgrowth of already present potentially pathogenic strains (*Edlund* and *Nord*, 1991). The anaerobic microflora outnumbers the aerobic and facultative anaerobic microflora with a factor of 1000:1. Experimental animal studies and studies on human volunteers and patients imply that the anaerobic part of the normal microflora plays a dominant role in protecting against potentially pathogenic microorganisms (*van der Waaij* et al., 1986). Most endogenous infections are caused by Gram-negative aerobic microorganisms, but Gram-positive and anaerobic infections originating from the gastrointestinal tract are also a severe problem in immunocompromised patients and in patients undergoing gastrointestinal surgery.

The intestinal microflora can be affected by incomplete absorption of orally administered agents and by secretion of the antimicrobial agent by the salivary glands, in the bile and from the intestinal mucosa. Antimicrobial agents are often inactivated to variable extent in the intestines by bacterial enzymes or by binding to bacteria and other faecal components. Nevertheless, the remaining activity in the gastrointestinal tract of many antimicrobial agents is sufficiently high to disturb the ecological balance. A decrease in the number of microorganisms due to antimicrobial therapy may lead to several unwanted effects (*Edlund* and *Nord*, 1991). One is overgrowth of already present microorganisms with natural resistance such as yeasts, which can cause systemic infections in immunocompromised patients and *Clostridium difficile*, which can lead to life-threatening diarrhoea and colitis. Secondly, establishment of new resistant pathogenic bacteria, mainly aerobic Gram-negative rods, which can colonise other areas of the host, may occur. A third effect is that bacterial overgrowth also promotes transfer of genetic elements carrying resistance factors among bacteria and bacterial groups.

It is of great clinical importance to gain knowledge about the ecological effects of different antimicrobial agents on the gastrointestinal microflora. During the last 15 years the impact of different antimicrobial agents on the human gastrointestinal microflora has been studied by several investigators. In a previous article we have reviewed the literature dealing with this subject (*Nord* and *Edlund*, 1990). Since then, several new antimicrobial agents and new

routes of administration of older agents have been investigated. We therefore find it necessary to update the previous data concerning ecological impact of antimicrobial agents.

IMPACT OF PENICILLINS ON THE INTESTINAL MICROFLORA

Phenoxymethylpenicillin

The impact of phenoxymethylpenicillin on the intestinal microflora was investigated by *Heimdahl* and *Nord* (1979a). Ten volunteers were given 800 mg phenoxymethylpenicillin capsules b.i.d. for 7 days. No antibiotic activity was found in the faecal samples during the observation period and no changes in the intestinal microflora were noticed (Table 1).

Ampicillin

Knothe and *Wiedemann* (1965) investigated the effect on the intestinal microflora of peroral daily administration of 1000 to 3000 mg ampicillin during five days to ten volunteers. The numbers of *Escherichia coli*, enterococci, bifidobacteria and anaerobic Gram-negative rods were significantly reduced, while an increased number of ampicillin-resistant *Citrobacter, Klebsiella,* and *Proteus* species was observed (Table 1).

Leigh (1979) studied the impact of peroral administration of ampicillin. Ten volunteers received 500 mg t.i.d. for five days. An increase in the numbers of enterobacteria was observed in six volunteers and two volunteers had overgrowth of *Candida* species. Five volunteers developed diarrhoea, which lasted for 1-2 days (Table 1).

Ampicillin/sulbactam

The influence of ampicillin plus sulbactam on the colonic microflora in patients undergoing colorectal surgery was studied by *Kager* et al. (1982). Ampicillin and sulbactam was given intravenously to 21 patients in 500 mg doses of each agent every eight hour for two days. Ampicillin was detected in faeces in the range of 0.1 to 21.6 mg/kg in 11 patients. No changes in the number of streptococci, enterococci, and enterobacteria were observed while anaerobic cocci, bifidobacteria, eubacteria, lactobacilli, and *Bacteroides* decreased in numbers. No new colonising aerobic or anaerobic bacteria were recovered during the investigation period. After two weeks the anaerobic microflora was normalised in all patients (Table 1).

In another study *Kager* et al. (1983a) investigated the effect of ampicillin and sulbactam in dosages of 1000 and 2000 mg, respectively, every eight hour for two days on the colon microflora in ten patients undergoing colorectal surgery. The numbers of streptococci, enterococci, enterobacteria, anaerobic cocci, anaerobic Gram-positive and Gram-negative rods decreased significantly during the prophylaxis period. The aerobic and anaerobic microflora was normalised in all patients after two weeks (Table 1).

Amoxicillin

The effect of 2000 mg amoxicillin per day for 15 or more days on the intestinal microflora in eight patients with respiratory tract infections was investigated by *Gipponi* et al. (1985). The total levels of microorganisms were reduced in four patients and three patients had increased numbers of *Candida* (Table 1).

The impact on the intestinal microflora in ten healthy volunteers receiving 250 mg amoxicillin t.i.d. perorally for five days was investigated by *Leigh* (1979). The number of enterobacteria was significantly increased in four vol-

unteers during the administration period. None of the volunteers developed diarrhoea (Table 1).

Forty-four patients received 250 mg amoxicillin t.i.d. orally for seven days as treatment for lower respiratory tract infections and the impact on the intestinal microflora was evaluated (*Christensson* et al., 1991). The intestinal microflora was partly influenced by amoxicillin treatment. There was a significant increase in the numbers of enterobacteria, anaerobic Gram-positive rods and *Bacteroides* (Table 1).

Brismar et al. (1993a) investigated the effect of amoxicillin on the normal intestinal microflora. Ten healthy volunteers were given 500 mg amoxicillin tablets every 8 hour for seven days. A minor decrease was observed in the numbers of streptococci, staphylococci and eubacteria. There was an overgrowth of *Klebsiella* in six volunteers and of *Enterobacter* in two volunteers. No overgrowth of *C. difficile* or yeast was observed (Table 1).

Amoxicillin/clavulanic acid

The effect of amoxicillin alone compared with amoxicillin plus clavulanic acid on the intestinal microflora was studied by *Mittermayer* (1983). Six volunteers received 500 mg amoxicillin and six volunteers 500 mg amoxicillin plus 125 mg clavulanic acid t.i.d. for seven days. The total number of aerobic and anaerobic bacteria was not affected by either treatment. The number of amoxicillin-resistant enterobacteria increased significantly in both groups of volunteers. Selection of amoxicillin-clavulanic acid-resistant enterobacteria occurred only in those volunteers receiving amoxicillin-clavulanic acid. No significant changes in the number of *Pseudomonas, Bacteroides*, or yeasts were observed during or after treatment (Table 1).

Lambert-Zechovsky et al. (1984a) studied the effect of amoxicillin-clavulanic acid on the intestinal flora in children. Seven patients received the antibiotic combination orally and four patients parenterally in a dosage of 27.5 mg/kg body weight q.i.d. during 10 to 11 days. Oral administration caused a greater impact on the microflora than did parenteral administration. An increase in amoxicillin-resistant *E. coli* strains was noticed after antibiotic treatment had stopped. No other significant changes in the intestinal microflora occurred (Table 1).

Wise and co-workers (1984) studied the effect of amoxicillin-clavulanic acid on the intestinal microflora in six subjects. The volunteers were given 500 mg amoxicillin and 250 mg clavulanic acid every eight hour for three days. No major changes in the intestinal flora were observed. One volunteer developed diarrhoea (Table 1).

The impact of amoxicillin-clavulanic acid on the intestinal microflora in eight volunteers was investigated by *Motohiro* et al. (1985). Four subjects were given 187.5 mg tablets and four subjects were given 375 mg tablets (ratio amoxicillin-clavulanic acid 2:1) t.i.d. for five days. The staphylococci were strongly suppressed while the number of enterobacteria increased significantly in both groups. The number of enterococci increased after the administration had stopped. There were no changes in the anaerobic intestinal microflora. Diarrhoea was observed in one subject in each group (Table 1).

Brumfitt et al. (1986) studied the effects on the intestinal microflora in six volunteers receiving 250 mg amoxicillin plus 125 mg clavulanic acid t.i.d. for seven days. Staphylococci were eliminated and the number of streptococci decreased significantly during the administration period. No other significant changes were observed (Table 1).

Table 1: Impact of penicillins on the intestinal microflora

Agent	Dose (mg/day)	Days of adminis-tration	Number of patients	Impact on: Entero-bacteria	Impact on: Aerobic Gr^+ cocci	Impact on: Anaerobic bacteria	Overgrowth of resistant strains: Entero-bacteria	Overgrowth of resistant strains: *C. dif-ficile*	Overgrowth of resistant strains: *Candida*	Reference
Phenoxymethyl-penicillin	800x2	7	10	-	-	-	-	-	-	*Heimdahl* & *Nord*, 1979a
Ampicillin	1000-3000	5	10	↓↓	↓↓	↓↓	-	-	-	*Knothe* & *Wiedemann*, 1965
	500x3	5	10	↑	-	-	+	-	+	*Leigh*, 1979
Ampicillin/ sulbactam	500x3	2	21	-	-	↓	-	-	-	*Kager* et al., 1982
	1000x3	2		↓	↓	↓	-	-	-	*Kager* et al., 1983a
	2000x3	2		↓	↓	↓	-	-	-	*Kager* et al., 1983a
Amoxycillin	2000	≥15	8	↓	↓	↓	-	-	+	*Gipponi* et al., 1985
	250x3	5	10	↑	-	-	-	-	-	*Leigh*, 1979
	250x3	7	44	↑	-	↑	-	-	-	*Christensson* et al., 1991
	500x3	7	10	-	↓	-	+	-	-	*Brismar* et al., 1993a
	500x3	7	6	-	-	-	-	-	-	*Mittermayer*, 1983
Amoxycillin/ clavulanic acid	500/125x3	7	6	-	-	-	+	-	-	*Mittermayer*, 1983
	27.5mg/kgx4	10-11	11	-	-	-	+	-	-	*Lambert-Zechovsky*, 1984a
	500/250x3	3	6	-	-	-	-	-	-	*Wise* et al., 1984
	187.5x3 (2/1)*	5	4	↑	↓	-	+	-	-	*Motohiro* et al., 1985
	375x3 (2/1)*	5	4	↑	↓	-	+	-	-	*Motohiro* et al., 1985
	250/125x3	7	6	-	↓	-	+	-	-	*Brumfitt* et al., 1986
Bacampicillin	400x3	7	12	-	-	↓	-	-	-	*Heimdahl* et al., 1979
	1600	≥15	8	-	-	-	-	-	-	*Gipponi* et al., 1985
Pivampicillin	700x4	3	10	↑	-	-	+	-	+	*Knothe* & *Lembke*, 1973
Azlocillin	5000x3	7-8	6	↓	↓	↓	+	-	-	*Nord* et al., 1986
Piperacillin	4000x3	2	20	↓	↓	↓	-	-	-	*Kager* et al., 1983b
Piperacillin/ Tazobactam	4000/500x3	4-8	20	↓	↓	-	-	-	-	*Nord* et al., 1993

Table 1: Impact of penicillins on the intestinal microflora (continued)

Agent	Dose (mg/day)	Days of adminis-tration	Number of patients	Impact on: Entero-bacteria	Impact on: Aerobic Gr+ cocci	Impact on: Anaerobic bacteria	Overgrowth of resistant strains: Entero-bacteria	Overgrowth of resistant strains: *C. difficile*	Overgrowth of resistant strains: *Candida*	Reference
Talampicillin	250x3	5	10	↑	-	-	+	-	-	*Leigh*, 1979
Pivmecillinam	600x4	7	10	↓↓	↑	↓	-	-	-	*Knothe*, 1976
	400x3	7	5	↓	↑	↓	-	-	-	*Knothe*, 1976
Ticarcillin/ clavulanic acid	5000/200x3	7	10	↓	↑	-	-	-	-	*Nord* et al., 1989

↓↓: strong suppression, >4 log10 CFU/g faeces.
↓: mild to moderate suppression, 2-4 log10 CFU/g faeces.
↑: increase in number of microorganisms during therapy.
-: no significant change.
*: ratio amoxycillin/clavulanic acid.

Bacampicillin

Heimdahl et al. (1979b) investigated the effect of bacampicillin on the colon microflora. Bacampicillin was given as tablets or syrup in doses of 400 mg t.i.d. for seven days to 12 subjects. No changes in the colon microflora were observed in the volunteers receiving tablets, while there was a decrease in the numbers of anaerobic bacteria in those volunteers taking syrup. No ampicillin was detected in the faecal samples during the investigation period and no increased resistance to ampicillin was observed in the colon microflora (Table 1).

The impact of bacampicillin on the intestinal microflora in eight patients receiving 1600 mg daily for at least 15 days was investigated by *Gipponi* (1985). Moderate microbial changes were observed in two of the patients. No *C. difficile* strains or toxins were isolated (Table 1).

Pivampicillin

The impact of pivampicillin on the intestinal microflora was investigated by *Knothe* and *Lembke* (1973). Ten volunteers received a dose of 700 mg q.i.d. for three days. Pivampicillin caused minor changes in the flora. The numbers of *E. coli* and *Candida* increased in seven respectively three volunteers (Table 1).

Talampicillin

Leigh (1979) studied the effect of talampicillin on the intestinal microflora. Ten volunteers were given talampicillin perorally in a dosage of 250 mg t.i.d. for five days. Six subjects had considerable increase in the numbers of enterobacteria and one volunteer developed diarrhoea (Table 1).

Azlocillin

The impact of parenteral azlocillin treatment on the intestinal microflora in six patients receiving 5000 mg azlocillin t.i.d. for seven to eight days was investigated by *Nord* et al. (1986). The numbers of *E. coli* and streptococci were suppressed markedly in two patients and in one patient the enterococci decreased. The numbers of anaerobic cocci, lactobacilli, clostridia and *Bacteroides* were also markedly decreased in two patients. No patients harboured *C. difficile* or had cytotoxin in their faecal samples during or after azlocillin treatment (Table 1).

Piperacillin

The influence of piperacillin on the colonic microflora in patients undergoing colorectal surgery was investigated by *Kager* et al. (1983). Piperacillin was given parenterally in doses of 4000 mg every eight hour for 48 hours to 20 patients undergoing colorectal surgery. Enterococci, streptococci, and enterobacteria decreased in five of the patients while anaerobic Gram-positive cocci and rods, fusobacteria and *Bacteroides* decreased in seven of the patients during the administration period. After the piperacillin treatment, the aerobic and anaerobic colon microflora returned to the pre-treatment levels in most patients (Table 1).

Piperacillin/Tazobactam

The effect of piperacillin/tazobactam on the intestinal microflora was studied in 20 patients with intra-abdominal infections (*Nord* et al., 1993). The patients received 4000 mg piperacillin combined with 500 mg tazobactam t.i.d. by intravenous injection during four to eight days. The numbers of enterobacteria and enterococci were slightly decreased during treatment. There was a minor decrease in the numbers of bifidobacteria, eubacteria, lactobacilli, clostridia and veillonella while the numbers of anaerobic Gram-positive cocci and *Bacteroides* were not influ-

enced by the treatment. None of the patients had *Clostridium difficile* or its cytotoxin in faeces or developed diarrhoea. After therapy, the aerobic and anaerobic microflora returned to normal levels in all patients (Table 1).

Pivmecillinam

Knothe (1976) investigated the impact on the intestinal microflora of pivmecillinam. Ten volunteers were given 600 mg q.i.d. and five volunteers were given 400 mg t.i.d. for seven days, respectively. The changes were more pronounced after the higher dose of pivmecillinam. The numbers of *E. coli*, lactobacilli, and *Bacteroides* were significantly decreased while the numbers of enterococci increased (Table 1).

Ticarcillin/clavulanic acid

The influence of ticarcillin/clavulanic acid on the intestinal microflora was investigated by *Nord* et al. (1989). Ten volunteers were given 5000 mg ticarcillin plus 200 mg clavulanate intravenously t.i.d. for seven days. The numbers of enterococci and streptococci slightly increased, while there was a small decrease in the number of enterobacteria. Minor changes in the anaerobic microflora were observed. No measurable faecal concentrations of ticarcillin or clavulanate were found (Table 1).

Comments

Administration of ampicillin led to a strong suppression of both the aerobic and the anaerobic intestinal microflora. Phenoxymethylpenicillin and acid-resistant derivates of ampicillin like amoxicillin, bacampicillin, pivampicillin and talampicillin caused only minor suppression of the normal microflora, although overgrowth of resistant enterobacteria were observed. Administration of broad-spectrum penicillins, like azlocillin, piperacillin, pivmecillinam and ticarcillin resulted in marked changes in the intestinal microflora with strong suppression of the aerobic Gram-negative bacteria and often overgrowth of enterococci. These agents affected the anaerobic microflora only to a minor degree. None of the penicillins caused any major overgrowth of *C. difficile* or yeasts.

IMPACT OF PARENTERAL CEPHALOSPORINS ON THE INTESTINAL MICROFLORA

Cefazolin

Vogel and *Knothe* (1985) investigated the impact of cefazolin on the aerobic intestinal microflora in five patients receiving cefazolin (60-80 mg/kg per day). Treatment with cefazolin did not induce any changes in the aerobic flora except for colonisation with *Pseudomonas* species (Table 2).

Cefbuperazone

The influence of cefbuperazone on the intestinal microflora in patients undergoing colorectal surgery was studied by *Kager* et al. (1986). Ten patients were given cefbuperazone intravenously in a dose of 1000 mg at induction of anaesthesia, followed by a subsequent dose of 1000 mg 12 hours after the first dose. The cefbuperazone concentration in the faecal samples varied between 0 and 27.0 mg/kg. Streptococci, enterococci and enterobacteria were suppressed significantly during the prophylaxis period. Among the anaerobic bacteria, cocci, bifidobacteria, eubacteria, lactobacilli, clostridia, fusobacteria and *Bacteroides* decreased markedly

Table 2: Impact of parenterally administered cephalosporins on the intestinal microflora

Agent	Dose (mg/day)	Days of adminis-tration	Number of patients	Impact on: Entero-bacteria	Impact on: Aerobic Gr^+ cocci	Impact on: Anaerobic bacteria	Overgrowth of resistant strains: Entero-bacteria	Overgrowth of resistant strains: *C. dif-ficile*	Overgrowth of resistant strains: *Candida*	Reference
Cefazolin	60-80mg/kg	4-11	5	–	-	a	+	-	-	*Vogel & Knothe*, 1985
Cefbuperazone	1000x2	1	10	↓	↓	↓	-	-	-	*Kager* et al., 1986
Cefepime	1000x2	8	8	↓	-	-	-	-	-	*Bächer* et al., 1992
Cefmenoxime	4000	3	15	↓	-	-	-	-	+	*Knothe* et al., 1985
Cefoperazone	2000x2	7-14	28	↓↓	↑↓	↓↓	-	+	-	*Alestig* et al., 1983
	100mg/kg	4-7	16	↓↓	↓↓	↓	-	-	+	*Lambert-Zechovskky*, 1984b
				↓↓	↑	a	+	-	+	*Guggenbichler&Kofler*,1984
Cefotaxime	100mg/kg	a	26	↓	↑	-	+	-	-	*Lambert-Zechovskky*, 1985
	60-80mg/kg	4-11	11	-	-	a	+	-	-	*Vogel & Knothe*, 1985
	a	a	6	↓	-	a	-	-	-	*Guggenbichler&Kofler*,1984
Cefotiam	6000	3	15	↓	-	-	+	-	+	*Knothe* et al., 1985
Cefoxitin	2000x4	2	20	↓	↑	↓	+	-	-	*Kager* et al., 1981a
	6000-12000	8-23	6	↓	↑	↑	+	+	-	*Mulligan* et al., 1984
Ceftazidime	4000	1	8	↓	-	-	-	-	-	*Knothe* et al., 1985
Ceftizoxime	4000	1	8	↓	-	-	+	-	-	*Knothe* et al., 1985
Ceftriaxone	1500x2	7-13	12	↓↓	↓↓	↓	-	+	+	*Nilsson-Ehle* et al., 1985
	?	?	9	↓↓	-	a	+	-	+	*Guggenbichler&Kofler*,1984
	2000	1	10	↓↓	-	-	-	-	-	*Cavallaro* et al., 1992
	1000	5	10	a	a	↓	-	+	-	*Welling* et al., 1991
Moxalactam	2000	1	10	↓	↑	↓	-	-	-	*Kager* et al., 1984
	2000x3	1	10	↓	↑	↓	-	-	-	*Kager* et al., 1984

↓↓: strong suppression, >4 log10 CFU/g faeces.
↓: mild to moderate suppression, 2-4 log10 CFU/g faeces.
↑: increase in number of microorganisms during therapy.
-: no significant change.
a: no data available

during the same period. The microflora was normalised in all patients after four weeks (Table 2).

Cefepime

The impact of cefepime on the intestinal microflora was studied by *Bächer* et al. (26). Eight volunteers received cefipime 1000 mg bid by constant infusion over 30 min for eight days. A decrease in the number of *E. coli* and bifidobacteria in faeces was observed, whereas *Bacteroides* spp. and *Clostridia* spp. showed a slight increase. The number of intestinal bacteria returned to normal 20 to 48 days after the study was completed (Table 2).

Cefmenoxime

The impact of cefmenoxime on the intestinal flora was evaluated by *Knothe* et al. (1992). Fifteen volunteers were given 4000 mg cefmenoxime daily intravenously for three days. The numbers of enterobacteria, bifidobacteria, and lactobacilli decreased significantly while the numbers of clostridia and *Candida* increased (Table 2).

Cefoperazone

The effect of cefoperazone on the intestinal microflora was investigated by *Alestig* et al. (1983). Twenty-eight patients were treated with 2000 mg cefoperazone b.i.d. intravenously for seven to 14 days. Enterobacteria, staphylococci and streptococci were significantly suppressed in numbers and the enterococci increased in most patients during and after cefoperazone treatment. There was a major decrease in the numbers of anaerobic cocci, *Bacteroides*, fusobacteria, bifidobacteria, eubacteria, and lactobacilli. Eight patients had *C. difficile* and its cytotoxin and five of these patients developed diarrhoea (Table 2).

The effect of cefoperazone on the intestinal microflora in 16 children was studied by *Lambert-Zechovsky* et al. (1984b). Streptococci, staphylococci, and enterobacteria were markedly reduced in numbers in 13 patients. Selection of cefoperazone-resistant yeast was observed in seven patients (Table 2).

Guggenbichler and *Kofler* (1984) analysed the impact of cefoperazone treatment on the aerobic intestinal microflora in five children with narcotising enterocolitis or septicaemia. Cefoperazone eliminated all susceptible enterobacteria within the first 24 hours and overgrowth with enterococci and *Candida* was seen. Resistant *Serratia, Klebsiella* and *Enterobacter* strains were isolated from two patients (Table 2).

Cefotaxime

Lambert-Zechovsky et al. (1985) studied the impact of cefotaxime on the intestinal microflora in 26 hospitalised children. Cefotaxime was given intravenously in doses of 100 mg/kg/day. The numbers of enterobacteria decreased while there was an increase in resistant enterococci during the cefotaxime treatment. Intestinal colonisation of *Pseudomonas* strains also occurred. No significant changes in the anaerobic microflora were observed (Table 2).

The impact of cefotaxime on the aerobic microflora in 11 patients was investigated by *Vogel* and *Knothe* (1985). Cefotaxime was given in a dosage of 60-80 mg/kg/day. Colonisation with *Pseudomonas* was frequently found. No other major ecological alterations were observed during treatment (Table 2).

The influence of cefotaxime treatment on the faecal aerobic microflora in six children with septicaemia, pulmonary or urinary tract infections was investigated by *Guggenbichler* and *Kofler* (1984). The numbers of aerobic bacteria decreased moderately during treatment but no emergence of resistant strains was observed. No cefotaxime was found in the faecal samples (Table 2).

Cefotiam

The impact of cefotiam in a dose of 6000 mg/day for three days on the intestinal microflora was determined by *Knothe* et al. (1985). The numbers of enterobacteria and lactobacilli were significantly suppressed while the numbers of *Pseudomonas* and *Candida* increased. No other changes in the microflora were observed (Table 2).

Cefoxitin

The impact of cefoxitin prophylaxis on the colon microflora in patients undergoing colorectal surgery was investigated by *Kager* et al. (1981). Cefoxitin was administered intravenously in doses of 2000 mg every six hour for two days to 20 patients. The cefoxitin concentrations in the faecal samples were between 1.5 and 35.5 mg/kg. In the aerobic microflora, cefoxitin-sensitive *E. coli* and other enterobacteria decreased significantly while cefoxitin-resistant enterococci, enterobacteria, and *Pseudomonas* proliferated. Among the anaerobic bacteria, *Bacteroides fragilis* and fusobacteria decreased significantly. After the antibiotic prophylaxis had stopped all cefoxitin-resistant bacterial strains decreased while the suppressed *E. coli* and *B. fragilis* strains increased (Table 2).

The effect of cefoxitin treatment on the intestinal microflora in six patients was studied by *Mulligan* et al. (1984). The patients received cefoxitin in daily doses of 6000 to 12000 mg for eight to 23 days. There were a proliferation of enterococci, coagulase-negative staphylococci, cefoxitin-resistant enterobacteria, *Pseudomonas* spp. and *Bacteroides fragilis*. *C. difficile* strains were isolated from five patients (Table 2).

Ceftazidime

Knothe et al. (1985) studied the effect of ceftazidime on the gut flora in eight volunteers. The volunteers received ceftazidime intravenously in a dose of 4000 mg for one day. The enterobacteria and the lactobacilli decreased considerably, while no effect on other microorganisms in the flora could be observed (Table 2).

Ceftizoxime

The influence of ceftizoxime on the intestinal flora in eight volunteers receiving 4000 mg ceftizoxime during one day was investigated by *Knothe* et al. (1985). Ceftizoxime administration significantly reduced the numbers of susceptible enterobacteria while *Citrobacter* and *Proteus* increased in numbers. No effect on enterococci, lactobacilli, and *Bacteroides* was observed (Table 2).

Ceftriaxone

Nilsson-Ehle, et al. (1985) studied the influence of ceftriaxone on the intestinal microflora in 12 patients with acute bacterial infections. The patients received 1500 mg ceftriaxone b.i.d. for seven to 13 days. The numbers of aerobic bacteria decreased significantly in all patients except two. These two patients had the lowest biliary clearance of ceftriaxone. In six patients overgrowth with *Candida* and *Torulopsis* was noticed during the treatment period. The aerobic microflora was normalised in all patients except one after one month. In the anaerobic microflora the cocci, bifidobacteria, eubacteria, *Bacteroides*, and fusobacteria decreased significantly. The two patients with low biliary excretion had only minor changes in the anaerobic flora. One patient had *C. difficile* and cytotoxin in faeces during and after treatment and had concomitant diarrhoea (Table 2).

The effect of ceftriaxone on the aerobic intestinal microflora in nine children with septicaemia was investigated by *Guggenbichler* and *Kofler* (1984). The aerobic susceptible Gram-negative bacteria was eradicated from the microflora

within 48 hours while *Klebsiella, Enterobacter, Citrobacter, Serratia,* and *E. coli* strains acquired resistance during therapy. Enterococci and *Candida albicans* dominated the aerobic microflora during treatment. Reappearance of aerobic Gram-negative bacteria was observed in all patients except two after the treatment had stopped (Table 2).

The influence of ceftriaxone on intestinal flora was investigated by *Cavallaro* et al. (1992) in ten patients undergoing colorectal surgery. Ceftriaxone were given intravenously in one 2000 mg dose before anaesthesia. The aerobic faecal microflora was considerably affected, enterobacteria were eliminated or strongly suppressed in all patients, while there was only minor changes in the number of aerobic Gram-positive bacteria. The anaerobic microflora showed only minor alterations. No new colonising microorganisms were isolated during the investigation period and no colonisation with ceftriaxone-resistant bacteria was observed (Table 2).

Welling et al (1991) studied the effect of ceftriaxone on the anaerobic intestinal microflora. Ten healthy volunteers received 1000 mg of ceftriaxone intramuscularly for five days. Ceftriaxone treatment resulted in a significant decrease in the total number of anaerobic microorganisms. Two patients were colonised by toxin negative *C. difficile* strains during the administration period (Table 2).

Moxalactam

Kager et al. (1984) studied the effect of single-dose as compared to three-dose prophylaxis with moxalactam on the colon microflora in patients undergoing colorectal surgery. Twenty patients were given an initial dose of 2000 mg moxalactam at the induction of anaesthesia. Ten of the patients received two subsequent doses at eight-hour intervals. The moxalactam concentrations in faeces varied between 0.2 and 23.0 mg/kg. There were no differences between the patients receiving one dose of moxalactam and those receiving three doses. Enterobacteria were markedly suppressed during the prophylaxis period, while streptococci and enterococci proliferated from day three to day five. Among the anaerobic bacteria, cocci, lactobacilli, bifidobacteria, clostridia, *Bacteroides*, and fusobacteria decreased significantly during the same period. After two weeks the microflora was normalised in all patients (Table 2).

Comments

Parenterally administered cephalosporins seemed in general to cause moderate effects on the intestinal microflora mostly in form of suppression of enterobacteria. However, administration of cefoperazone and ceftriaxone also led to a considerably decrease in the numbers of aerobic Gram-positive and anaerobic microorganisms. Most parenterally administered cephalosporins led to overgrowth of resistant enterobacteria and also sometimes to colonisation of *C. difficile* and yeasts.

IMPACT OF ORAL CEPHALOSPORINS ON THE INTESTINAL MICROFLORA

Cefaclor

Finegold et al. (1987) investigated the impact of cefaclor on the intestinal microflora in six volunteers receiving cefaclor orally 250 mg t.i.d. for 14 days. The aerobic flora was not significantly altered by cefaclor administration. In the anaerobic microflora bifido-

bacteria were eliminated in two subjects. Three volunteers were colonised by *C. difficile*, but none developed colitis (Table 3).

Cefaclor was given orally to 10 volunteers in doses of 250 mg t.i.d. for seven days by *Nord* et al. (1987). The aerobic intestinal microflora was not affected by cefaclor administration, while minor changes in the anaerobic intestinal microflora was observed. There was no new colonisation with cefaclor-resistant bacteria (Table 3).

Forty patients with lower respiratory tract infections were treated with 250 mg cefaclor orally t.i.d. for seven days, and the ecological alterations in the intestinal microflora were evaluated (*Christensson* et al., 1991). The numbers of streptococci, staphylococci and anaerobic cocci decreased significantly, while enterococci, enterobacteria, *Bacteroides* and *Candida albicans* increased significantly in numbers during cefaclor treatment (Table 3).

Cefixime

Finegold et al. (1987) evaluated the influence of 400 mg orally administered cefixime daily for 14 days on the intestinal microflora in six healthy subjects. A significant decrease in the numbers of *E. coli* occurred. In four subjects the enterococci increased markedly. In the anaerobic microflora, bifidobacteria disappeared from two subjects, clostridia from three subjects, and *B. fragilis* from one subject. *C. difficile* was isolated from four subjects without severe gastrointestinal symptoms. No development of resistance among the aerobic or anaerobic bacteria was seen (Table 3).

The ecological effects on the normal intestinal microflora in ten healthy persons after 200 mg cefixime tablets bid for seven days was studied by *Nord* et al. (1988). The numbers of streptococci and *E. coli* decreased while the numbers of enterococci increased during the administration of cefixime. In the anaerobic microflora, the numbers of cocci, clostridia and *Bacteroides* were suppressed significantly. *C. difficile* was isolated from 5 volunteers but cytotoxin was only detected in one volunteer. The intestinal microflora was normalised within two weeks after treatment had stopped (Table 3).

Cefpodoxime-proxetil

Brismar et al. (1993a) investigated the effect of cefpodoxime-proxetil on the normal intestinal microflora. Ten healthy volunteers were given 200 mg cefpodoxime-proxetil tablets every 12 hour for seven days. The numbers of streptococci, enterobacteria and clostridia were strongly reduced in the faecal flora, while there was an overgrowth of enterococci. Beta-lactamase activity was detected in six subjects. Three volunteers had high concentrations of cefpodoxime in faeces and no detectable beta-lactamase activities. These subjects were colonised by *C. difficile* and yeast (Table 3).

Cefprozil

Lode et al. (1992) studied the multiple-dose pharmacokinetics of orally administered cefprozil and its impact on intestinal microflora of healthy volunteers in a randomised double-blind placebo controlled trail. Eight volunteers received cefprozil 500 mg b.i.d. for eight days. Analysis of the faecal microflora showed a limited ecological impact of cefprozil on the intestinal microflora, such as a moderate decrease in enterobacteria and a slight increase in enterococci, staphylococci and *Bacteroides* during the study. *C. difficile* strains were detected in three volunteers but no toxins could be found. Four days after the administration period the number of all bacteria was normalised (Table 3).

Table 3: Impact of perorally administered cephalosporins on the intestinal microflora

Agent	Dose (mg/day)	Days of adminis-tration	Number of patients	Impact on:			Overgrowth of resistant strains			Reference
				Entero-bacteria	Aerobic Gr^+ cocci	Anaerobic bacteria	Entero-bacteria	*C. dif-ficile*	*Candida*	
Cefaclor	250x3	14	6	-	-	-	-	+	-	*Finegold* et al., 1987
	250x3	7	10	-	-	-	-	-	-	*Nord* et al., 1987
	250x3	7	40	-	↓	-	+	-	+	*Christensson* et al., 1991
Cefixime	400	14	6	↓	↑	↓	-	+	-	*Finegold* et al., 1987
	200x2	7	10	↓	↑	↓↓	-	+	-	*Nord* et al., 1988
Cefpodoxime-proxetil	200x2	7	10	↓↓	↓↑	↓	-	+	+	*Brismar* et al., 1993a
Cefprozil	500x2	8	8	↓	↑	-	-	-	-	*Lode* et al., 1992
Ceftibuten	400x1	10	14	↓	↑	-	-	+	+	*Brismar* et al., 1993b
Cefuroxime-Axetil	600x3	3	6	↓	↓	↓	-	-	+	*Wise* et al., 1984
	250x2	10	10	-	↑	-	-	+	+	*Edlund* et al., 1993
Cephradine	1000x2	7	6	-	-	-	-	-	-	*Brumfitt* et al., 1986
Loracarbef	200x2	7	20	-	-	-	-			*Nord* et al., 1991

↓↓: strong suppression, >4 log10 CFU/g faeces.
↓: mild to moderate suppression, 2-4 log10 CFU/g faeces.
↑: increase in number of microorganisms during therapy.
-: no significant change.

Ceftibuten

The effect of ceftibuten on the normal intestinal microflora was studied in fourteen healthy subjects given 400 mg ceftibuten tablets once daily for ten days (*Brismar* et al., 1993b). The numbers of *E. coli* and anaerobic cocci were partly reduced, while there was an overgrowth of enterococci during the administration period. Six volunteers were colonised by *C. difficile* during days 4 to 17. Beta-lactamase activity was detected in faecal samples from eight volunteers (Table 3).

Cefuroxime-axetil

The effect of cefuroxime-axetil on the intestinal microflora in six healthy subjects was studied by *Wise* et al. (1984). The volunteers were given 600 mg cefuroxime-axetil orally every eight hours for three days. The number of enterobacteria decreased in three volunteers who developed diarrhoea. The enterococci were also significantly suppressed and the numbers of *Candida* increased in two of these three volunteers. *Bacteroides*, peptococci, and peptostreptococci decreased significantly in three volunteers (Table 3).

Edlund et al (1993) studied the impact of cefuroxime-axetil on the normal intestinal microflora. Ten healthy volunteers were given 250 mg cefuroxime-axetil tablets b.i.d. for ten days. There was an overgrowth of enterococci and staphylococci while the levels of bifidobacteria and clostridia decreased during the administration period. The numbers of enterobacteria, eubacteria and *Bacteroides* were unaffected. Low cefuroxime concentrations in faeces corresponded to high beta-lactamase activities and minor alterations in the normal microflora, while high cefuroxime concentrations in faeces corresponded to low beta-lactamase activities and considerable ecological disturbances in the intestinal microflora (Table 3).

Cephradine

Brumfitt et al. (1986) studied the effects on the intestinal microflora in six volunteers receiving 1000 mg cephradine b.i.d. for seven days. Staphylococci were eliminated during administration while no other significant changes were observed (Table 3).

Locarbef

Nord et al. (1991) studied the effect of Loracarbef on the normal intestinal microflora. Twenty healthy volunteers received loracarbef capsules 200 mg bid for seven days. In the intestinal aerobic microflora the numbers of enterococci and streptococci increased slightly, while staphylococci, micrococci, corynebacteria, bacillus and enterobacteria were not affected. The numbers of bifidobacteria and eubacteria in the anaerobic microflora decreased, while no other bacterial groups were affected. One week after withdrawal of loracarbef, the intestinal microflora had returned to normal. No new colonising loracarbef resistant microorganisms were observed during the investigation period (Table 3).

Comments

Orally administered cephalosporins often resulted in minor decreases in the number of enterobacteria. However cefixime and high doses of cefuroxime-axetil led to alterations in the anaerobic microflora. Most orally administered cephalosporins were associated with an increase in the numbers of enterococci and colonisation with *C. difficile*.

IMPACT OF MONOBACTAMS AND CARBAPENEMS ON THE INTESTINAL MICROFLORA

Aztreonam

The impact of aztreonam on the colonic microflora in 20 patients undergoing colorectal surgery was studied by *Kager* et al. (1985). Aztreonam was given intravenously in a dose of 1000 mg at induction of anaesthesia, followed by subsequent doses of 1000 mg at 8 hours intervals over 48 hours. Enterobacteria were significantly suppressed during the antimicrobial prophylaxis period while enterococci proliferated. There was a significant increase of staphylococci in 10 patients. Three of these patients developed post-operative infections with staphylococci. In the anaerobic microflora only minor changes were observed. The microflora was normalised in all patients after two weeks (Table 4).

De Vries-Hospers et al. (1984) studied the effect on the intestinal microflora of orally administered aztreonam in 10 volunteers with three regimens: 60, 300, and 1500 mg daily for five days. The enterobacteria decreased significantly in all patients. Patients given the highest dose had increased numbers of aztreonam resistant enterococci and yeast. The anaerobic intestinal microflora was not significantly affected. The faecal concentrations increased with the dose of aztreonam and varied between 0.1 and 100 mg/kg faeces (Table 4).

Jones et al. (1984) studied the impact of aztreonam on the intestinal microflora of 18 patients with haematological malignancies. Nine patients received 1000 mg aztreonam t.i.d. and nine patients received 2000 mg aztreonam t.i.d. for seven to nine days. There was a significant decrease in the aerobic Gram-negative rods during the administration of aztreonam. The impact on anaerobic bacteria was variable. Most isolates of *Bacteroides* species persisted but all strains of *B. fragilis* were eliminated. Eight of 12 *Clostridium* strains disappeared in the patients receiving 2000 mg t.i.d. No *C. difficile* strains were isolated during the investigation period (Table 4).

Van der Waaij (1985) investigated the effect of oral administration of different dosages of aztreonam on the intestinal microflora in 10 volunteers. The aerobic Gram-negative flora was suppressed in most persons receiving 60 to 1500 mg aztreonam daily. The anaerobic microflora remained unchanged during and after treatment with doses of 60 to 300 mg/day while the highest daily dose of 1500 mg aztreonam caused a slight suppression of anaerobes in four volunteers (Table 4).

Imipenem

The impact of imipenem/cilastatin treatment on colon microflora was studied by *Nord* et al. (1984). Ten patients received 500 mg imipenem combined with 500 mg cilastatin q.i.d. by intravenous infusion for 6 to 11 days. The numbers of enterobacteria and enterococci decreased slightly during the treatment period. There was also a minor decrease in the numbers of anaerobic cocci and *Bacteroides* during the treatment period. *C. difficile* or its cytotoxin was not present in faecal samples during or after imipenem therapy and no patient developed diarrhoea. No colonisation with imipenem-resistant bacteria was observed during the investigation period (Table 4).

In a study by *Kager* et al. (1989) imipenem/cilastatin were given intravenously, as prophylaxis, to twenty patients undergoing colorectal surgery. Ten patients received a dose of 500/500 mg and 10 patients were given

Table 4: Impact of monobactams and carbapenems on the intestinal microflora

Agent	Dose (mg/day)	Days of adminis-tration	Number of patients	Impact on:			Overgrowth of resistant strains			Reference
				Entero-bacteria	Aerobic Gr+ cocci	Anaerobic bacteria	Entero-bacteria	*C. dif-ficile*	*Candida*	
Aztreonam	1000x3	2	20	↓	↑	-	-	+	-	*Kager* et al., 1985
	60	5	10	↓	-	-	-	-	-	*de Vries-Hospers* et al., 1984
	300	5	10	↓↓	-	-	-	-	-	*de Vries-Hospers* et al., 1984
	1500	5	10	↓↓	↑	-	-	-	+	*de Vries-Hospers* et al., 1984
	1000x3	7-9	9	↓↓	-	-	-	-	-	*Jones* et al., 1984
	2000x3	7-9	9	↓↓	-	↓	-	-	-	*Jones* et al., 1984
	20x3	5	10	↓	-	-	-	-	-	*van der Waaij* et al., 1985
	100x3	5	10	↓↓	-	-	-	-	-	*van der Waaij* et al., 1985
	500x3	5	10	↓↓	-	-	-	-	-	*van der Waaij* et al., 1985
Imipenem/ cilastatin	500/500x4	6-11	10	↓	↓	↓	-	-	-	*Nord* et al., 1984
	1000/1000x4	2	10	↓	-	↓↓	-	-	-	*Kager* et al., 1989
Meropenem	500x3	7	10	↓	↑	↓	-	-	-	*Bergan* et al., 1991

↓↓: strong suppression, >4 log10 CFU/g faeces.
↓: mild to moderate suppression, 2-4 log10 CFU/g faeces.
↑: increase in number of microorganisms during therapy.
-: no significant change.

1000/1000 mg of imipenem/cilastatin every six hour for 48 hours. The aerobic intestinal bacteria were suppressed significantly during the imipenem prophylaxis period. Among the anaerobic bacteria, cocci, bifidobacteria, eubacteria, lactobacilli, clostridia, fusobacteria, and *Bacteroides* decreased markedly during the same period. The microflora was normalised after two weeks. There were no differences between the patients receiving different dose regimens of imipenem. No postoperative infections occurred (Table 4).

Meropenem

Bergan et al. (1991) studied the effect of meropenem on the intestinal microflora of health volunteers. Ten subjects were given 500 mg meropenem by intravenous infusion over 30 min t.i.d. for seven days. The number of enterobacteria and streptococci decreased during the administration period, while the numbers of enterococci increased. There was a decrease in the number of clostridia, *Bacteroides* and Gram-negative cocci, while the numbers of Gram-positive cocci and rods were unchanged. The intestinal microflora returned to normal in all volunteers within two weeks after the termination of meropenem administration (Table 4).

Comments

Administration of aztreonam resulted in elimination or strong suppression of intestinal enterobacteria. Imipenem and meropenem caused moderate reductions in the numbers of both aerobic and anaerobic bacteria. None of these agents were associated with colonisation of *C. difficile*.

IMPACT OF MACROLIDES ON INTESTINAL MICROFLORA

Erythromycin

Heimdahl and *Nord* (1982) studied the effect of erythromycin on the intestinal microflora in healthy volunteers. Ten subjects received 500 mg erythromycin b.i.d. for seven days. The number of enterobacteria was significantly suppressed in all subjects and enterococci and streptococci were eliminated in three subjects. In the anaerobic colon microflora *Bacteroides* strains were eliminated in four subjects, fusobacteria in three subjects, and *Veillonella* in two subjects. New colonisation with erythromycin resistant enterobacteria, staphylococci, or yeast occurred in all patients. New resistant clostridial strains colonised the colonic microflora in three subjects (Table 5).

Clarithromycin

Brismar et al. (1991) compared the effects of clarithromycin and erythromycin on the normal intestinal microflora. Ten healthy volunteers received 250 mg of clarithromycin orally b.i.d. for seven days, and ten other volunteers received 1000 mg of erythromycin ethylsuccinate orally b.i.d. for seven days. In the clarithromycin group, the numbers of streptococci and enterobacteria decreased while in the erythromycin group streptococci, enterococci and enterobacteria decreased and staphylococci increased during antibiotic administration. The anaerobic intestinal microflora was also affected. The alterations were more pronounced in the volunteers receiving erythromycin than in those having clarithromycin (Table 5).

Dirithromycin

The impact of dirithromycin on the normal intestinal microflora was evalu-

Table 5: Impact of macrolides, tetracyclines, nitroimidazoles and clindamycin on the intestinal microflora

Agent	Dose (mg/day)	Days of adminis-tration	Number of patients	Impact on:			Overgrowth of resistant strains			Reference
				Entero-bacteria	Aerobic Gr^+ cocci	Anaerobic bacteria	Entero-bacteria	*C. difficile*	*Candida*	
Erythromycin	500x2	7	10	↓↓	↓	↓↓	+	-	+	*Heimdahl & Nord*, 1982
	1000x2	7	10	↓	↓↑	↓	+	-	+	*Brismar* et al., 1991
Clarithromycin	250x2	7	10	↓	↓	-	+	-	-	*Brismar* et al., 1991
Dirithromycin	500x1	7	20	↓↓	↑	↓	+	-	-	*Eckernäs* et al., 1991
Roxithromycin	150x2	5	6	↓	-	-	-	-	-	*Pecquet* et al., 1991
Tetracycline	250x4	8-10	15	-	-	-	+	-	+	*Bartlett* et al., 1975
Doxycycline	200+100x1*	8-10	15	-	↑	-	-	-	+	*Bartlett* et al., 1975
	100x1	7	10	↓	↓	-	+	-	-	*Heimdahl & Nord*, 1983
Metronidazole	400x3	5-7	10	-	-	-	-	-	-	*Nord*, 1990
Tinidazole	150x2	7	10	-	-	-	-	-	-	*Heimdahl* et al., 1980
	800+400x2**	2	20	-	↑	↓	-	-	-	*Kager* et al., 1981b
Clindamycin	150x4	7	10	-	↑	↓↓	-	+	-	*Heimdahl & Nord*, 1982
	600x3	3	15	-	↓↑	↓↓	-	+	-	*Kager* et al., 1981c

↓↓: strong suppression, >4 log10 CFU/g faeces.
↓: mild to moderate suppression, 2-4 log10 CFU/g faeces.
↑: increase in number of microorganisms during therapy.
-: no significant change.
*: 200 mg was given as a loading dose.
**: 800 mg was given as a loading dose.

ated by *Eckernäs* et al. (1991). Twenty healthy volunteers received 500 mg of dirithromycin orally once daily for seven days. The numbers of enterobacteria decreased significantly in the aerobic intestinal microflora, while streptococci and staphylococci increased. New colonising dirithromycin resistant enterobacteria were isolated during and after treatment. In the anaerobic microflora, the numbers of Gram-positive cocci, bifidobacteria, eubacteria and *Bacteroides* decreased, while the number of clostridia and lactobacilli increased (Table 5).

Roxithromycin

The ecological impact on the intestinal microflora of six volunteers when roxithromycin was given orally 150 mg twice every day for five days was studied by *Pecquet* et al. (1991). The faecal concentrations of active roxithromycin were in the range of 100 to 200 mg/kg faeces. The total number of enterobacteria decreased, while the rest of the aerobic and the anaerobic microflora was only affected to a minor degree. No overgrowth of roxithromycin resistant microorganisms was observed (Table 5).

Comments

Administration of macrolides resulted in suppression of both the aerobic and anaerobic intestinal microflora as well as in overgrowth of resistant microorganisms. Erythromycin and dirithromycin seemed to cause greater ecological alterations in the intestinal microflora compared to roxithromycin.

IMPACT OF TETRACYCLINES ON INTESTINAL MICROFLORA

Bartlett et al. (1975) compared the effect of tetracycline and doxycycline of the aerobic and anaerobic faecal flora in 30 healthy volunteers. Fifteen volunteers received 200 mg doxycycline the first day followed by 100 mg in a single daily dose for eight to 10 days. The other 15 subjects received tetracycline hydrochloride (250 mg q.i.d.) for eight to 10 days. Neither tetracycline hydrochloride nor doxycycline had a major impact on the total numbers of aerobic or anaerobic bacteria. Nine subjects acquired new aerobic strains during antibiotic administration. In the volunteers receiving tetracycline, enterococci, *Citrobacter freundii*, and *C. albicans* were recovered and, in those volunteers taking doxycycline, *S. aureus*, enterococci, and *C. albicans* were found. Patients receiving tetracycline hydrochloride had a mean increase of 10^4 resistant *E. coli* strains/g faeces compared to 10^1 resistant *E. coli* strains/g faeces in patients receiving doxycycline. This difference between the two tetracyclines was significant (Table 5).

The influence of doxycycline on the colon microflora was investigated by *Heimdahl* and *Nord* (1983). Ten volunteers received doxycycline orally in doses of 100 mg once daily for seven days. The number of enterococci and streptococci decreased 23 log cycles in eight volunteers and the number of enterobacteria also decreased 2-3 log cycles in five volunteers. Three subjects were colonised by new doxycycline resistant strains such as *K. pneumoniae, Proteus mirabilis,* and *E. cloacae.* Among the anaerobic bacteria, fusobacteria were eliminated during the administration period. A marked emergence of resistance to doxycycline among both aerobic and anaerobic bacteria in the colon microflora was observed (Table 5).

Comments

Administration of tetracycline and doxycycline caused no or only minor suppression of the normal intestinal microflora. However, both agents led to a major overgrowth of resistant aerobic and anaerobic microorganisms during the administration period.

IMPACT OF NITROIMIDAZOLES ON INTESTINAL MICROFLORA

Metronidazole

Metronidazole was given orally to 10 patients as tablets in a dose of 400 mg t.i.d. for 5-7 days (*Nord*, 1993). The aerobic microorganisms were only slightly affected during and after the treatment. Only minor changes in the anaerobic microflora occurred at the same period. The microflora was normalised in all patients after the treatment was terminated (Table 5).

Tinidazole

Heimdahl et al. (1980) investigated the effect of orally administered tinidazole on the intestinal microflora. Tinidazole was given in doses of 150 mg b.i.d. for seven days to 10 volunteers. No tinidazole was detected in the faecal samples and no changes in the colon microflora were noticed (Table 5).

Kager et al. (1981b) investigated the impact of tinidazole prophylaxis on the intestinal microflora in patients undergoing colorectal surgery. Tinidazole was given intravenously to 20 patients in an initial dose of 800 mg given at the induction of anaesthesia and then in doses of 400 mg every 12 h for two days. Staphylococci and enterococci proliferated during the tinidazole prophylaxis period while anaerobic bacteria decreased significantly. No bacterial strains resistant to tinidazole were recovered (Table 5).

Comments

Orally administered metronidazole and tinidazole did not cause any significant alterations in the intestinal microflora while parenterally administered tinidazole reduced the number of anaerobic bacteria and caused overgrowth of staphylococci and enterococci.

IMPACT OF CLINDAMYCIN ON INTESTINAL MICROFLORA

Heimdahl and *Nord* (1982) investigated the effect of orally administered clindamycin on the intestinal microflora. Clindamycin capsules (150 mg) were given to 10 subjects q.i.d. for seven days. Pronounced changes in the aerobic and anaerobic intestinal microflora occurred. Among the aerobes clindamycin-resistant enterococci proliferated and among the anaerobes the number of cocci and Gram-negative rods significantly decreased. In four volunteers, clindamycin-resistant clostridia were recovered and one of the volunteers developed *C. difficile*-associated diarrhoea (Table 5).

Kager et al. (1981c) studied the effect of clindamycin prophylaxis on the colon microflora in 15 patients undergoing colorectal surgery. An initial dose of 600 mg clindamycin was given as a short-term infusion during the induction of anaesthesia followed by six subsequent doses of 600 mg at eight hours intervals. Enterococci and streptococci decreased postoperatively during the first two days and then proliferated during the following three days. In the anaerobic colon flora anaerobic cocci, Gram-positive rods, and Gram-negative rods decreased significantly. The aerobic and anaerobic colon microflora was

normalised in most patients after two weeks (Table 5).

Comments

The numbers of anaerobic intestinal microorganisms were strongly suppressed during administration of clindamycin. Resistant clostridia and enterococci were frequently isolated.

IMPACT OF QUINOLONES ON INTESTINAL MICROFLORA

Ciprofloxacin

The effect of ciprofloxacin on the intestinal microflora were tested in 12 male healthy subjects taking 500 mg of ciprofloxacin orally bid for seven days by *Brumfitt* et al. (1984) In the aerobic colon microflora enterobacteria were eliminated on day seven, and the numbers of streptococci and staphylococci were significantly reduced. Anaerobic bacteria were little affected quantitatively but acquired resistance to ciprofloxacin. One week later the colonic microflora had returned to a state similar to that found before treatment (Table 6).

In another investigation ciprofloxacin was given in a dose of 500 mg b.i.d. to 15 patients with acute leukaemia during remission induction treatment for a mean duration of 42 days (*Rozenberg-Arska* et al., 1985). Enterobacteria were eliminated within three to five days. *Bacteroides* and *Clostridium* species were not affected, but the numbers of anaerobic non-sporeforming Gram-positive rods and anaerobic cocci were decreased. Nine ciprofloxacin-resistant *Pseudomonas* and *Acinetobacter* species were recovered but without colonisation or subsequent infection. Four of the five infections in the patients were caused by Gram-positive cocci (Table 6).

The colonic microflora in 12 volunteers receiving 400 mg ciprofloxacin orally b.i.d. for seven days were studied by *Enzensberger* et al. (1985). *E. coli* was eliminated in all volunteers after two days of treatment. No selection of resistant enterobacteria could be observed. Anaerobic bacteria were not significantly affected and there was no selection of *C. difficile* strains (Table 6).

The pharmacokinetics of ciprofloxacin and the effect of repeated dosages on the colon microflora in volunteers were investigated by *Bergan* et al. (1986). Twelve volunteers received 500 mg ciprofloxacin tablets b.i.d. for five days. The numbers of enterobacteria and enterococci decreased markedly, whereas the changes in the anaerobic microflora were minor. The colon microflora became normalised within fourteen days after the drug was discontinued. No new colonisation of ciprofloxacin-resistant bacteria was observed (Table 6).

The impact of ciprofloxacin on the intestinal microflora with regard to colonisation resistance was investigated by *van Saene* et al. (1986). Twelve volunteers received 50 mg ciprofloxacin q.i.d. for six days. In all volunteers enterobacteria were eliminated from faeces while the number of enterococci were slightly affected. A minor increase of *Candida* spp. was noticed. No new ciprofloxacin resistant bacteria were recovered. One week after treatment the flora had returned to normal (Table 6).

Holt et al. (1986) studied the effect of ciprofloxacin on the faecal microflora of six volunteers. The volunteers received 500 mg ciprofloxacin daily for five days. There was a marked reduction of enterobacteria in all volunteers during the administration period. Two volunteers were colonised by resistant coagulase-negative staphylococci or corynebacteria. The total counts of an-

Table 6: Impact of quinolones on the intestinal microflora

Agent	Dose (mg/day)	Days of adminis-tration	Number of patients	Impact on:			Overgrowth of resistant strains			Reference
				Entero-bacteria	Aerobic Gr^+ cocci	Anaerobic bacteria	Entero-bacteria	*C. dif-ficile*	*Candida*	
Ciprofloxain	500x2	7	12	↓↓	↓	-	-	-	-	*Brumfitt* et al., 1984
	500x2	mean 42	15	↓↓	-	↓	+	-	-	*Rozenberg-Arska* et al., 1985
	400x2	7	12	↓↓	-	-	-	-	-	*Enzenberger* et al., 1985
	500x2	5	12	↓↓	↓	↓	-	-	-	*Bergan* et al., 1986
	50x4	6	12	↓↓	↓	-	-	-	+	*van Saene* et al., 1986
	500x1	5	6	↓↓	-	-	-	-	-	*Holt* ete al., 1986
	250x2	5-10	7	↓↓	-	↓	-	-	-	*Esposito* et al., 1987
	500x1	5-10	7	↓↓	-	↓	-	-	-	*Esposito* et al., 1987
	500x2	5	14	↓↓	↓	↓	-	-	-	*Ljungberg* et al., 1990
	750x2+400x2	2	21	↓↓	↓	↓	-	-	-	*Brismar* et al., 1990
	250x2	3	17	↓↓	-	-	-	-	-	*Wiström* et al., 1992
Enoxacin	400x2	7	10	↓↓	-	-	-	-	+	*Edlund* et al., 1987a
Norfoxacin	200x1	7	10	↓↓	-	-	-	-	-	*Meckenstock* et al., 1985
	400x2	7	10	↓↓	-	-	-	-	-	*Meckenstock* et al., 1985
	100x2	5	10	↓↓	-	-	-	-	-	*de Vries-Hospers* et al., 1985
	200x2	5	10	↓↓	-	-	-	-	-	*de Vries-Hospers* et al., 1985
	400x2	5	10	↓↓	↓	-	-	-	-	*de Vries-Hospers* et al., 1985
	400x2	8	10	↓↓	-	-	-	-	-	*Leigh* et al., 1985
	200x2	5	6	↓↓	-	-	-	-	-	*Pecquet* et al., 1986
	400x2	5	6	↓↓	↓	-	-	-	-	*Pecquet* et al., 1986
	200x2	7	10	↓↓	-	-	-	-	-	*Edlund* et al., 1988
Ofloxacin	200x2	5	5	↓↓	↓	-	-	-	+	*Pecquet* et al., 1987
	400x1	1	24	↓↓	↓	↓	-	-	-	*Edlund* et al., 1988
Pefloxacin	400x2	7	15	↓↓	-	-	-	-	-	*van Saene* et al., 1986
Lomefloxacin	400x1	7	10	↓↓	-	-	-	-	-	*Edlund* et al., 1990

↓↓: strong suppression, >4 log10 CFU/g faeces.
↓: mild to moderate suppression, 2-4 log10 CFU/g faeces.
↑: increase in number of microorganisms during therapy.
-: no significant change.

aerobic bacteria were almost unaffected during the administration period (Table 6).

Esposito et al. (1987) studied the alterations in the intestinal microflora of 14 patients with liver cirrhosis by ciprofloxacin therapy for intercurrent urinary tract infections or respiratory tract infections. The patients received 250 mg twice daily or 500 mg once daily. A marked decrease in enterobacteria was noticed with both doses. From day three to six of therapy enterobacteria disappeared completely and returned to normal levels two weeks after termination of treatment. No changes in the aerobic Gram-positive microflora or the anaerobic microflora were noticed. Two patients were colonised by *C. albicans* during therapy (Table 6).

The effect of ciprofloxacin on the intestinal microflora in young and elderly volunteers were studied by *Ljungberg* et al. (1990). Seven young and seven elderly, healthy volunteers received 500 mg ciprofloxacin b.i.d. for five days. The number of enterococci, streptococci, staphylococci, and enterobacteria decreased markedly in both age groups. The effects on the anaerobic bacteria were less pronounced. Despite larger absolute bio-availability of the first dose in the elderly (77% vs. 63%; $p<0.05$), the effect of ciprofloxacin on the microflora was similar in the two groups of volunteers (Table 6).

Brismar et al. (1990) investigated the effect of ciprofloxacin on the colonic microflora in patients undergoing colorectal surgery. Ciprofloxacin was given orally in two doses of 750 mg each with a 12-h interval starting 24 h prior to surgery, 400 mg of ciprofloxacin was given intravenously at the induction of anaesthesia, and 400 mg of ciprofloxacin was given 12 h later to 21 patients undergoing elective colorectal surgery. During the ciprofloxacin administration period, the numbers of streptococci, enterococci, and enterobacteria decreased markedly. In the anaerobic microflora both Gram-positive and Gram-negative bacteria were suppressed during the first three days. No postoperative infections occurred (Table 6).

The ecological effects of three days ciprofloxacin treatment (250 mg b.i.d.) of travellers' diarrhoea in 17 patients travelling to Mexico were studied by *Wiström* et al. (1992). A significant suppression of enterobacteria was observed and a minor increase in the numbers of anaerobic cocci and bifidobacteria was found two to three days after treatment, compared with placebo treated and asymptomatic travellers. The mean time to cure was 26 h for ciprofloxacin and 60 h for placebo-treated patients ($p=0.03$) (Table 6).

Enoxacin

Edlund et al. (1987a) studied the effect of enoxacin on the intestinal microflora of ten healthy volunteers. The subjects received 400 mg enoxacin orally b.i.d. for seven days. Enterobacteria was strongly suppressed in numbers during the enoxacin administration, while enterococci, streptococci, staphylococci, micrococci, and *Bacillus* spp. were not significantly affected. Low numbers of yeast, mostly *C. albicans*, were detected during the administration period. The anaerobic flora was only slightly affected by the administration of enoxacin. No emergence of resistance was noticed during the investigation period. The intestinal microflora became normal within two weeks after withdrawal of enoxacin. The mean concentration of enoxacin on day seven was 348 mg/kg faeces (Table 6).

Norfloxacin

Meckenstock et al. (1985) investigated the effect of norfloxacin on the faecal flora of ten healthy volunteers.

The volunteers were given 200 mg once daily or 400 mg b.i.d. for seven days with an appropriate interval between the two treatment periods. The Gram-negative aerobic microflora was eliminated by the higher dose and strongly suppressed by the lower dose, while enterococci and anaerobic bacteria were not markedly affected (Table 6).

De Vries-Hospers et al. (1985) evaluated selective decontamination of the intestinal microflora by administration of norfloxacin. Ten healthy volunteers received three different dosages of norfloxacin, 100 mg, 200 mg and 400 mg b.i.d., for five days. Aerobic Gram-negative rods were eliminated from the faecal samples with all the three dosages tested. Enterococci tended to decrease during the administration period. No major changes in the anaerobic microflora were seen (Table 6).

The pharmacokinetics of norfloxacin and the effect on the faecal flora was studied by *Leigh* et al. (1985). Ten healthy volunteers were given 400 mg twice daily for a total of 15 doses. Gram-negative aerobic bacteria were eliminated but there was no effect on the anaerobic bacteria. Replacement with Gram-positive organisms was seen frequently but re-establishment of the normal faecal flora was found 14 days after treatment had stopped. No resistant strains of Gram-negative aerobic bacteria were detected (Table 6).

Pecquet et al. (1986) studied selective decontamination of the digestive tract by norfloxacin. Twelve human volunteers were treated with 400 mg or 800 mg of oral norfloxacin daily for five days. Enterobacteria were eliminated while streptococci were partly suppressed. The anaerobic intestinal microflora was not affected by administration of norfloxacin (Table 6).

Edlund et al. (1987b) studied the impact of norfloxacin on the intestinal microflora and its multiple-dose pharmacokinetics. Ten healthy volunteers were given 200 mg norfloxacin orally b.i.d. for seven days. The number of enterobacteria was strongly depressed while only minor changes in the aerobic Gram-positive flora were observed. The anaerobic colonic flora was not significantly affected (Table 6).

Ofloxacin

The impact of ofloxacin on the intestinal microflora in human volunteers was investigated by *Pecquet* et al. (1987). Five volunteers were given 400 mg ofloxacin daily for five days. Enterobacteria were eliminated in faeces four days after the treatment had started. Six days after the end of ofloxacin administration, the enterobacteria had not yet returned to pre-treatment levels. Enterococci decreased significantly during ofloxacin treatment, but increased again to pre-treatment numbers within four days after the end of treatment. All five volunteers were colonised by low numbers of *Candida* spp. after four days of treatment. The number of anaerobic bacteria were not significantly affected (Table 6).

Edlund et al. (1988) evaluated the effect of ofloxacin on the intestinal microflora in 24 patients undergoing gastric surgery. A single oral dose of 400 mg ofloxacin was given to each patient two to four hours before surgery. Enterobacteria were eliminated in 12 patients and strongly suppressed in eight patients. The numbers of enterococci, lactobacilli, bifidobacteria, eubacteria, *Veillonella* and *Bacteroides* were also suppressed. Anaerobic cocci and clostridia remained unaffected during the investigation period. The intestinal microflora returned to normal four weeks after the administration of ofloxacin (Table 6).

Pefloxacin

The effect of pefloxacin on the intes-

tinal flora in human volunteers with regard to colonisation resistance was studied by *van Saene* et al. (1986). Fifteen healthy volunteers received 400 mg pefloxacin tablets bid for seven days. Enterobacteria were eliminated in all subjects three days after the first dose. Recolonisation with enterobacteria was seen one week after the end of administration. *E. faecalis* decreased slightly in numbers while *Candida* spp. did not change during the observation period. The anaerobic microflora was not affected by pefloxacin administration (Table 6).

The influence of pefloxacin, 400 mg b.i.d. for ten days on microbial colonisation resistance in six health volunteers was investigated by *Vollaard* et al. (1992). There was an elimination or strong reduction in the numbers of enterobacteria during the administration period while enterococci decreased slightly in numbers. In three volunteers impairment of colonisation resistance was indicated by a significant increase in the faecal concentration of yeasts (Table 6).

Lomefloxacin

The influence of lomefloxacin on the intestinal microflora was studied by *Edlund* et al. (1990). Ten volunteers were given 400 mg lomefloxacin orally once daily for 7 days. The numbers of enterobacteria were strongly reduced or eliminated on days 2-9, while the aerobic Gram-positive microflora did not alter in number during the investigation period. In the anaerobic intestinal microflora only minor changes were seen. The intestinal microflora was normalised two weeks after administration of lomefloxacin had stopped (Table 6).

Comments

Administration of quinolones resulted in elimination or strong suppression of intestinal enterobacteria. Ciprofloxacin and ofloxacin also affected enterococci and anaerobic microorganisms to a minor degree. The quinolones did not induce overgrowth of resistant bacteria or yeasts.

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THE INTERACTION OF THE MUCOSAL IMMUNE SYSTEM OF THE GALT WITH INDIGENOUS BACTERIA AND ENTERIC VIRUSES

JOHN J. CEBRA[1,2], NICOLAAS A. BOS[2], ETHEL R. CEBRA[1,2], DAVID R. KRAMER[1], FRANS G. M. KROESE[2], KHUSHROO E. SHROFF[1], and ROBERTA D. SHAHIN[1]

[1]Department of Biology, University of Pennsylvania, Philadelphia PA, USA, and
[2]Department of Histology and Cell Biology, Immunology Section, University of Groningen, Groningen, The Netherlands

SUMMARY

Our observations of gnotobiotic and antigen-free adult mice and of conventionally-reared neonatal mice following their colonisation with microbial flora or infection with enteric viruses suggest that these latter mucosal antigenic stimuli play a major role in the development and maintenance of the normal elements of the mucosal immune system in Peyer's patches, intestinal lamina propria, and the intra-epithelial leukocyte compartment. We have developed a number of novel assay procedures to evaluate a gut mucosal immune response including: (1) detection of germinal centre reactions in Peyer's patches by fluorescence-activated cell sorting; (2) tissue fragment cultures for Peyer's patches and small intestine to detect secreted specific and total antibodies; (3) single or clonal B cell microcultures to assess frequencies and isotype potential of specific B cells; and (4) detection of endogenous coating of gut bacteria by IgA antibodies by the host. Using these methods we have shown that initial stimulation of the gut mucosal immune system results in transient germinal centre reactions in Peyer's patches and sufficient secretory IgA antibody to shield or attenuate subsequent or continued gut immune responses. Maternal antibodies, passively acquired by suckling, can both protect neonates and forestall their active mucosal responses to both intestinal commensal microbes and to pathogenic enteric viruses. It appears that acute, novel enteric stimulation - for instance with enteric viruses or cholera toxin - can overcome the hypo-responsiveness to naturally occurring 'bystander' antigens and reactivate mucosal immune responses to them. Finally, although the B1 B cell subset, including specificities cross-reactive with bacterial antigens, seems an attractive complement to the B2 B cells, acutely primed in the Peyer's patches, in populating the gut lamina propria with IgA plasma cells, we have failed to demonstrate a significant contribution for these B1 cells in physiologically normal, immunocompetent mice.

INTRODUCTION

Some years ago our laboratory implicated clusters of lymphoid follicles (Peyer's patches, PP) found in the small intestinal mucosa as major sites for the

development of IgA-committed pre-plasmablasts and for priming of a subsequent IgA 'memory' response (*Craig* and *Cebra*, 1971; *Gearhart* and *Cebra*, 1979; *Fuhrman* and *Cebra*, 1981; *Lebman* et al., 1987). Following effective gut mucosal stimulation by antigens there is an efflux of specific IgA plasmablasts from PP into the efferent lymph and circulation, followed by an accumulation of these cells in exocrine gland interstitia and the lamina propria (LP) of the respiratory and gastrointestinal tracts (*Pierce* and *Gowans*, 1975; *McWilliams* et al., 1975). We still do not know whether the selective lodging of IgA plasmablasts in secretory tissue exhibits any finer preference, for instance a biased accumulation of specific cells in respiratory LP vs. gastro-intestinal LP following exposure of the upper respiratory tract to antigens or vice versa (*Pierce* and *Cray*, 1981). Further, although vascular addressins on the luminal surface of high endothelial venules (HEVs) and corresponding 'homing' receptors on lymphocytes that could account for selective egress of cells from the circulation into LP have been identified, we do not know whether IgA-plasmablasts utilise this recognition system (*Phillips-Quagliata*, 1992). Even if they do, the further bases for their *accumulation* in secretory sites also remains unknown although lymphokines (LKs) abundant at these sites (IL-5, IL-6) may both stimulate their maturation to plasma cells and halt their migration (*Tagochi* et al., 1990).

Together with the development of specific IgA pre-plasmablasts in PP following effective antigenic stimulation of the gut mucosa with cholera toxin there is also a rise in the frequency of specific IgA memory cells (*Fuhrman* and *Cebra*, 1981). Although the dissemination of IgA memory cells to distal lymphoid tissues becomes evident following gut mucosal priming (*Fuhrman* and *Cebra*, 1981), the frequency of these cells remains highest in gut-associated lymphoid tissue (GALT) relative to other sites for long periods thereafter (*Cebra* et al., 1984). The likelihood and magnitude of subsequent secondary mucosal IgA responses positively correlates with the frequency of IgA memory cells in PP following exposure of the gut to antigen (*Fuhrman* and *Cebra*, 1981). We have previously operationally defined IgA-memory cells as those B cells which responded to specific antigens and TH cells in splenic fragment cultures to generate a clone that exclusively secretes IgA antibodies (*Lebman* et al., 1987). These clonal precursor cells from PP are small, non-dividing B cells which are $sIgA^{+}$, $sIgD^{-}$, and $sIgM^{-}$ and *do not* express the characteristic marker of murine germinal centre (GC) B cells, i.e., they do not bind high levels of the lectin, peanut agglutinin and thus they are PNA^{low}.

The sites of generation of IgA memory cells and of IgA pre-plasmablasts within PP have not been unequivocally defined but the chronically present GC are likely locations. There are more $sIgA^{+}$ B cells in the GC of PP than at any site in any other lymphoid tissue (*Lebman* et al, 1987). Many of these $sIgA^{+}$ PNA^{high} B cells are dividing (*Lebman* et al, 1987) and most, if not all, have lost at least one copy of their $C\mu$ and $C\gamma l$-genes, indicating that they have undergone irreversible switch-recombination (*Weinstein* et al., 1991). Recently, we have shown that GC reactions can be stimulated *de novo* in formerly germfree (GF) mice using oral reovirus infection (*Weinstein* and *Cebra*, 1991). These GC are transient and wax and wane with a time course similar to that observed in draining lymph nodes (LN) after local, parenteral reovirus infection. However, the outcome in terms of Ig isotype-switching is very different at the two sites: There is

Table 1: Isotype patterns expressed by clones from anti-inulin specific B cells taken from formerly germfree mice varying times after colonisation

	Time after Colonisation	Bacterial colonisers	# clones	Clones making (%): A-only	some M	M-only
Sp1[2]	1 week	lactobacilli	10	0	100	70
PP	1 week	"	4	0	100	50
Sp1	1 week	lactobacilli	24	0	92	54
PP	2 week	"	5	20	40	0
Sp1	3 week	lactobacilli	44	0	100	9
PP	3 week	"	18	5	89	0
Sp1	3-18 week	Sch./Convent.[1]	45	29	18	0
MLN	"	"	25	36	20	4
PP	"	"	19	42	11	0
Sp1	52 week	Sch./Convent.[1]	47	9	49	0
MLN	"	"	40	20	55	5
PP	"	"	17	47	12	0

[1] Mice were colonised with Schaedler's commensal bacteria and then conventionalised in a non-SPF animal facility.
[2] Spl = spleen; PP = Peyer's patches; MLN = mesenteric lymph nodes.

prompt expression of Cα germ-line (GL) transcripts in GC of PP, followed by expression of $sIgA^+$ B cells and the potential to secrete IgA in culture while LN GC B cells fail to exhibit this preferential switching to IgA expression but rather develop the potential to express IgG isotypes. These findings favour a micro-environmental difference between GALT and peripheral LN sites as accounting for the preferred switching to IgA rather than a difference in physiologic state due to the usual chronic antigenic stimulation of the gut mucosa (*Weinstein* and *Cebra*, 1991). Thus, our system may permit analysis of those micro-environmental factors (DCs, APC, T_H cells, CKs, such as TGFβ, IL-10, etc.) that may favour preferred switch-recombination to IgA expression.

Mice reared under conventional conditions (CNV) harbour an intestinal flora (*Dubos* et al., 1965) and display chronic GC reactions in their PP (*Butcher* et al., 1982). Thus, PP are unlike other lymphoid tissues, which ordinarily contain quiescent B cell follicles without GCs. Analysis of PP from CNV adult mice indicates a frequency of antigenic-specific clonal B cell precursors similar to that found in other lymphoid tissues except that - for some specificities - a much higher proportion are already IgA memory cells. The specificities of precursors accounting for the 'naturally occurring' IgA memory cells include: Anti-phosphocholine (PC), anti-β2→1 fructosyl (Inulin, In), anti-β-galactosyl (β-Gal), etc. (*Potter*, 1971). In fact, most of the identifiable specificities are reactive with bacterial antigens (see *Cebra* et al., 1980). If one examines gnotobiotic mice (germfree, GF) or antigen-free (AF), (*Bos* et al., 1993), one finds for both that PP are vestigial, that their clonal B cell precursors against bacterial determinants are

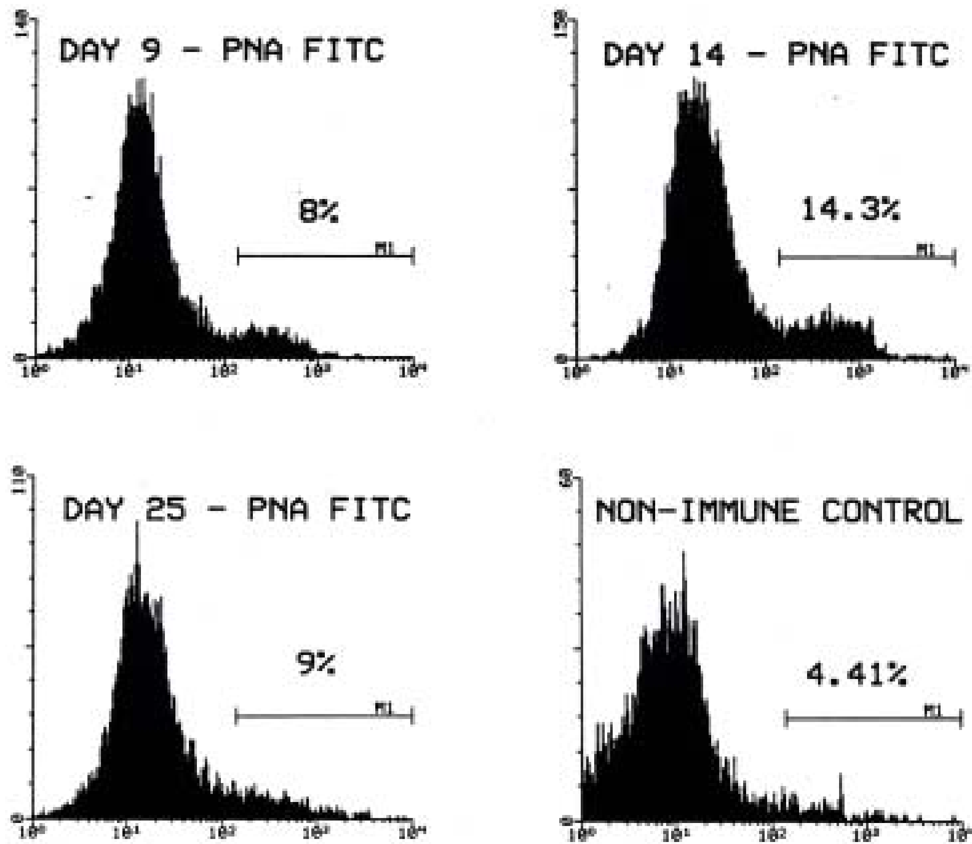

Figure 1: Development of germinal centre reactions in germfree C3H mice colonised with *Morganella morganii*.
(The waxing and waning of the germinal centre reactions in formerly germfree mice after mono-association with *Morganella morganii* by the oral route. Peyer's patch cell suspensions were stained with fluorescein-labelled peanut agglutinin, which preferentially binds to germinal centre cells compared with other cells of the B lineage. Except for the non-colonised control mice, all others harbour high densities of intestinal *M. morganii*).

of much lower frequencies than in CNV mice and few, if any, of these are committed to IgA expression (*Cebra* et al, 1980, 1986).

The AF mice not only lack discernible PP structures, but also show an almost complete lack of IgA and IgM plasma cells in their intestinal lamina propria (*Bos* et al., 1993). These findings suggest a role for the intestinal microflora in the normal development of the humoral mucosal immune system.

Our early studies with GF mice, colonised with the Schaedler 'cocktail' of indigenous bacteria, mostly facultative anaerobes (*Schaedler* et al., 1965), or mono-associated with *Morganella morganii* (*M. morganii*), an occasional commensal of mice (*Potter*, 1971), indicated that:

1. A rise in frequency of B cell clonal precursors occurred both in PP and elsewhere in the first few weeks after colonisation and these were specific for bacterial antigenic determinants;
2. At first most of these precursors were able to generate clones making IgM antibodies and then IgM plus

Table 2: Frequency of anti-inulin and anti-PC clonal precursors in spleens of neonates primed at birth

Donor	# cells analysed ($x10^{-6}$)	Frequency/10^6 cells to: In	PC
3 wks unprimed	1344	1.49	8.88
3 wks In-Hy primed[1]	460	7.07	10.10
1 wk unprimed	80	<0.3	n.d.
1 wk In-Hy primed[1]	80	9.38	n.d.
5 wks unprimed	400	8.91	15.30

[1] 3 day old neonatal mice were primed by intraperitoneal injection of 100 µg of inulin-conjugated to haemocyanin (In-Hy). The clonal precursor frequencies were determined using the splenic fragment assay and Hy-primed recipients and antigenic challenge with either In-Hy or phosphocholine-conjugated Hy (PC-Hy).

other isotypes; eventually precursors appeared with the characteristics of IgA-memory cells, i.e., they generated clones that exclusively expressed IgA antibodies. The latter were always in highest proportion in PP.

Figure 1 summarises some of these studies.

One might consider that neonatal mice exhibit changes in their gut mucosal immune system similar to those observed upon colonising GF mice with enteric bacteria. Neonatal mice are colonised at birth by contamination from their dams. However, they receive maternal IgG antibodies perinatally, first by transplacental passage and later, after birth by transport of suckled IgG antibodies across gut enterocytes. They also accumulate maternal IgA antibodies from milk in their gut lumen. These processes raise questions about whether:

1. Neonates are developmentally competent to mount an active, preferential IgA response in their PPs, and
2. Whether perinatally acquired maternal antibodies can interfere with or modulate this response.

Some years ago we analysed the potential of PP cells from neonatal mice born of CNV parents. The general finding was that the 'spontaneous' development of IgA memory cells specific for antigenic determinants associated with indigenous flora was delayed until 10-12 weeks post partum (*Cebra* et al., 1986; *Shahin* and *Cebra*, 1981). However, in the case of one particular bacterial determinant, $\beta 2 \rightarrow 1$ fructosyl (In), we showed that neonates could be primed by parenteral immunisation with In conjugated to either lipopolysaccharide (In-LPS) or haemocyanin (In-Hy) to exhibit a 'premature' rise of anti-In specific B cells in spleen (*Shahin* and *Cebra*, 1981; see Table 2). Thus, delayed mucosal responsiveness is not due to the absence or paucity of In-reactive B cells. However, these observations still leave unanswered whether the microenvironment of neonatal PPs is underdeveloped in its ability to respond to antigens delivered by the mucosal route or whether maternal antibodies acquired in the milk act to shield the neonatal gut from antigenic stimulation.

Finally, in the past few years, an extra-GALT source of IgA plasma cells that can populate intestinal LP has been proposed (*Kroese* et al., 1989). This is the $CD5^+$ B cell population (B1 cells) that develops perinatally in the liver and

persists throughout life in the peritoneal cavity of some species as an apparently self-renewing population (*Hayakawa* and *Hardy*, 1988). These B1 cells may be stimulated by 'internal' or 'self' antigens and may provide a measure of 'natural' immunity vs. cross-reactive bacterial determinants via their substantial contribution to circulating IgM (*Forster* and *Rajewsky*, 1987; *Lalor* and *Morahan*, 1990; *Riggs* et al., 1990). The actual, physiologic role in mucosal immunity of this potential source of IgA plasma cells, especially in immunocompetent neonatal and adult animals, has yet to be determined and quantified. Further, the possible stimulation of these B1 cells by *exogenous* antigens impinging on the gut mucosa has yet to be evaluated, although preliminary data suggest that cells of this lineage cannot generate GC reactions and therefore may not benefit from the GC microenvironment that facilitates isotype switching and affinity maturation processes (*Linton* et al., 1992).

Special techniques and assays developed to analyse the Rut mucosal immune response

1: *Analysis of the GC reaction in PP of formerly GF mice by fluorescence-activated cell sorting (FACS).*

Our approach was to develop acute, *de novo* GC reactions in PP of GF mice orally infected with reovirus serotype 1 (*Weinstein* and *Cebra*, 1991). Ordinarily, the PP follicles of GF mice do not contain detectable GCs. If the virus-infected mice are kept in isolators under otherwise GF conditions the GC reactions in PP wax and wane while the intestinal virus infection is completely resolved. The GC reactions and antibody expression by B cells from PP of these mice were compared with those from LNs of conventionally reared syngeneic mice inoculated in footpads with infectious virus. GC reactions were initially detected by FACS for the appearance of B cells, which bound high levels of the lectin, peanut agglutinin (PNA^{high}). The PNA^{high} marker has been found to distinguish most GC B cells from others and, in PP of mice, the prevalent phenotype of these is PNA^{high} surface kappa low (SK^{low}). Following acute local antigen stimulation with infectious reovirus, GC B cells appear first at day 6, reach maximum numbers by day 10-12, and decline during the day 14-21 period following infection at both PP and LN sites. The earliest and most persistent GC cells display the phenotype PNA^{high} $s\kappa^{high}$ at both sites, while cells with the PNA^{high} $s\kappa^{low}$ phenotype are only prevalent around the time of maximal GC reaction. While the time courses of the acute GC reaction are similar at the PP and LN sites, the two conspicuously differ in several respects: (1) B blasts appear with the phenotype $sIgA^{+}$ by day 6 and persist through day 19 in PP but not LN and most of these are PNA^{high} $sIgA^{+}$, while a very small component of $sIgG1^{+}$ cells can be detected in LN but not in PP; (2) upon secondary local re-infection with reovirus the GC reactions in PPs and LNs exhibit similar time courses but in the LN they are exaggerated while in the PP they are attenuated compared with the primary reactions; and (3) more $sIgA^{+}$ cells which are PNA^{low} appear in PPs and many more $sIgG^{+}$ cells appear in LNs after re-infection but not *vice versa*. We have found an excellent quantitative correlation between the proportion of PNA^{high} B cells or the appearance of $sIgA^{+}$ or $sIgG1^{+}$ B cells detected by FACS analysis and the magnitude (number and size) of GC reactions or the presence of cells bearing non-IgM/IgD isotypes observed microscopically after immunohistochemical staining of tissue sections.

2: *Tissue fragment cultures to evaluate the immune status of the small intestine.*

Heretofore, humoral mucosal immune responses in animal models have been evaluated by determining antibody titres in secretions (milk, saliva, tears, tracheal lavage, intestinal washes, etc.) or, much less commonly, by enumerating antigen-binding, IgA plasma cells in sections or cell suspensions of intestinal LP. These are cumbersome and tedious procedures and the former suffers from problems of uncertain dilution, need for internal standards, and the possibility of enzymatic degradation of antibodies. We have developed a simple PP fragment culture and a complementary (small intestinal) lamina propria fragment culture which we believe accurately reflect the immune status of the small intestine at the time the tissue is sampled with respect to displaying a humoral mucosal immune response (*Logan* et al., 1991; *Kramer* and *Cebra*, 1992; *Shroff* and *Cebra*, 1993). At varying times following oral (or intraduodenal) exposure to antigens, animals are sacrificed, their PP are dissected from small intestine, fragments of these tissues are extensively washed in antibiotic containing solution and these are cultured at high O_2 for up to 10 days (*Logan* et al., 1991). Net increase in specific IgA antibodies from day 1 to day 7-10 culture fluids attest to specific mucosal responses and the continued viability of cultures. Total Ig and IgA output of these cultures provides an internal standard against which the specific responses may be normalised. Generally, LP cultures exhibit a 2-3 day lag over PP cultures in the time course of specific IgA antibody responses (*Kramer* and *Cebra*, 1992).

3: *A dispersed B cell microculture which scores and distinguishes antiRen-specific IRA memory cells and IRA-preplasmablasts based on their secretion of IRA antibody in single cell or clonal cultures.*

One of our major objectives has been to establish single B cell clonal microcultures that would enable assay of the functional potential of subsets of B cells to produce IgA. Heretofore, only the *ex vivo* splenic fragment culture has permitted the successful clonal outgrowth of IgA memory cells and this type of culture has not allowed definition of cellular interactions and CK requirements for the expression of IgA. In addition, no *in vitro* cultures heretofore available have supported the expression of IgA secretion by preplasmablasts from GCs.

Our original T/B microculture was based on clonal culturing of B cells responsive to thymus-independent antigens (*Schweitzer* and *Cebra*, 1988), as practised by the Nossal laboratory using antigen-specific B cells enriched by panning on haptenated gelatine (*Nossal* and *Pike*, 1978), except that we used cloned, antigen (conalbumin)-specific D10.G.4.1 T_H2 cells and haptenated antigen as stimuli (*Schrader* et al., 1990). Small numbers (10-20) of enriched, antigen-specific B cells give antibody secreting clones when placed in microcultures (10 μL) with non-limiting numbers of T_H2 cells (1500-3000) and their specific antigen conjugated to the hapten corresponding to the B cell specificity. These responses are clonal, Ia haplotype restricted, and exhibit requirements for hapten-carrier linkage if the T_H cells are 'rested' prior to use by brief (48 hr.) culture in the absence of antigen, APC, and LKs. The resting period results in a marked decline in cytoplasmic mRNA for IL-4 and IL-5 as detected by *in situ* hybridisation. An antigen-independent, haplotype-restricted version of these clonal microcultures has been developed using inputs of 0.5-2 purified F_1, k x b haplotype, B cells and the alloreactivity of

D10 cells vs. I-A^b. In either type of microculture, primary B cells can be stimulated to proliferate and generate clones that display isotype switching. However, IgA expression is rare among the clonal antibody products (6 ± 2% of antibody-secreting clones express IgA (*Schrader* et al., 1990). Further, B cells shown to include many IgA-memory cells, such as PP B cells enriched on phosphocholine (PC)-gelatine and tested in splenic fragment cultures, failed to generate clones secreting solely IgA in microculture (*Schrader* et al., 1990; *George* and *Cebra*, 1991). Even addition of exogenous IL-5 and IL-6 failed to markedly enhance IgA expression in clonal microculture and addition of TGFβ markedly reduced the cloning efficiency of B cells without increasing IgA expression (*Cebra* et al., l991a).

In an attempt to make T/B microcultures more supportive of IgA expression, we added either peritoneal macrophage, NIH/3T3, BALB/3T3, or dendritic cells (DC) as 'filler' or 'feeder' cells (*Schrader* et al., 1990; *Schrader* and *Cebra*, 1993). Although all types of added cells increased the frequency of responding B cells, only DC prepared from either spleen by the Steinman procedure or from PP by the Spalding method markedly potentiated IgA expression in both antigen-dependent and allo-stimulated clonal B cell microcultures. We found that as few as 400 DC per culture resulted in a marked increase in the proportion of antigen-dependent clones making anti-PC or anti-GlcNAc antibodies of the IgA isotype (to 30-50%). The IgA antibody-secreting clones included a sizeable fraction that secreted IgA antibodies exclusively (*Schrader* et al., 1990). A similar result was found for allostimulated B cells when DC were added (*Schrader* et al., 1990; *George* and *Cebra*, 1991). Neither the source - spleen or PP - of either the B cells or the DC affected the clonal expression of IgA when DC were added to the culture. If $sIgD^+$ B cells were prepared from either spleen or PP by FACS, then addition of DC to clonal microcultures resulted in a large fraction of clones that expressed IgA as one of several isotypes of antibody or Ig. Thus, addition of DC to these cultures revealed intraclonal isotype switching to IgA (*Schrader* et al., 1990).

Using allostimulated PP B cells we were also able to demonstrate that addition of DC to about one B cell and T_H cells allowed the outgrowth of IgA memory cells (*George* and *Cebra*, 1991). These IgA memory cells partitioned into the non-GC subset of PP cells prepared by FACS with a surface phenotype of low levels of peanut agglutinin binding (PNA^{low}) and high levels of sIg (kappa chain) ($s\kappa^{high}$). They were also markedly enriched by selecting the $sIgA^+$ cell fraction by FACS. When plated into alloreactive T_H-dependent microcultures containing DC these IgA memory cells gave clones that exclusively secreted IgA (*George* and *Cebra*, 1991).

Other features of the DC effect on T-dependent microcultures were that: (1) TH cells were required for generating antibody-producing clones, but their division during the culture period was not necessary as 2,000 R irradiation did not affect their efficacy; (2) the input of T_H cells could be reduced from about 3,000 without DC to about 375 with DC and comparable frequencies of responding clones were observed; and (3) the presence of DC promoted more vigorous cultures which survived longer and produced more total antibody than in their absence.

Although germinal centres developing in lymphoid tissues local to sites of antigen stimulation have been implicated as sites of generation of pre-plasmacells and memory B cells (*Cebra* et al., 1991b), direct demonstration of this role

has been frustrated by our inability to assay the functional potential of GC B cells. Necessary frequency analyses for specificity and isotype potential of GC B cells *in vitro* have heretofore been unsuccessful - GC cells usually die rapidly in culture - and assessment of their functional potential by adoptive transfer has been confounded by the lack of homing receptors on most GC B cells and the impossibility of defining the exact surface phenotype of progenitor cells responsible for any Ig expression in the host (*Cebra* et al., 1991b). Thus, when we developed a microculturing technique supportive of IgA expression we applied it to GC B cells from PPs, purified by FACS to enrich for PNA^{high} $s\kappa^{low}$ cells (*George* and *Cebra*, 1991). These cultures included DC and allospecific D10 cells reactive with $I\text{-}A^{b}$ molecules on the 0.5-2 PP GC cells ($I\text{-}A^{b/k}$) added. Under these conditions we found relatively low frequencies of responding cells and these gave clones that were not particularly distinctive of the PP site: Secretion of IgM, IgG1, and, only occasionally some IgE and IgA. Since about half to two-thirds of PP GC B cells from conventionally reared mice bore sIgA, we supposed that these were not responding with Ig secretion in our microcultures. However, we did find that our microcultures allowed extensive proliferation of GC B cells (*George* and *Cebra*, 1991). Because proliferation and differentiation have often been observed to be seemingly antagonistic processes, we assessed the effect of blocking division on Ig secretion by GC B cells from PPs (*George* and *Cebra*, 1991). If division of GC B cells is blocked by either x-irradiation (1600 R) or aphidicholin, a specific inhibitor of DNA polymerases, a high proportion of the positive cultures (60-70%) exclusively express IgA. About 30-40% of the division-blocked GC B cells responded with Ig secretion and both DC and T_H2 cells are required for such expression. Incorporation of ^3H-thymidine by B cells in such cultures is, of course, blocked and Ig-positive cultures expressing multiple Ig isotypes become rare. Experiments demonstrating GC B/T_H2 haplotype restriction and assay of secreted IgM by the F_1 input B cells using anti-allotypes strongly argue against B cell contaminants of the DC being the source of IgA in these cultures (*George* and *Cebra*, 1991). Finally, purification of $sIgA^+$ cells from PPs, distributed about 1:1 between GC and non-GC populations, enriches for both the uncommon memory IgA B cells (13-14%) that give clones exclusively secreting IgA in the standard microculture [see above] and also, especially for those that secrete IgA only when their proliferation is blocked. We hypothesise that our microculture assay of GC B cells from PPs, when their division is blocked, reveals a subset of cells committed to secretion. Such cultures demonstrate that, although many GC B cells die *in situ* by apoptosis, death need not be their immediate fate if their division is blocked and they are provided with necessary supportive cells and signals. These cultures also emphasise the extraordinary preference for the expression of IgM and IgA isotypes by GC B cells in PP and support the role of PP GC in the process of selective isotype switching to IgA expression.

4: *Assay for coating of gut bacteria* in vivo *with host IRA antibodies.*

Recently, we have begun to employ a rather simple but elegant assay for the occurrence, waxing, waning, continuation, or reappearance of gut mucosal IgA responses against bacterial colonisers. This assay was developed by Drs. D. and L. van der Waaij, Groningen, for human patients (personal communication). Faecal samples are periodically

collected, the bacterial suspension is washed, stained for IgA antibodies, and analysed by FACS. In our case, where colonisers consist of a single or few, known bacterial species the assay is more readily interpretable and permits non-destructive, constant monitoring of the gut mucosal IgA response.

RESULTS

The gut humoral, mucosal immune response to Gram-negative bacterial colonisers

We sought to determine and compare the effectiveness of various enteric bacteria at stimulating a mucosal IgA response and of cholera toxin (CT) at potentiating some of these responses. *Potter* (1971) had found that *M. morganii*, an occasional Gram-negative commensal of mice, reacted with certain anti-phosphocholine (PC) monoclonal antibodies and we had used this organism to colonise germfree mice and stimulate the appearance of specific anti-PC IgA memory cells in their PP (*Cebra* et al., 1980). Two, putative samples of *M. morganii* were used to successfully colonise GF mice within a few days after oral administration. PP fragment culture analyses of formerly GF mice mono-associated with one of these samples - now reclassified as *Proteus rettgeri* - failed to detect any specific anti-PC response up to 10 weeks after colonisation (*Logan* et al., 1991; *Shroff* and *Cebra*, 1993). FACS analysis failed to show any indication of GC reactions. Cholera toxin, a potent mucosal antigen in its own right, has been found to potentiate mucosal IgA responses to unrelated antigens when orally co-administered (*Elson* and *Ealding*, 1984). When the unresponsive, mono-associated mice were given 50 μg of CT orally, 6 weeks after colonisation, they made a prompt mucosal IgA response to CT as well as to the PC-determinant of their enteric bacteria. This response, detected in PP fragment cultures, was accompanied by obvious GC reactions in the PP. GF mice colonised only with the other sample of *M. morganii* - the authentic culture - made a prompt mucosal IgA anti-PC response at 10-14 days, as shown by PP fragment cultures (*Shroff* and *Cebra*, 1993) and FACS analyses of their PP cells. Figure 1 shows the waxing and waning of PNA^{high} GC cells over a 28-day period. Of note is that the colonisation at high bacterial density persists in these mice. So, having obtained a specific anti-PC IgA antibody response in PP fragment cultures of *M. morganii* immunised mice, we attempted to analyse the events leading to IgA commitment in GCs and the relationship between generation of IgA memory and secretory plasma cells. Roughly 40% of sIgA^{+} cells in PP have elevated mRNA levels for alpha chain of IgA and most of these are PNA^{high} (*Weinstein* et al., 1991). It is likely that the survivors amongst these become secretory IgA plasma cells. sIgA^{+} B cells from PP should contain both GC B cells that have switched to IgA expression (pre-plasmablasts) and non-GC, IgA memory cells. Using flow cytometry we enriched for PNA^{high} cells, and separately, for sIgA^{+} cells from formerly GF mice mono-associated with *M. morganii* 15 days previously. The enriched cells were analysed in clonal B cell microcultures. Our findings were (*Shroff* and *Cebra*, 1993) that:

1. PC-specific B cells are present in both the PNA^{high} and sIgA^{+} subsets at rather high frequencies at the time of maximal GC reactions in PP (~ 1%);
2. IgA memory cells produce clones expressing only IgA in T/B/DC cultures of some sIgA^{+} B cells; and

3. Such IgA memory cells are rare in cultures of PNA^{high} enriched cells but both these and some sIgA^{+} cells give secreted IgA antibody when put into T/B/DC cultures in the presence of aphidicholin to block their division, prevent apoptosis, and permit IgA secretion.

We harvested *M. morganii* from the intestine of formerly GF mice at the time of maximal GC reactions (day 15). These were stained for host IgA coating their surface. Faecal *M. morganii* stained for host IgA, cultured *M. morganii* coated in vitro with MPC603, a monoclonal antibody against the PC-determinant of *M. morganii* also stained for IgA, and a control sample of cultured bacteria only stained with fluorochrome-labelled anti-murine IgA was negative. The FACS analysis indicates that a significant proportion of faecal bacteria have become endogenously coated with murine IgA.

We expect that a detailed comparison of murine host responses to gut colonisation with these two very closely related enteric bacteria at the single cell level and exploitation of this first example of the use of CT to overcome non-responsiveness to a chronically present enteric antigen, may provide some insight into a long-standing mystery: why the host does not appear to continuously respond to its commensal bacteria. Further, more comprehensive, functional assays, using clonal B cell microculture, should be informative of the quantitative and temporal development of antigen-specific IgA memory cells and pre-plasmablasts in relationship to the GC reaction in PP.

Are neonatal PP competent to generate a preferential IgA response?

Reoviruses given intraduodenally or orally cause sub-clinical enteric infections in immunocompetent adult mice but are potent stimulators of a mucosal IgA antibody response (*London* et al., 1987). Type 1 reovirus has a tissue tropism for M-cells overlying PP and can generate a rapid response in conventionally reared or GF mice as evidenced by PP fragment cultures (*Weinstein* and *Cebra*, 1991). These cultures generate specific antibody when established 3-6 days after *in vivo* infection and show peak responses if initiated 6-14 days post infection. We have previously found that neonatal mice show a delay in the development of IgA memory B cells vs. bacterial determinants associated with their normal gut flora; the frequencies of these specific IgA memory cells do not reach adult levels until 10-12 weeks of life (*Cebra* et al., 1986). In order to determine whether this delay was due to a delay in the development of fully functional PPs, able to confer preferential switching to IgA expression on locally stimulated B cells, we challenged 10 day old neonatal mice orally with reovirus 1 and compared their responses to 12 week old mice using PP and LP fragment cultures (*Kramer* and *Cebra*, 1992). Ten-day-old mice are the youngest that can contain reovirus infections to the gut and forestall often fatal sequelae such as hepatitis, meningo-encephalitis, biliary atresia or severe diarrhoea. A comparison of the fragment cultures clearly showed the development of a mucosal IgA response, peaking at about 6 days in PP followed by a progressive rise in LP, and that the time course was the same in 10 day or 10-12 week old mice (*Kramer* and *Cebra*, 1992). We next sought to examine the influence of the maternal immune system on the development of humoral mucosal immunity by the pups. Using reciprocal crosses of congenic BALB/c and CB.17 scid mice we have generated immunocompetent F1 pups that are born to and reared by either immunocompetent BALB/c or immuno-

incompetent CB.17 scid dams. Upon infecting both groups orally with active reovirus 1 we found no differences in the magnitude or kinetics of the developing, reovirus specific IgA antibody responses in PP or LP cultures (*Kramer* and *Cebra*, 1992). PP cultures from both groups produced virus specific IgM when initiated 3 days post infection (p.i.) and began to produce specific IgA if cultured at day 6 p.i. Control, non-challenged littermates of both groups were consistently negative for reovirus-specific antibodies. However, the non-challenged F_1 pups of BALB/c immunocompetent dams did not exhibit detectable 'total' or 'non-specific' IgA Ig in either PP or LP cultures until days 19-22 of life, while non-challenged pups born to CB.17 scid dams made detectable total IgA as early as day 13 of life (*Kramer* and *Cebra*, 1992). In close correlation with this finding, pups born of scid mothers had abundant LP and MLN IgA plasma cells by days 19-22 while pups born of immunocompetent mothers had very few (*Cebra* et al., 1993).

Of relevance to this present discussion was our observations that reovirus infected F_1 pups born of immunocompetent mothers also showed a premature increase in output of 'natural' IgA, similar to the spontaneous earlier appearance of 'natural' IgA exhibited by pups born of scid mothers. Presently, we are making similar reciprocal crosses to those described above except we are using GF parents. Mono-association of dams and pups with a particular bacterial coloniser should allow us to evaluate whether reovirus infection of neonates can overcome a specific suppression mediated by maternal antibodies or whether the virus infection potentiates a non-specific/polyclonal IgA response.

To further evaluate the role of maternally acquired passive immunity to reovirus in orally challenged neonates, dams were pre-immunised with different serotypes of reovirus by different routes (*Cuff* et al., 1990). Pups were challenged at 48 hr. after birth with Type 3 reovirus, which ordinarily causes death via meningo-encephalitis of all pups within 10 days ($3x10^6$ PFU/dose). Female mice immunised with homotypic virus via the oral route developed the most potent response. Infected neonates born and nursed by these females developed no signs of disease, and no virus was recoverable from their small intestines, livers, or brains following infection. Neonates born to females immunised with homotypic virus by the subcutaneous route manifested no evidence of meningo-encephalitis or virus dissemination, yet virus could be recovered from their intestines. Dams immunised with heterotypic virus by either the subcutaneous or the oral route also conferred protection vs. the fatal consequences of the disease. However, virus was recovered from both small intestines and livers of infected neonates. Based on results from foster-nursing experiments, it appears that factors obtained *both* during suckling and transplacental transfer contribute to protection. However, the most complete resistance to infection is via suckling on dams orally immunised with homotypic (serotype 3) virus.

A consequence of this protection is that pups from previously orally infected, immunocompetent dams do not exhibit an active mucosal IgA antibody response when they themselves are orally challenged. Foster nursing experiments indicate that this suppression of active, local mucosal immunity is not mediated by maternal antibodies delivered to the foetus before birth but rather by antibodies obtained during suckling.

Table 3: Transfer of BALB/c (Igh^a) PeC cells into non-irradiated, congenic CB.17 (Igh^b) adult or neonatal recipients

Age recipient[1]	#PeC transferred	Time to assay (days)	Donor B cells in PeC (%)	Tissue assayed[2]	IgA^a/ IgA (counts)[3]	IgA^a/ IgA (%)
Exp. 1						
adult	$4x10^6$	8	8	--	---	---
adult	$4x10^6$	13	5	LP	13/1016	1.2
adult	$4x10^6$	23	--	LP	2/1028	0.2
Exp. 2						
adult #1	$5x10^6$	1	28	--	---	–
adult #2	$5x10^6$	50	4	MLN	3/460	
adult #2	$5x10^6$	50	4	LP	14/181	7.6
adult #3	$5x10^6$	50	4	MLN	0/37	<3.0
adult #3	$5x10^6$	50	4	LP	55/3050	1.8
adult #4	$5x10^6$	85	--	MLN	0/38	<3.0
adult #4	$5x10^6$	85	--	LP	0/53	<2.0
adult #5	$5x10^6$	85	--	MLN	1/98	1.0
adult #5	$5x10^6$	85	--	LP	0/226	<0.5
Exp. 3						
neonate A	$1x10^6$	13	–	MLN	0/38	<3.0
neonate A	$1x10^6$	13	--	LP	0/524	<0.2
neonate B	$1x10^6$	13	--	MLN	1/61	1.6
neonate B	$1x10^6$	13	--	LP	0/1344	<0.1
neonate C[5]	$1x10^6$	13	--	MLN	0/0	–
neonate C[5]	$1x10^6$	13	--	LP	0/12	–
neonate D	$2x10^6$	16	53	MLN	0/23	<4.0
neonate D	$2x10^6$	16	53	LP	0/174	<0.6
neonate E	$2x10^6$	16	69	MLN	0/3	–
neonate E	$2x10^6$	16	69	LP	0/93	<1.0
neonate F[5]	$2x10^6$	16	26	MLN	0/0	–
neonate F[5]	$2x10^6$	16	26	LP	0/10	–
neonate G[5]	$2x10^6$	16	69	MLN	0/0	–
neonate G[5]	$2x10^6$	16	69	LP	0/23	<4.0

[1] Adult recipients 6-8 months of age; neonates 5 days old.
[2] MLN = mesenteric lymph nodes; LP = intestinal lamina propria.
[3] By fluorescence microscopy on cytospots with 10^5 cells/spot.
[4] Zynaxis dye not detectable but IgM^a B cells detectable in PeC.
[5] Assay of neonates done on groups of three, pooled; the neonates in these groups born of CB.17 (scid/scid) male x CB.17 (scid/scid) female. Groups A-C had 3 IgM^a/430 IgM (0.7%) and D-G had 8 IgM^a/193 IgM (1.9%) in spleens.

An attempt to evaluate the contribution of B1 B cells to the population of IRA plasma cells in the gut lamina propria

In an attempt to estimate the normal, physiologic contribution of B1 (PeC) B cells to the pool of IgA plasma cells in mesenteric lymph nodes (MLN) and LP of immunocompetent mice, we transferred inocula of BALB/c (Igh^a) peritoneal cavity (PeC) cells into congenic CB.17 (Igh^b) recipients. Both Ig-allotype markers and Zynexis vital dye labelling were used to determine the success and extent of engraftment.

Table 3 shows that adult recipients of inocula sufficient to account for an appreciable proportion of PeC B cells in

the host displayed, with one exception, only a few percent of MLN or LP IgA plasma cells of donor origin at 13, 23, 50, and 85 days after transfer. Donor PeC B cells could be detected in the PeC of recipients up to 50 days after transfer.

Newborn mice have few IgA or IgM plasma cells in MLN or LP for the first three weeks of life if they are born of immunocompetent mothers (F_1 from CB.20 [scid/scid] male x CB.17 [+/+] female. Neonates born of immunocompromised mothers (F_1 from CB.20 [+/+] male x CB.17 [scid/scid] female) exhibit appreciable numbers of IgA plasma cells in MLN and LP by two weeks of life (*Kramer* and *Cebra*, 1992; *Cebra* et al., 1993). We transferred 1-2x10^6 BALB/c PeC (Igh^a) cells, into five-day-old F_1 neonates derived from these reciprocal crosses. Table 3 shows that, although engraftment was successful, the transferred PeC B cells made little if any contribution to the developing population of MLN or LP IgA plasma cells after 13 or 16 days (by 18-21 days of life).

Congenic transfers of PeC B cells into immunocompetent adult or neonatal (5 day old) recipients suggest a quantitatively minor role for B1 B cells in maintaining the steady state level of IgA plasma cells in the MLN and intestinal lamina propria of immunocompetent mice. A caveat is whether the congenic host treats the Igh allotype-different inocula as 'self' or not.

DISCUSSION

For decades we have all pondered the enigma of non-or hypo-responsiveness of the host to copious, ever-present commensal bacteria (*Berg* and *Savage*, 1975; *Dubos* et al., 1965; *Wold* et al., 1989) and food antigens (*Wold* et al., 1989; *Thomas* and *Parrot*, 1974; *Nedrud* and *Sigmund*, 1991). Of course, this hypo-responsiveness may only be apparent, since most studies fail to comprehensively assess all elements of both the mucosal and systemic immune response. Alternatively, if real, the anergy may be constitutive - i.e., dependent on actual delivery of antigen across the gut mucosa - or it may be specifically inducible - i.e., dependent on the occurrence of a prior humoral and/or cellular immune response. We believe that our analyses of GF, AF, and CNV neonatal mice indicate a major role for the intestinal flora in the development and maintenance of the physiologically normal steady state of the elements of the mucosal immune system in the Peyer's patches, intestinal lamina propria, and the intraepithelial leukocyte compartment (IEL). However, our observations suggest a subtle distinction between the continuously ongoing activity of the humoral and cellular mucosal responses in the gut and what is displayed by spleen or peripheral lymph nodes upon chronic stimulation with the same set of antigenic determinants due to a persistent infection or repeated, deliberate parenteral introduction of antigens. We have observed a waxing and waning of GC reactions in PP of formerly GF mice similar to what one detects in draining lymph nodes of parenterally injected mice (*Kreal* et al., 1990; *Coico* et al., 1983). Such transient GC reactions are seen after oral reovirus infection, upon colonisation of GF mice with enteric bacteria, or after oral administration of CT (*Shroff* and *Cebra*, 1993), provided that the animals were maintained in otherwise protected isolators. Although the reovirus infection and CT antigens appear to be completely resolved or cleared, the coloni-

sation by enteric bacteria persists. Furthermore, re-exposure of the gut mucosa to the previously resolved antigens - such as reovirus - results in successively less robust GC reactions and humoral, mucosal immune responses (*Weinstein* and *Cebra*, 1991). These observations suggest that the humoral mucosal immune response works! Antigens are excluded, primary infection is prevented and the local mucosal immune system is shielded from intense restimulation. This outcome is very different from observations after secondary or tertiary stimulation of draining lymph nodes by parenteral introduction of antigens (*Kreal* et al., 1990; *Coico* et al., 1983).

The effectiveness of IgA antibodies in the gut lumen at shielding the local mucosal immune system from stimulation is supported by our analyses of neonates born of immune, immunocompetent dams versus pups born of non-immune and/or immuno-incompetent mothers. The former pups can be passively protected by suckling but their own active mucosal immune responses are forestalled by maternal antibodies. So, if pre-existing IgA antibodies in the gut lumen are so effective at forestalling local immune responses, why does the gut appear to be in a chronic state of inflammation and the PP, lamina propria, and IEL appear to be undergoing chronic stimulation? Several possibilities come to mind. One is that the chronically present GC reactions in PP of CNV mice may be maintained by an ever-changing array of gut luminal antigens, possibly supplied by new microbial colonists (or food antigens) or by antigenic variation of existing microbes. Our observations of the effect of CT on local responses to *P. rettergi*, after established colonisation, and of oral reovirus on the increase in 'natural' IgA output by neonatal gut suggest that occasional exposure to novel, effective gut mucosal antigens may activate or re-activate mucosal responses to 'bystander', currently ineffective antigens.

The normal, physiologic role of B1 B cells in contributing to the intestinal IgA plasma cell pool remains enigmatic. Although IgA or IgM plasma cells derived from anti-microbial specific (cross-reactive) B1 B cells presented an attractive possibility our present, imperfect efforts have not supported it. However, we have now developed an efficient system for generating IgA hybridomas in scid mice derived from $sIgM^{high}$, $sIgD^{low}$ B1 cells from peritoneal cavity or $sIgA^{+}$ B2 cells from PP. Recipient scid mice develop copious IgA plasmablasts in their mesenteric lymph nodes and these are effective fusion partners for generating hybridomas. Presumably, use of CNV scid or GF scid mouse hosts, and oral challenges with microbial colonisers or enteric viruses should allow us to assess the relative potentials of B1 and B2 B cells to contribute to mucosal, humoral immunity.

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IMMUNOSTIMULATING EFFECT OF THE INTESTINAL MICROFLORA

J. BEUTH, H.L. KO, L. TUNGGAL, and G. PULVERER

Institute of Medical Microbiology and Hygiene, University of Cologne, Cologne, Germany

SUMMARY

Mucosal surfaces are habitats for the physiological microflora and are closely related to the mucosal immune compartment (mucosa-associated lymphoid tissue, MALT). Recently, considerable evidence has been accumulated showing that various members of the physiological microflora liberate low molecular weight metabolites which, apparently, are essential for the adequate immune response of the host. Antibiotic decontamination (e.g. of the BALB/c-mouse gastrointestinal tract) results in a lack of generation of immunopriming microbial metabolites leading to immunosuppression. Biochemical analysis of the microbial metabolites revealed reproducible chromatographic fractions which selectively influence maturation, proliferation and activation of mononuclear immune cells.

ANTIMICROBIAL CHEMOTHERAPY: INITIAL ENTHUSIASM AND OBJECTIONS

About fifty years ago when antimicrobial substances became available for treatment of infectious diseases it was not realised that these drugs as well may affect bacteria other than those causing the infection. The importance of microorganisms not involved in infectious diseases (e.g. those of the physiological microflora) has recently been shown since, apparently, they guarantee the adequate function of certain organs such as the gastrointestinal (GI) tract, skin and immune system (*Roszkowski* et al., 1988; *van der Waaij*, 1982; *Pulverer* et al., 1990a). The attention of many physicians had primarily been focused on the therapy of the infection without giving sufficient notice to side effects, e.g. microbial dysbiosis and immunocompromisation, respectively. Data derived from experiments involving the GI-tract of humans and animals provide some outline of the immune responses associated with the intestinal mucosal compartment. The mucosa-associated lymphoid tissues (MALT), the primary source of immunologic function, extend beyond the intestine and consist of the gut-associated (GALT), bronchial-associated (BALT) and duct-associated lymphoid tissues (DALT). Thus, virtually every mucosal surface of the body has the ability to respond to and to induce effector cells capable of protecting the host from potentially harmful organisms or antigens (Kagnoff, 1987a, 1987b). Our understanding of these various defence mechanisms and how they equip the host for its continuing conflict against pathogenic organisms and potentially harmful substances deposited on mucosal surfaces has a wide range of

biological and medical applications. For example, studies on mucosal immunity might lead to more effective methods of immunoprophylaxis against agents responsible for a wide range of infectious, neoplastic and autoimmune diseases.

ANTIBIOTICS AND INTESTINAL MICROFLORA

The indigenous microflora of the GI-tract plays a major role in the ecological flora-associated colonisation resistance (*van der Waaij*, 1982; *Gorbach* et al., 1988). *Van der Waaij* (1982, 1985, 1988) intensively studied the changes in colonisation resistance after antimicrobial treatment. Recently, the influence of mezlocillin and other selected antibiotics on the physiological aerobic and anaerobic digestive tract microflora of BALB/c-mice was shown in detail by *Roszkowski* et al. (1986a,1988). Bacteriological analysis of the GI-tract microflora demonstrated a stable spectrum of aerobic and anaerobic bacteria. However, a 7 days treatment of BALB/c-mice with mezlocillin (dosage and timing scheme of the antibiotic treatment were calculated according to therapy in human medicine) caused elimination of most species of the endogenous intestinal microflora. Especially representatives of the anaerobic flora (e.g. *Bacteroides* sp., *Clostridium* sp., *Lactobacillus* sp., *Propionibacterium* sp.) were completely eradicated (*Roszkowski* et al., 1988; *Pulverer* et al., 1990a). Since mezlocillin was the only antimicrobial drug tested that eliminated Gram-negative as well as Gram-positive aerobes and anaerobes respectively, it was chosen for further investigations. Subsequent studies on the dynamics of changes of the intestinal microflora showed that aerobic bacteria could no longer be cultivated after 24 h of mezlocilllin treatment. After termination of the mezlocillin therapy still all aerobes and anaerobes were absent from the GI-tract for at least 24 h, followed by a slow recovery of the physiological microflora during the following days. Even four days after finishing antimicrobial chemotherapy the number of (an)aerobes in the digestive tract was definitely lower as compared to non-treated control mice.

Correlating to the decontamination, the concentration of *E. coli*-endotoxin in caecal contents showed a decreasing tendency during mezlocillin treatment. However, a significant enhancement of *E. coli*-endotoxin could be detected during the recovery phase three to seven days after terminating antibiotic administration (*Roszkowski* et al., 1987).

Further investigations on the pharmacokinetics of mezlocillin demonstrated that:

a) three days of systemic treatment were sufficient to decontaminate the GI-tract of BALB/c-mice,
b) oral administration as well resulted in a total digestive tract decontamination in BALB/c-mice,
c) no serum level was detectable after oral administration of mezlocillin,
d) as compared to humans, serum levels of mezlocillin were rather low in BALB/c-mice after parenteral administration,
e) elimination of mezlocillin from mouse serum was relatively fast, and
f) drug concentration in the digestive tract of BALB/c-mice was rather high and long lasting (*Roszkowski* et al., 1986b, 1987).

Since mezlocillin proved to be a drug with constant GI-tract decontaminating effects (at least in BALB/c-mice) it was chosen for subsequent investigations on the interaction of the physiological microflora and immune responses.

ANTIMICROBIAL CHEMOTHERAPY AND NEOPLASTIC DISEASE

Cancer with concomitant bacterial infection is a well-known clinical problem. Not only neoplastic processes but also anti-tumour chemotherapy can induce immunosuppressing effects leading to infectious diseases (*Fainstein* and *Bodey*, 1983). Therefore, many cancer patients have to be treated with antimicrobial chemotherapy and generally such treatment lasts longer than in cases of bacterial infections in non-cancer patients (*Bodey* et al., 1966; *Fainstein* and *Bodey*, 1983). Thus, it seems appropriate to ask whether antibacterial chemotherapy can interfere with the host-tumour relationship. For this purpose the BALB/c-mouse/sarcoma L- 1 model was chosen because it allows reproducible test modalities. The effect of mezlocillin treatment on tumour development in BALB/c-mice offered great variations according to the experimental schedule. Thus, enhancement of tumour growth was observed when mezlocillin was administered to animals prior to tumour implantation. However, application of mezlocillin immediately after tumour implantation manifested a totally opposite effect and resulted in a significant reduction of tumour growth (*Roszkowski* et al., 1984a, 1985a).

Enhancement of tumour growth after mezlocillin pre-treatment might be the result of unspecific immunosuppressive activities of the drug (suppression of natural killer [NK] cell and cytotoxic T-cell activities). Administration of the antibiotic after tumour implantation may provide different possibilities and co-existence of at least two phenomena:

1. stimulation of host immune system, and,
2. direct effects on tumour cells.

Assuming that mezlocillin possesses cytotoxic activities the resultant effect might be the inhibition of tumour growth. Such a phenomenon can be observed in conventional anticancer chemotherapy since many cytotoxic drugs induce a strong immunosuppression and a simultaneous anti-tumour effect. However, mezlocillin did not affect the growth behaviour of sarcoma L-1 cells and other tumour cell-lines *in vitro* when it was added to cell cultures. This result obviously is in contrast to the hypothesis that mezlocillin possesses cytotoxic/cytostatic activities. It might be possible, however, that metabolites of the antibiotic, which eventually are produced *in vivo*, possess cytotoxic properties. So far, no evidence for such a possibility could be demonstrated. On the other hand, mezlocillin may be able to alter the antigenicity of tumour cells and make them more susceptible to the host defence. However, this hypothesis as well could not be confirmed in experiments on specific anti-tumour immunity (*Roszkowski* et al., 1984a, 1985a, 1986b).

When analysing and discussing these data we speculated that antibiotic-induced modification (eradication) of the physiological microflora (e.g. of the GI-tract) might be responsible for this tumour-modulating phenomenon. Two hypotheses arouse and were extensively investigated:

a) the physiological microflora interacts with the immune system, accordingly antibiotic decontamination may enhance drug induced immunosuppression, or
b) the physiological microflora liberates growth factor-like substances.

So far, many data are in favour of these postulations and still encourage further studies.

ANTIBIOTICS AND IMMUNE SYSTEM

Chemotherapy of bacterial infections can no longer be regarded as a simple interaction between drugs and bacteria. Undoubtedly, the immune system of the host is also strongly involved. Recently it was suggested that the GI-tract microflora of experimental animals might influence the development of immune responses (*van der Waaij*, 1985; *Roszkowski* et al., 1988; *Pulverer* et al., 1990a). In BALB/c-mice certain antimicrobial drugs could be shown to modulate host defence mechanisms (*Roszkowski* et al., 1984b, 1985b, 1985c) resulting in substantial suppression of cellular and humoral immune responses (*Roszkowski* et al., 1985c, 1989). The most striking finding of these studies was the potent and long lasting suppressive effect of mezlocillin.

Interestingly, the immunosuppressive effects of mezlocillin obviously are associated with changes in the endogenous intestinal microflora of BALB/c-mice since direct interactions between the antibiotic and certain cells of the immune system could not be found. Recent data confirm that gastrointestinal tract decontamination affects the immune system since peritoneal macrophages harvested 24 h after terminating antimicrobial treatment showed a significant reduction of certain functions, e.g. chemiluminescence response, chemotactic, bactericidal and anti-tumour activities (*Roszkowski* et al., 1988; *Pulverer* et al., 1990a). Of special interest was the observation that the basic macrophage activity (background activity in chemiluminescence assays) seemed to be suppressed after GI-tract decontamination.

Apparently, the GI-tract microflora provides a trigger mechanism for a moderate (but constant) priming of certain immune cells such as peritoneal macrophages. In the meantime considerable evidence has been accumulated showing that the physiological microflora (e.g. of the GI-tract) interacts with a variety of immune functions (*Kagnoff*, 1987a, 1987b; *Roszkowski* et al., 1988; *van der Waaij*, 1988; *Pulverer* et al., 1990a). In addition to its suppressive influence on macrophage activity, humoral immune responses and anti-tumour activity, decontamination of BALB/c-mice induced a significant atrophy of thymus and spleen and significantly decreased lymphocyte function (*Roszkowski* et al., 1988; *Pulverer* et al., 1990a) *in vitro* (^{3}H-TdR incorporation) and *in vivo* (^{125}IUdR uptake). However, oral or intraperitoneal administration of a heat killed vaccine (mixed from 9 *Bacteroides* species isolated from fresh faeces of healthy, non-treated BALB/c-mice) or of an immunomodifying *P. avidum* strain could - at least partly - reverse the suppressive effects of the mezlocillin therapy. These observations suggested that the adequate function of the immune system might be closely correlated to the presence of the physiological microflora (e.g. of the GI tract).

In the course of studying the underlying (patho)physiological mechanism we found that certain species of the indigenous GI-tract microflora liberate low molecular weight metabolites which apparently trigger basic immune responses (*Pulverer* et al., 1990a, 1990b). Accordingly, the hypothesis was discussed that eradication of the GI-tract microflora might suppress local and/or systemic immune responses since the generation of immunotriggering microbial metabolites ceased.

PHYSIOLOGICAL MICROFLORA LIBERATES IMMUNOMODULATING METABOLITES

Previous studies suggested that the physiological microflora (e.g. of GI-tract) exert a stimulus on certain immune functions since antibiotic decontamination of experimental animals resulted in immunosuppression and modification of anti-tumour immunity (*Pulverer* et al., 1990a; *Roszkowski* et al., 1984a, 1985a, 1988). In the course of investigations (to clarify the mechanisms) certain members of the BALB/c-mouse GI-tract microflora (e.g. *Bacteroides* sp., *Clostridium* sp., *Lactobacillus* sp., *Pronionibacterium* sp.) were found to liberate low molecular weight metabolites (MW <6.500 D). To substantiate the assumption that microbial metabolites might prime basic immune responses, cultivation procedures were established to provide optimal conditions for their generation and release. In BALB/c-mice, antibiotic decontamination of the GI-tract reproducibly resulted in considerable immunosuppression, apparently due to the lack of a specific stimulus. The substitution of metabolites liberated from microorganisms of the GI-tract such as *Bacteroides* sp. and *Propionibacterium* sp. to digestive tract decontaminated animals (route and interval of administration analogue to the antibiotic) reconstituted the cellular function (peritoneal macrophage phagocytic activity) and lymphatic tissue weight (thymus and spleen).

To confirm the hypothesis that the human physiological microflora interacts with the immune system, certain bacteria of human origin were tested for their ability to liberate immunomodulating metabolites. Two species (*P. acnes* and *S. saprophyticus*) could be shown to release considerable amounts of low molecular weight metabolites. Substitution of those metabolites (liberated from bacteria of human origin) to antibiotic-decontaminated (and immunocompromised) BALB/c-mice reconstituted the function of their immune systems.

Sephadex chromatography revealed a uniform arrangement of peaks for microbial metabolites of different origin including those liberated from strains of BALB/c-mouse GI-tract microflora (*Bacteroides* sp., *Clostridium* sp., *Lactobacillus* sp., *Propionibacterium* sp.) and those from *P. acnes* and *S. saprophyticus* of human sources (*Pulverer* et al., 1990b). Apparently, the generation and release of microbial metabolites seems to be a unique property of various members of the physiological microflora resulting in a moderate but constant priming of the immune system (mucosa- associated lymphoid tissues, MALT).

To investigate the immunomodulating potency with another well established experimental model (*Scollay* et al., 1984a; *Reichert* et al., 1986a), microbial metabolites from *P. acnes* and *S. saprophyticus* were administered to hydrocortisone-treated BALB/c-mice. Hydrocortisone-resistant thymocytes generally have been used to investigate the functional maturity since the vast majority of thymocytes surviving the administration of hydrocortisone are of a mature phenotype (*Reichert* et al., 1986a). Intrathymic T-cell differentiation is a process in which immature thymocytes expand and develop by undergoing complicated maturational events leading to the acquisition of immunocompetence and subsequent emigration to the periphery (*Scollay*, 1984; *Scollay* et al., 1984b). The thymic microenvironment is thought to exert local influences, which may contribute to the T-cell maturation process (*Reichert* et al., 1986b). Quantitative analyses re-

vealed a significantly decreased number of thymocytes after hydrocortisone-treatment in BALB/c-mice. However, administration of microbial metabolites apparently stimulated the cell proliferation and maturation since the number of thymocytes increased significantly as compared to non-treated animals.

Administration of microbial metabolites (released from *S. saprophyticus* or *P. acnes* of human sources) to non-treated BALB/c-mice as well manifested some immunopotentiation which positively correlated with a remarkable increase of thymus weight. However, weight gain of spleen was less pronounced (*Pulverer* et al., 1990b).

Recently it has been shown that T-lymphocyte antigens undergo characteristic changes in their surface density expression as T-cells mature in thymus and lymphoid tissues (*Ledbetter* et al., 1980; *Micklem* et al., 1980; *Reichert* et al., 1986a, 1986b). Quantitative investigations on Lyt-1 (pan T-cells), Lyt-2 (T-cytotoxic/suppressor cells), L3T4 (T-helper/inducer cells) expression has been facilitated by the use of monoclonal antibodies. Directly fluorescence-conjugated anti-Lyt-1, anti-Lyt-2 and anti-L3T4 monoclonal antibodies were each used alone and in combination in FACS (Fluorescence-Activated Cell Sorter) staining experiments. The T-cell receptor first appears during thymic ontogeny (*Ceredig* et al., 1983; *Fitch*, 1986). Roughly 80% of thymocytes are Lyt-2^+/L3T4^+ and a small proportion are Lyt-2^-/L3T4^-, cells belonging to these thymocyte subsets are thought to be immature (*Ceredig* et al., 1983; *Scollay* et al., 1984a). In contrast, approximately 15% of thymocytes and nearly all peripheral T-cells express the mature Lyt-2^-/L3T4^+ (T-helper/inducer) or Lyt2^+/L3T4^- (T-suppressor/cytotoxic) phenotype (*Ceredig* et al., 1983; *Scollay* et al., 1984a). Administration of microbial metabolites (liberated from *P. acnes* and *S. saprophyticus*) to BALB/c-mice apparently provides a stimulus for the development of lymphoid cells. Accordingly, the numbers of T-helper/inducer cells evidently increased in thymus after metabolite injections whereas T-cytotoxic/suppressor cells did not undergo considerable changes. A calculation of the helper/inducer-suppressor/cytotoxic cell ratio suggested that the administration of microbial metabolites preferably stimulated the proliferation of T-cells with helper/inducer phenotype (*Pulverer* et al., 1990b). The exact mechanisms for this selection process have not yet been clarified, however, a variety of growth factors and interleukins similarly affect effector tissues (*O'Garra*, 1989). A further characterisation of the involvement of antigen receptors and/or other cell surface molecules during T-cell development and their activity will provide additional insight into events that determine the T-cell repertoire.

CONCLUSION AND FUTURE ASPECTS

Mucosal surfaces are habitats of the physiological microflora and are closely related to the mucosal immune compartment (mucosa- associated lymphoid tissues, MALT) which interacts with the systemic immune compartment on separate levels of host defences (*Tomasi* et al., 1980; *Bienenstock* and *Befus*, 1984). It is the first line of defence and has the ability to block antigen-access to the systemic compartment of the host by producing local responses (*Walker* and *Isselbacher*, 1977; *Challacombe* and *Tomasi*, 1980). However, antigens (e.g. microbial metabolites) can gain access to the MALT and trigger (local)

immune responses. In addition, some antigens are able to produce systemic tolerance (*Challacombe* and *Tomasi*, 1980). If these particular antigens gain access to the local immune systems, a suppression of the systemic immune response may be induced by suppressor cells which were activated in the MALT and then translocated to the systemic immune compartment (tolerance (*Challacombe* and *Tomasi*, 1980; *Richman* et al., 1981).

Recently, considerable evidence has been accumulated showing that the physiological microflora liberates low molecular weight metabolites which, apparently, prime certain immune responses. Investigations on suppression and reconstitution of immune functions depending on the presence of the physiological microflora (respectively on microbial metabolites liberated from members of the physiological microflora) favoured the hypothesis that symbiotic microorganisms (respectively their metabolic products) are essential for adequate immune functions. Since those events are generally stimulated and regulated by T-helper/inducer cells, this activity may be explained by the production of growth and differentiation factors. These properties of interleukins and related molecules (e.g. microbial metabolites) indicate a key role in the positive and negative regulation of antigen-specific cellular and humoral immune responses and in the ontogeny of the immune system.

Preliminary investigations suggested that microbial metabolites may be considered to be potential growth factors (e.g. for fibroblasts, epithelial cells, bone marrow cells, tumour cells) as well as differentiation factors (e.g. for lymphoid cells, bone marrow cells). Accordingly, a wide range of biological and medical applications of these metabolites should be considered, e.g.

1) specific immunomodulation (with special emphasis on anti-infectious and anti-neoplastic immunity,
2) therapeutical administration of growth and differentiation factors (interleukin-like molecules),
3) specific adjuvans in patients treated with decontaminating antimicrobial drugs (omnispectrum therapy) and last but not least
4) a contribution to current knowledge on interactions of the physiological microflora and immune responses.

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ENTERIC BACTERIAL TRANSLOCATION: CURRENT PERSPECTIVES FROM *IN VIVO* AND *IN VITRO* MODELS

CAROL L. WELLS[1,2] and STANLEY L. ERLANDSEN[3]

Departments of Laboratory Medicine & Pathology[1], Surgery[2], and Cell Biology & Neuroanatomy[3], University of Minnesota, Minneapolis, Minnesota 55455, USA

SUMMARY

Bacterial translocation was initially viewed with scepticism, but it is now commonly accepted that this process is associated with a wide variety of clinical conditions. There is evidence that bacterial movement across the intestinal epithelium has a role in the induction of normal immune response, as well as in the induction of inflammatory bowel disease, reactive arthritis, endotoxaemia, and sepsis. The term bacterial translocation has been generally applied to the process by which normal intestinal microbes migrate out of the intestinal tract and cause systemic infections in high-risk patients. Patients at greatest risk for these complicating infections include post-surgical patients, trauma patients, and immunosuppressed patients such as organ transplant recipients and cancer patients. In the past decade, studies involving humans and laboratory animals have helped to clarify some of the clinical conditions associated with bacterial translocation. Results from human studies have strongly implicated normal intestinal bacteria as aetiologic agents of systemic disease. Laboratory animal models have helped to clarify the factors that either increase or decrease the recovery of translocating bacteria from extra-intestinal tissues, and translocating microbes have been observed in the cytoplasm of intact intestinal epithelial cells. Thus, the absorptive enterocyte appears to be at least one portal of entry for translocating bacteria, and cultured enterocytes appear to be a relevant model to dissect the initial events involved in bacterial adherence and uptake by the intestinal epithelium. Future correlations of results from *in vivo* and *in vitro* models should provide information that will not only further clarify the physiological factors controlling bacterial translocation, but will suggest new treatment regimens to reduce the costly morbidity associated with complicating infections caused by translocating bacteria.

INTRODUCTION

In its broadest terms, intestinal bacterial translocation can be defined as the passage of bacteria (both live and dead), and bacterial products (such as exotoxins, endotoxins, and cell wall fragments), from the intestinal lumen to extra-intestinal sites. The term bacterial translocation has been most often used

to describe the process by which normal flora microbes migrate out of the intestinal lumen and cause complicating systemic infections and/or endotoxaemia in hospitalised patients. High-risk patients include post-surgical patients, trauma patients, and immunosuppressed patients such as cancer patients and organ transplant recipients. To target new treatment regimens and reduce costly morbidity, investigators are attempting to clarify the clinical conditions and physiological mechanisms involved in bacterial translocation. Clarification of these factors may have broad implications, because the process of bacterial movement from the intestinal lumen is likely inherent in a number of physiological processes, as described below.

Enteric bacteria and induction of intestinal immune responses

The intestinal epithelium is subject to a constant barrage of antigenic stimuli, originating not only from food antigens, but from the many antigens in a complex microflora that includes over 400 different species of bacteria (*Moore* and *Holdeman*, 1975). These bacteria are relatively sparse (10^2 to 10^3 per ml) in the proximal small bowel and become extremely dense (approximately 10^{12} per gram) in the colon (*Gebbers* and *Laissue*, 1984). The obvious site of host interaction with this antigenic burden is the intestinal epithelium.

According to current concepts for induction of mucosal immunity, soluble and particulate antigens, including bacteria and their products, penetrate the intestinal epithelium to be processed by antigen presenting cells, such as tissue macrophages. These processed antigens are presented to uncommitted gut lymphoblasts which migrate through the thoracic duct and "home" to the intestinal mucosa (via selective endothelial recognition mechanisms) as mature lymphocytes (*Bland* and *Kambarage*, 1991). As a result, approximately 80% of all immunoglobulin-producing cells in the human body are found in the intestinal mucosa (*Brandtzaeg* et al., 1991). In addition, it may not be coincidental that animal studies of bacterial translocation repeatedly culture viable intestinal bacteria from the draining mesenteric lymph nodes of a small percentage of normal animals (*Wells* and *Erlandsen*, 1992b).

Although initial investigations implicated Peyer's patch M cells as the primary site of antigen uptake (*Sneller* and *Strober*, 1986), there is accumulating evidence that other cell types may not only participate in this process, but may be the primary site of antigen uptake. Peyer's patches have been reported to take up inert particles (carbon, India ink, latex beads) and viable pathogenic bacteria, such as *Salmonella* spp., *Listeria monocytogenes*, and *Vibrio cholerae* (*Wells* et al., 1988c). However, there is growing evidence that absorptive enterocytes can also function as fixed phagocytes for the uptake of particulate antigens (*Falkow* et al., 1992). Inert particles (ferritin and latex beads) and viable microbes (*Salmonella* spp., *L. monocytogenes*, *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis*, *Candida albicans*) have been observed within enterocytes (*Wells* and *Erlandsen*, 1992b). Interestingly, major histocompatibility complex class II glycoproteins have been localised in the apical cytoplasm of absorptive enterocytes, suggesting that enterocytes can function as antigen presenting cells (*Mayer* and *Shlien*, 1987; *Bland* et al., 1991). By sheer numbers, absorptive enterocytes may be the most efficient route available for the uptake and processing of luminal antigens. At the risk of further complicating this discussion, it should be mentioned that Paneth cells have been shown to ingest and degrade certain bacteria and protozoa, and may

also function as fixed phagocytes in the intestinal epithelium (*Erlandsen* and *Chase*, 1972a, 1972b).

Mayer and *Shlien* (1987) suggested that M cells are the site of antigen sampling only for those antigens with specific receptors on M cells. Curiously, the bacteria reported to be taken up by M cells are frankly pathogenic species, such as *Salmonella* spp. and *L. monocytogenes*. No species of normal microbial flora has been observed to penetrate into Peyer's patches, but definitive studies have not been done.

Enteric bacteria and inflammatory bowel disease

Although the aetiology of inflammatory bowel disease remains elusive, there is evidence that an infectious agent is at least partially involved. To date, no animal model for ulcerative colitis or Crohn's disease perfectly reproduces the human disease, but several models have characteristics of one or both diseases and might provide insights into the disease aetiology.

It has been proposed that Crohn's disease might be caused by an increase in mucosal permeability, causing luminal antigens to enter the lamina propria. Consequently, *Morris* et al. (1989) instilled a hapten (to elicited an inflammatory response) suspended in 50% ethanol (to increase mucosal permeability) into the rat colon; the resulting chronic inflammatory response had a thickened bowel wall, granulomas, and inflammatory infiltrate. *Mee* et al. (1979) irritated the rabbit colon with formalin, and parenterally injected the common enterobacterial antigen, followed by soluble immune complexes; chronic inflammation occurred only in the colonic regions treated with formalin. *Sartor* et al. (1985) produced chronic, granulomatous inflammation after surgical injection of *Streptococcus* (*Enterococcus*) *faecium* cell wall fragments into the rat bowel wall. *Sartor* et al. (1988) subsequently noted that peptidoglycan-polysaccharide complexes (the primary structural component of the cell walls of nearly all bacterial species) could initiate or enhance intestinal inflammation. Local infusion of N-formyl-methionyl-leucyl-phenylalanine (an inflammatory peptide produced *in vitro* by all species of intestinal bacteria investigated to date) resulted in experimental colitis (*Chester* et al., 1985).

Highlighting the importance of the intestinal flora in colitis, oral carageenan caused caecal ulcerations in conventionally reared, but not germfree guinea pigs (*Onderdonk* et al., 1977). Parenteral immunisation and oral feeding of *Bacteroides vulgatus* augmented this carageenan-induced colitis, and the outer membrane antigens of *B. vulgatus* appeared responsible for the effect (*Breeling* et al., 1988). Mycobacteria may also be involved in Crohn's disease. *Mycobacterium paratuberculosis* has been isolated from several patients with Crohn's disease and is the causative agent of John's disease, a chronic enteritis of ruminants with clinical and histopathological features in common with Crohn's disease. *Hamilton* et al. (1989) inoculated *M. paratuberculosis* into germfree athymic mice and functionally normal controls; a progressive infection, characterised by the presence of acid-fast bacilli and granulomas in the intestinal mucosa and the liver, was noted only in the athymic mice.

It may be unrealistic to propose a single aetiology for Crohn's disease or ulcerative colitis. Both diseases are heterogeneous and vary greatly in their clinical presentation. Evidence is accumulating that both diseases may result from a complex interplay of mechanical damage to the bowel wall, the presence of bacterial antigens within the intestinal mucosa (possibly causing an overstimulation of T helper cells in the lam-

ina propria), and an inherent immune defect in the host. Thus, inflammatory bowel disease may be associated with increased intestinal permeability, coupled with mucosal penetration of whole bacteria, bacterial fragments, or bacterial products.

Enteric bacteria and reactive arthritis

The microbes involved in septic arthritis are many and diverse (*Calin*, 1987), e.g., *Mycoplasma* spp., *Chlamydia trachomatis*, fungal agents, and viral agents. Taxonomically diverse bacterial agents have been implicated in septic arthritis, and include *Hemophilus influenza*, *Staphylococcus aureus*, *Neisseria gonorrhoeae, N. meningitidis*, *Streptococcus pneumoniae*, *Salmonella* spp., *Moraxella osloensis*, *Kingella kingae*, *Streptobacillus moniliformis*, *Pseudomonas* spp., *Borrelia burgdorferi* (Lyme disease), *Peptostreptococcus* spp., *Bacteroides* spp., *Clostridium* spp., *Yersinia enterocolitica*, and *Mycobacterium* spp.

Reactive arthritis can be defined as arthritis triggered by prior infection. There is evidence that the synovitis associated with both Reiter's syndrome and ankylosing spondylitis is related to the presence of tissue damaging immune complexes within the synovium, possibly associated with microbial antigens (*Keat*, 1983). Reiter's syndrome is often preceded by enteric infection with *Shigella* spp., *Salmonella* spp., *Y. enterocolitica*, or *Campylobacter jejuni*, although viable bacteria are not cultured from arthritic joints. Ankylosing spondylitis is associated with *Klebsiella pneumoniae* in the bowel flora (*Ogasawara*, 1986). Approximately 70-90% of ankylosing spondylitis and Reiter's syndrome patients is HLA-B27$^+$, while only 10% of normal Caucasians have this marker. Antibody to HLA-B27 cross-reacts with certain *K. pneumoniae* strains, and there is evidence that *K. pneumoniae* can modify the lymphocytes of asymptomatic HLA-B27$^+$ persons resulting in active ankylosing spondylitis. There is also an association between HLA-B27, *Y. enterocolitica* enterocolitis, and reactive arthritis (*Toivanan* et al., 1985).

Similar to inflammatory bowel disease, there is evidence that arthritis can be induced or augmented by cell wall fragments of normal intestinal bacteria. *Stimpson* et al. (1988) induced arthritis in rats by simple intraperitoneal injection of cell wall fragments of *S. faecium* and *Peptostreptococcus productus*, two prominent members of the normal intestinal flora; in further studies, it was shown that parenteral lipopolysaccharide could reactivate the arthritis produced by these cell wall fragments. Another common bacterial antigen, termed the 65K protein, has also been linked to arthritis (*Kaufman*, 1988). This bacterial cell wall protein is found on a variety of *Mycobacterium* spp. and many Gram-positive and Gram-negative, pathogenic and non-pathogenic species, including *E. coli*. The wide distribution of the 65K protein suggests that a variety of bacteria, rather than a single species, may be involved in the induction of arthritis.

Thus, in a scenario similar to that described above for inflammatory bowel disease, there is evidence that certain arthritic syndromes may involve a leakage of bacterial cell wall components across the intestinal mucosa, coupled with gradual accumulation of bacterial cell wall fragments or immune complexes in the tissues of a genetically predisposed host.

Enteric bacteria, endotoxaemia, and sepsis

There is substantial evidence that translocating intestinal bacteria cause a significant proportion of complicating

systemic infections in hospitalised patients. Typical aetiologic agents include *E. coli*, *Proteus* spp., *Klebsiella* spp., other members of the *Enterobacteriaceae*, *Pseudomonas aeruginosa*, enterococci, streptococci, and the yeast *Candida albicans*. Gram-negative bacteraemia is especially problematic because, despite advances in antimicrobial therapy, the mortality remains between 20% and 45% (*de la Torre* et al., 1985; *Gransden* et al., 1990; *Uzun* et al., 1992; *Geerdes* et al., 1992). Also, antibiotic-resistant *E. faecalis* is emerging as a major aetiologic agent of complicating nosocomial infections (*Moellering*, 1992), possibly due to widespread use of cephalosporins in hospitalised patients. Curiously, strictly anaerobic bacteria, such as *Bacteroides* spp. and *Clostridium* spp., outnumber other aerobic and facultative species by 100:1 or 1000:1 in the intestinal tract, yet anaerobic bacteria rarely cause complicating infections in high risk patients, and rarely translocate out of the intestinal tract in animal models of bacterial translocation.

Bacterial endotoxin appears to play a role in the pathogenesis of "sepsis syndrome", a clinical condition with the manifestations of systemic infection, but without successful isolation of a microbe from blood culture. Sepsis or sepsis syndrome is accompanied by hypertension and hypermetabolism that has been speculated to predispose to multiple organ failure, a poorly defined syndrome with a mortality of 50% to 75% (*Carrico* et al., 1986). According to one theory, the gut is a reservoir of potential pathogens; these potential pathogens, or their toxins, enter the circulation as a result of changes in the composition of the intestinal flora and/or altered barrier function.

Interest in the association between bacterial endotoxin and sepsis was renewed following a report by *Deitch* et al. (1987), who noted that parenteral endotoxin stimulated the translocation of normal flora bacteria from the intestinal lumen to the draining mesenteric lymph nodes of laboratory rodents. This finding had broad implications because alterations in gut barrier function, permitting leakage of endotoxin, could theoretically be initiated by any event facilitating intestinal ischaemia, e.g., shock, trauma. Investigators began to speculate that endotoxin-induced bacterial translocation might be the initial event in a wide variety of clinical conditions that predispose to sepsis, multiple organ failure, and death. The exact mechanism of endotoxin-induced bacterial translocation remains unclear. Parenteral endotoxin has been associated with intestinal bacterial overgrowth as well as alterations in intestinal histology, primarily villous oedema (*Deitch* et al., 1987; *Wells* et al., 1992c). There is recent evidence that even lethal doses of endotoxin do not consistently cause increased bacterial translocation, and that translocation may be associated with certain threshold level of intestinal bacterial overgrowth (*Wells* et al., 1992c).

CLINICAL EVIDENCE OF BACTERIAL TRANSLOCATION

Clinical evidence of bacterial translocation is largely circumstantial and can be divided into three categories: (a) the use of prophylactic antibiotics designed to eliminate selected populations of intestinal bacteria in high risk patients, coupled with a decrease in complicating infections in these patient populations; (b) prospective studies documenting that the predominant bacterial strain carried in the faecal flora is the same strain subsequently isolated from systemic infections; and (c) the recovery of viable intestinal bacteria from draining mesen-

teric lymph nodes, implying that bacteria can migrate out of the intestinal lumen. As noted below, there is substantial evidence that normal intestinal bacteria are important aetiologic agents of complicating infections in immunosuppressed, post-surgical, and trauma patients.

Selective antibiotic decontamination of the digestive tract

In the early 1970's, *van der Waaij* and colleagues (1971, 1972a, 1972b) introduced the concept of "colonisation resistance". According to this theory, strictly anaerobic bacteria do not normally translocate, and function to limit the intestinal colonisation and translocation of potentially pathogenic species such as *E. coli*. Physicians began to administer prophylactic antibiotics designed to decontaminate the intestinal tract of high-risk patients.

Over the ensuing 20 years, a substantial number of clinical studies have concluded that selective antimicrobial decontamination, typically targeted at elimination of *Enterobacteriaceae*, results in a significant decrease in the incidence of complicating infection. An excellent review of this literature has been written by *van Saene* et al. (1990). Typically, the alimentary tract is decontaminated using a mixture of non-absorbable antimicrobials (such as polymyxin E, tobramycin, amphotericin B), with an initial short-term course of a parenteral agent, such as cefotaxime. Using this approach, fourteen out of fifteen controlled studies reported a significant reduction in infection. Six of ten studies reported decreased mortality; however, *van Saene* et al. (1990) emphasised that it is imperative to distinguish between overall mortality and infection-specific mortality, and that the relationship between infection and mortality still remains unclear.

Association between intestinal colonisation and development of sepsis

Prospective studies have reported that a predominant strain of faecal bacteria is often the aetiologic agent of subsequent systemic infection. *Tancrede* and *Andremont* (1985) identified and quantified bacteria in 4,347 stool specimens from 688 cancer patients receiving no antimicrobial therapy; 60 patients developed 64 episodes of Gram-negative bacteraemia that appeared to be caused by a dominant faecal organism that translocated from the intestinal tract during a period of severe granulocytopenia. *Wells* et al. (1987a) also found evidence of faecal carriage in organ transplant recipients with gram-negative bacteraemia.

Recovery of viable bacteria from lymph nodes draining the intestinal, reproductive, and respiratory tracts

Direct evidence of bacterial movement across the intestinal mucosa is difficult to obtain in humans. Several investigators have cultured mesenteric lymph nodes obtained by surgery, attempting to recover viable bacteria in patients expected to have altered intestinal barrier function (Table 1). For ethical reasons, peri-operative antibiotics are typically used in these patients, thus underestimating the presence of viable bacteria; nonetheless, these results document the presence of viable intestinal bacteria in draining mesenteric lymph nodes.

Bacterial translocation may occur in other sites in addition to the intestinal tract. Normal vaginal flora has been recovered from the uterine lymph nodes of 34% (n=83) of patients undergoing surgery for gynaecological tumours (*Wells* et al., 1990c). There is also evidence that mononuclear phagocytes can

Table 1: Clinical studies documenting recovery of viable intestinal bacteria from draining mesenteric lymph nodes (MLN) obtained at surgery

Reason for surgery	No. patients with MLN bacteria / Total no. patients	(%)	Reference
Crohn's disease	15/46	(33%)	*Ambrose* et al. (1984)
Other elective abdominal surgery	2/43	(5%)	
Crohn's disease	7/28	(25%)	*Laffineur* et al. (1992)
Colorectal cancer	13/20	(65%)	*Vincent* et al. (1988)
Other digestive diseases	6/20	(30%)	
Intestinal obstruction, without necrosis	10/17	(59%)	*Deitch* (1989)
other abdominal surgery	1/25	(4%)	
Laparotomy for intestinal disease	3/4	(75%)	*Peitzman* et al. (1991)
laparotomy following trauma	0/25	(0%)	

transport particles from the terminal alveolus of the canine lung to the draining tracheo-bronchial lymph node (*Harmsen* et al., 1985). These latter two studies provide evidence for bacterial translocation in anatomical sites outside of the intestinal tract.

IN VIVO LABORATORY MODELS OF BACTERIAL TRANSLOCATION

Because direct evidence is difficult to obtain from humans, many investigators have used laboratory animals to clarify the clinical situations and pathogenic mechanism associated with bacterial translocation. Information in this area is emerging rapidly, with over one hundred articles published in the past five years.

Clinical conditions or treatments facilitating translocation

A representative (not exhaustive) listing of the diverse clinical conditions facilitating bacterial translocation in animal models is presented in Table 2. Several caveats should be mentioned concerning these experimental studies: (a) Data concerning immunosuppressive effects is conflicting (*Maddaus* et al., 1989), and there is evidence that the intestinal concentration and invasive ability of the colonising microbe may be more important than the immune status of the host (*Wells* et al., 1991c; *Jackson* et al., 1991); (b) Although several studies specifically monitor dietary influences, some animal models (such as parenteral endotoxin, burn wounds, surgery) have a malnutrition component that is typically not addressed; (c) Some models involve a histologically normal intestinal mucosa (e.g., antibiotic therapy), but other models induce alterations in intestinal histology (e.g., parenteral alimentation, burn wounds, parenteral endotoxin, intestinal ischaemia), and translocation may occur by different mechanisms across a normal or abnormal mucosa; (d) Insulin treatment of

Table 2: Selected references describing clinical conditions and treatments facilitating translocation of normal flora from the intestinal lumen to the draining mesenteric lymph nodes of experimental animals

Clinical condition/treatment	Reference
Antibiotic-induced intestinal bacterial overgrowth	*Berg*, 1981; *van der Waaij* et al., 1971, 1972a, 1972b; *Wells* et al., 1987b, 1988b, 1988d
Germfree animals colonised with aerobic /facultative bacteria	*Berg* and *Garlington*, 1979; *Steffen* et al., 1988; *Wells* et al., 1988a, 1991a
Subtherapeutic polymyxin B	*Dijkstra* et al., 1992
Neonatal animals	*Glode* et al., 1977; *Pluschke* et al., 1983
Cytomegalovirus	*Erickson* et al., 1991
Glucocorticoids	*Jones* et al., 1990a; *Alverdy* and *Aoys*, 1991
Interleukin-2	*Penn* et al., 1991
Surgery	*Salman* et al, 1992
Parenteral endotoxin	*Deitch* et al., 1987; *Wells* et al., 1992c
Experimentally induced diabetes	*Imai* and *Kurihara*, 1984; *Ohsugi* et al., 1991
Oral ricinoleic acid	*Morehouse* et al., 1986
Burn wounds	*Maejima* et al., 1984; *Alexander* et al., 1990; *Jones* et al, 1990a, 1990b, 1991; *O'Brien* et al., 1992; *Huang* et al., 1993
Obstructive jaundice/bile depletion	*Deitch* et al., 1990c / *Slocum* et al., 1992
Intestinal obstruction	*Maddaus* et al., 1989; *Deitch* et al., 1990b
Splenectomy/Hepatectomy	*Spaeth* et al., 1990c / *Wang* et al., 1992
Small bowel transplant	*Browne* et al., 1991; *Grant* et al., 1991
Haemorrhagic shock	*Baker* et al., 1988; *Rush*, et al., 1988; *Deitch* et al., 1990a
Mesenteric ischaemia	*Papa* et al., 1983; *Bennion*, et al., 1984; *Sheng* et al., 1992
Irradiation	*Brook* et al., 1984; *Souba* et al., 1990; *Kobayashi* et al., 1991
Immunosuppressive defects/agents	*Owens* and *Berg*, 1980; *Berg*, 1983; *Berg* et al., 1988
Parenteral alimentation	*Alverdy* et al., 1988; *Spaeth* et al., 1990a; *Keuppers* et al., 1993; *Helton* and *Garcia*, 1993
Oral liquid diet	*Alverdy* et al., 1990; *Spaeth* et al., 1990a, 1990b; *Mainous* et al., 1991; *Wells* et al, 1991b
Other dietary manipulations	*Li* et al., 1989; *Deitch* et al., 1990d; *Souba* et al., 1990; *Barton* et al., 1992; *Wells* et al., 1990d, 1992a
Intra-abdominal abscesses	*Wells* et al., 1986
Foreign materials	*Mora*,et al., 1991
Solid tumours	*Penn* et al., 1985

experimentally induced diabetes did not decrease the incidence of translocation,indicating that translocation may not result from diabetes (*Ohsugi* et al., 1991); (e) The effect of dietary manipulation is extremely variable, and while the studies listed in Table 2 reported a facilitating effect on bacterial translocation, other studies report no effect using similar dietary additives/alterations (e.g., *Barber* et al., 1990; *Wells* et al., 1990b) and (f) When monitored, intestinal bacterial overgrowth (similar to that reported to induce translocation on its own) is a component of many animal models including some dietary manipulations, parenteral alimentation, parenteral endo-

toxin, intestinal obstruction, liver resection. (Curiously, intestinal overgrowth has recently been reported to facilitate the absorption of bacterial cell wall polymers, namely the peptidoglycan associated with reactive arthritis, providing further evidence for related aetiologies in reactive arthritis, inflammatory bowel disease, and bacterial translocation [*Lichtman* et al., 1991]).

Factors influencing translocation in burn wounds

The burn wound model has received much attention in recent years, and this literature is too extensive to review here. Studies often attempt to address the mechanisms involved in bacterial translocation, and several findings deserve mentioning. Following burn wounds, increased bacterial translocation to the mesenteric lymph nodes is typically transient and diminishes after several days. There is evidence that the mechanism involved in bacterial translocation following burns is related to gut atrophy (*Jones* et al., 1990b; *Huang* et al., 1993) and mesenteric vasoconstriction (*Herndon* and *Zeigler*, 1993; *Jones* et al., 1991). The extent and duration of translocation can be increased with glucocorticoids (*Alverdy* and *Aoys*, 1991; *Jones* et al., 1990a), *P. aeruginosa* wound colonisation (*Manson* et al., 1992), cytomegalovirus (*Erickson* et al., 1991), and decreased with insulin-like growth factor 1 (*Huang* et al., 1993), a combination of fibroblast growth factor and sucralfate (*Gianotti* et al., 1993), prostaglandin E analogues (*Fukushima* et al., 1992), and inhibition of thromboxane synthetase (*Tokyay* et al., 1992). It is possible (even likely) that the physiological mechanisms involved in translocation associated with burn wounds, e.g., transient mesenteric ischaemia, may apply to other animal models listed in Table 2; if so, treatments modulating translocation in the burned animal may apply to other clinical conditions as well.

Role of the mononuclear phagocyte

Earlier studies from our laboratory demonstrated that mononuclear phagocytes could transport intestinal particles (1μ latex beads and *E. coli*) from the small intestine to the draining mesenteric lymph nodes of experimental animals (*Wells* et al., 1988d). We then proposed a direct relationship between the ability of an enteric organism to translocate and its ability to interact with mononuclear phagocytes. This postulate seemed reasonable because pathogenic enteric bacteria that readily translocate, such as *Salmonella* spp. and *L. monocytogenes*, are reported to survive and replicate within mononuclear phagocytes (*Moulder*, 1985). Unlike normal flora, these enteric pathogens translocate after simple oral inoculation into normal animals.

We studied ten strains of enteric bacteria with a documented spectrum of ability to translocate in mice, including *S. typhimurium*, *L. monocytogenes*, *E. coli*, *P. mirabilis*, *E. faecalis*, *B. fragilis*, and *Bacteroides* species. Peritoneal exudate cells from the same mouse strain were then used to study bacterial interactions with mononuclear phagocytes. Differences in oral infectivity (translocating ability) did not consistently correlate with the ability of these strains to be ingested by, or to survive within, mononuclear phagocytes (*Wells* et al., 1993a).

The above results do not imply that intestinal bacteria can not translocate within mononuclear phagocytes, only that this method of bacterial transport may not be a rate-limiting factor modulating the incidence of translocation. Assuming some bacteria do translocate within phagocytes, translocation may be

augmented by conditions that interfere with phagocyte function. There are several reports of immunosuppression facilitating translocation (*Berg*, 1983; *Berg* et al., 1988; *Gianotti* et al, 1992). Factors facilitating bacterial translocation in laboratory animals often have concurrent immunnosuppressive effects, e.g., burns, glucocorticoids, antibiotics. Thus, although transport by tissue phagocytes may not be a primary mechanism of bacterial translocation, bacterial interactions with phagocytes may play a significant role in some clinical conditions. This role may include limiting bacterial transport from the lumen to the draining lymph node, limiting systemic spread of bacteria that have translocated to the draining lymph node (lymphatic route) with its population of resident macrophages, or limiting the systemic spread of bacteria that have translocated to the liver (via the portal vein) with its population of Kupffer cells.

IN VITRO MODELS OF BACTERIAL TRANSLOCATION

Types of models

Mechanisms of pathogenesis of enteric bacteria are difficult to study *in vivo* because many factors in the intestinal environment confound interpretation of experimental results, such as peristalsis, epithelial sloughing, the presence of hundreds of different microbial species, an extensive 200 to 300 square meter surface area, etc. Using rodent models of microbial translocation, *C. albicans*, *E. coli*, *P. mirabilis*, and *E. faecalis* have been visualised within intact intestinal epithelial cells (*Cole* et al., 1988; *Alexander* et al., 1990; *Wells* et al., 1990a; *Wells* and *Erlandsen*, 1991a). Thus, the absorptive enterocyte may be at least one portal of entry for translocating bacteria, and bacterial uptake by "non-professional phagocytes" is becoming increasingly recognised as a mechanism of invasion for a wide variety of microbes (*Falkow* et al., 1992). However, direct observation of bacterial entry into the intestinal epithelium *in vivo* is difficult due to the typically low numbers of translocating microbes, and to the sampling problem inherent to the large surface area of the intestinal tract.

There are several alternatives to *in vivo* testing (*Neutra* and *Louvard*, 1989). Dispersed epithelial cells are readily obtained from the intestinal mucosa of experimental animals, but remain viable for an only few hours and are useful only for short-term physiologic experiments that do not require maintenance of polarity. Intact sheets of epithelium can be obtained by EDTA perfusion: Protein and glycoprotein synthesis continues, apical-basal polarity can be maintained, but basolateral surfaces loose their organisation within hours. Intestinal cell culture is an attractive alternative, and in recent years, numerous reports have described its use to study the interactions of enteric bacteria with intestinal epithelial cells. These reports have generally focused on frankly pathogenic bacteria such as *Salmonella* spp. and *L. monocytogenes*. Consequently, our understanding of the mechanisms involved in the epithelial adherence and uptake of these enteric pathogens has significantly expanded (*Falkow* et al., 1992). However, it is likely that this model can also be used to clarify the initial events involved in the translocation of normal enteric bacteria across the intestinal epithelium.

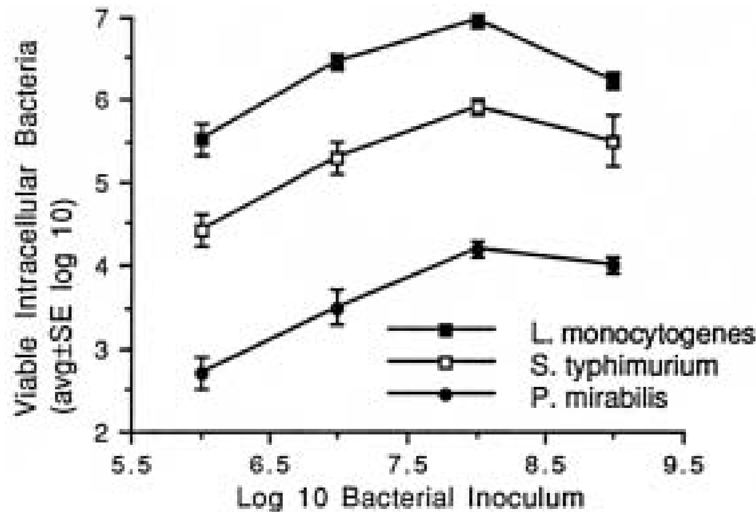

Figure 1: Internalisation of varying concentrations of *L. monocytogenes* ATCC 43249, *S. typhimurium* ATCC 10428, and *P. mirabilis* M13 incubated for one hour with Caco-2 cells, with intracellular bacteria quantified as described (*Wells* et al., 1993b). Each data point represent the results of three separate assays, each assay representing the average of triplicate determinations with a variability $<20\%$; any two data points differing by ≥ 1 $\log_{10}$ are significantly different by $p<0.01$, using a one-way analysis of variance followed by Scheffe's test for significant differences.

Cultured enterocytes as a relevant model

Of the dozens of intestinal adenocarcinoma cell lines available, most do not differentiate under standard culture conditions. Important exceptions are Caco-2 and HT-29 cells, the two cell lines most widely used to study the interactions of enteric bacteria with enterocytes (*Neutra* and *Louvard*, 1989; *Rousset*, 1986). Caco-2 cells are well polarised, are joined by tight junctions, and have well-developed microvilli. Although derived from human colonic epithelium, Caco-2 cells express some of the disaccharidases and peptidases typical of villous cells from the small intestine. Caco-2 cells also transport water and ions toward the basolateral surface and form domes on impermeable substrates. When grown in medium containing glucose and normal serum, HT-29 cells do not have polarity or other characteristics of differentiated cells. However, when grown without glucose, HT-29 cells are highly polarised, have several typical brush border enzymes, secrete an immunoreactive laminin, have transferrin receptors, and have histocompatibility antigen on the basolateral surface. Interestingly, fully differentiated HT-29 cultures contain two cell types resembling terminally differentiated absorptive cells and mucous-secreting goblet cells. Both Caco-2 cells and HT-29 cells are considered relevant models to study bacterial adherence and uptake by the intestinal epithelium.

Interactions of normal enteric bacteria with cultured enterocytes

There is a rapidly expanding literature describing interactions of enteric pathogens with cultured enterocytes, but only few studies involve normal enteric flora. Most studies involving cultured epithelial cells and normal enteric bacteria utilise cells of non-intestinal origin, such as HEp-2 (human la-

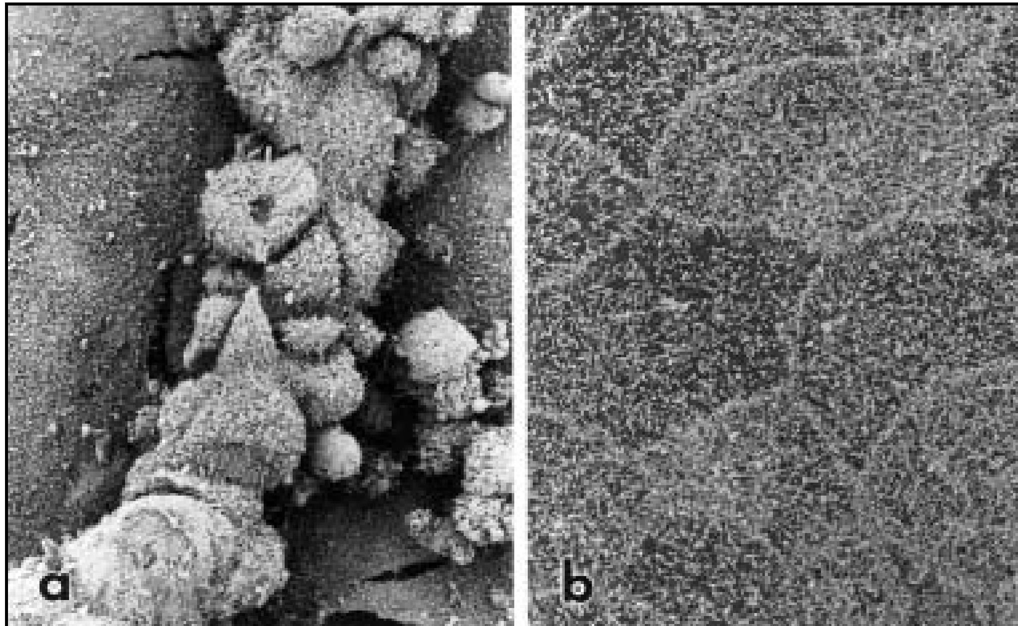

Figure 2: Low voltage scanning electron micrographs showing the variable surface topography of the intestinal epithelial cell line, Caco-2. a: Low magnification of enterocytes forming a three dimensional tunnel-like structure; b: Higher magnification of a monolayer area showing cellular outlines and relatively dense apical microvilli.

ryngeal epithelium), HeLa (human cervical epithelium), CHO (Chinese hamster ovary), and MDCK (Madin-Darby canine kidney) cells. The few studies involving normal enteric flora and cultured enterocytes, typically analyse only bacterial adherence and the process of bacterial internalisation is not studied.

Our laboratory is beginning to study the interactions of enteric bacteria with Caco-2 cells and HT-29 cells. Initial studies compared the Caco-2 internalisation of *L. monocytogenes*, *S. typhimurium*, *P. mirabilis*, *E. coli* (two strains), and *E. faecalis* (*Wells* et al., 1993b). As expected, *Salmonella* and *Listeria* were invasive in Caco-2 cells, but *P. mirabilis* and one of the two *E. coli* strains were also clearly internalised, albeit at significantly fewer numbers. Figure 1 presents the uptake of varying concentrations of *L. monocytogenes*, *S. typhimurium*, and *P. mirabilis* following incubation with Caco-2 cells. Additional electron microscopic studies demonstrated that surface fimbriae and flagella mediated bacterial attachment to Caco-2 microvilli, although bacteria without surface appendages were also adherent to the enterocyte surface (Figures 2-4). Compared to *Salmonella* and *Listeria*, internalised *P. mirabilis* and *E. coli* were relatively difficult to locate, but were consistently within membrane-bound vacuoles in the apical cytoplasm of Caco-2 cells (*Wells* et al., 1993b).

Thus, cultured enterocytes can be used to study the interactions of normal enteric flora with intestinal epithelial cells. However, progress in this area will likely be more difficult than studies involving frank pathogens (e.g., *Salmonella* and *Listeria*) due to the comparative rarity of the interactions of normal flora with the intestinal epithe-

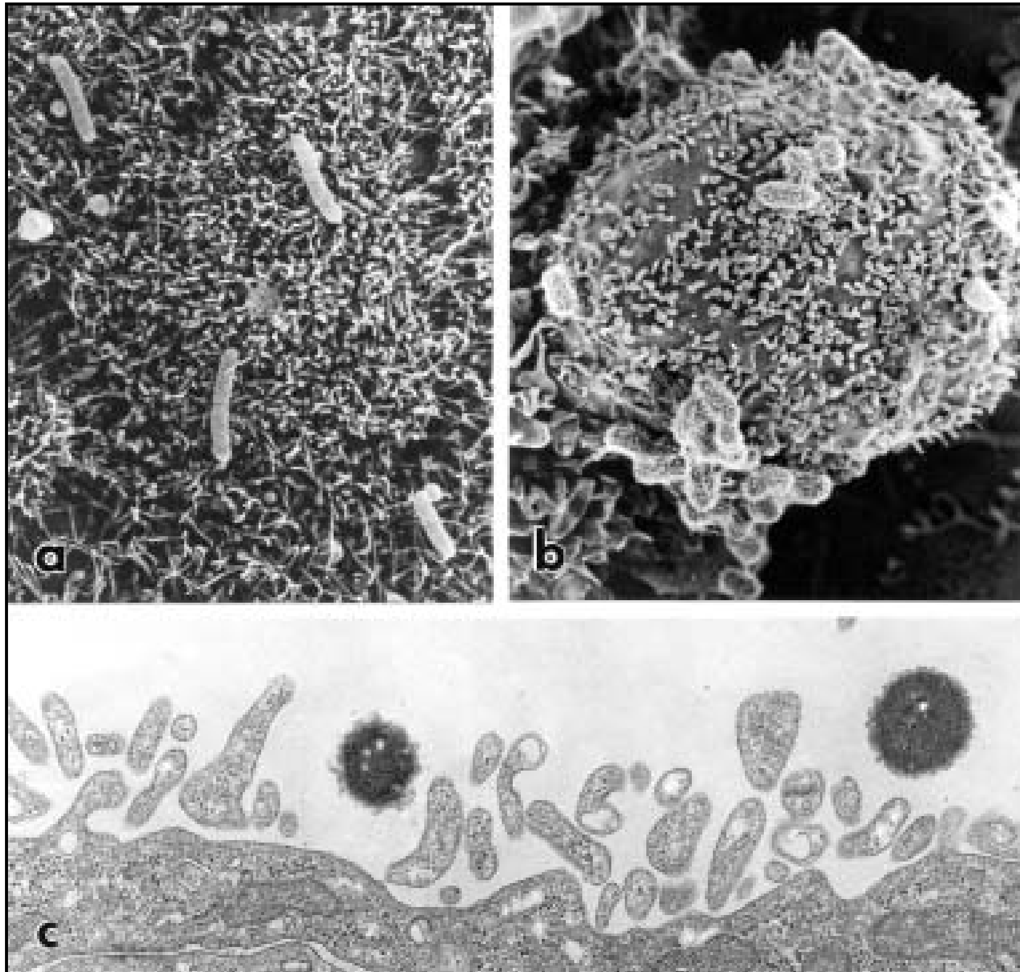

Figure 3: Surface interactions of enterobacteria with cultured Caco-2 cells. a and b: Low voltage scanning electron micrographs showing different patterns of bacterial adherence on the enterocyte surface: Diffuse adherence of *E. coli* C25 (a), and fimbriated and flagellated *P. mirabilis* M13 preferentially adherent on a single enterocyte (b); c: Transmission electron micrograph showing interactions of *E. coli* M21 with apical microvilli.

lium. Nonetheless, it is anticipated that this *in vitro* model will be useful in clarifying the initial events involved in epithelial adherence and internalisation of translocating bacteria.

CONCLUSION

Bacterial translocation has evolved from a process whose existence was viewed with scepticism, to a process that is clearly associated with a variety of clinical conditions. Bacterial movement across the intestinal epithelium is inherent in induction of normal immune response, as well as in induction of in-

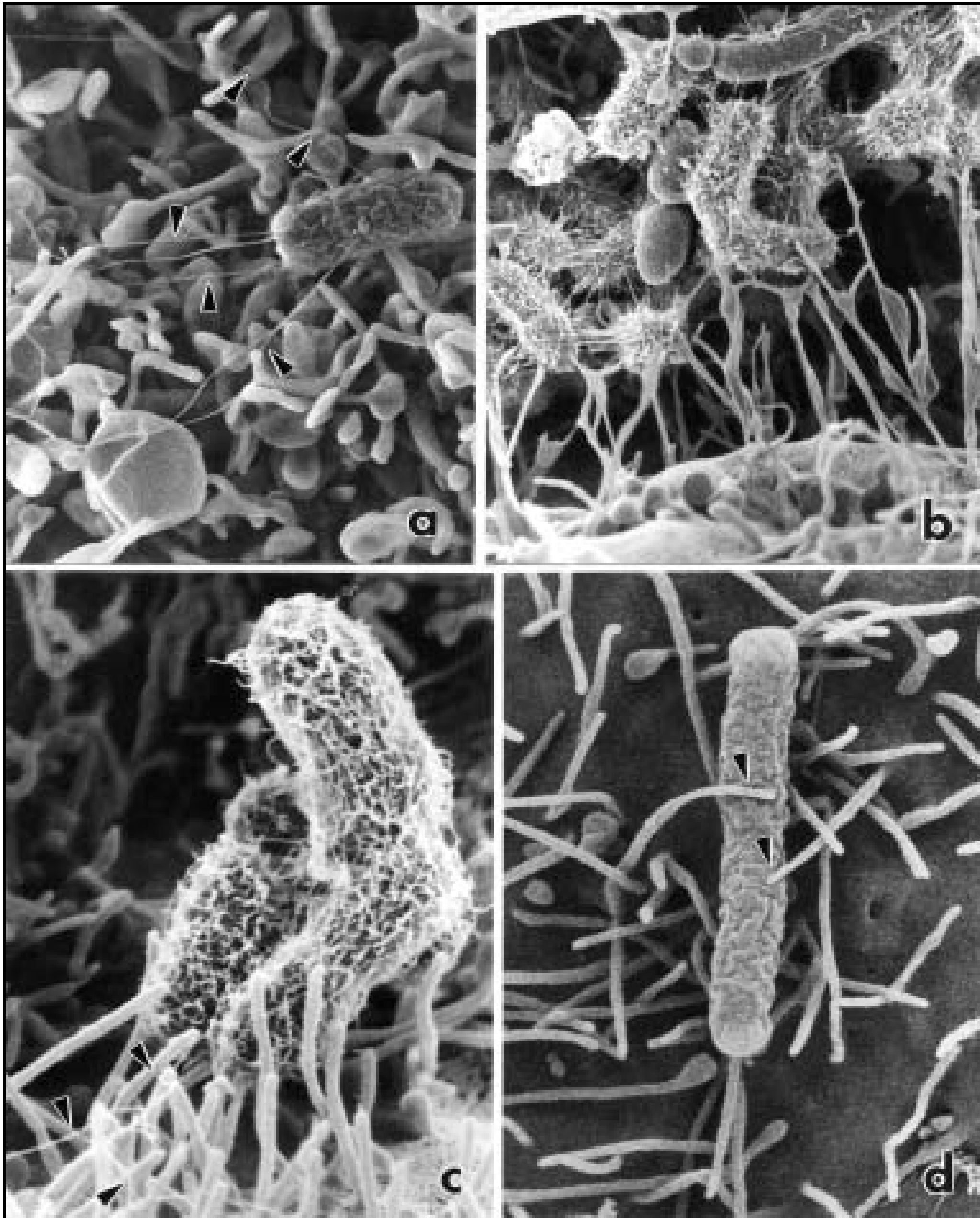

Figure 4: Low voltage scanning electron micrographs showing the interactions of enteric bacterial cell walls and surface appendages with apical microvilli of Caco-2 cells. a: Flagella (arrowheads) of *E. coli* M21 appear to aid in anchoring the bacterium to the enterocyte surface; b: Fimbriae and flagella of *P. mirabilis* M13 appear to mediate attachment of bacterial cells to one another, while microvilli appear to suspend bacteria above the enterocyte surface; note the variable distribution of surface appendages among individual bacteria in this pure culture of *P. mirabilis*; c: Higher magnification showing interactions of *P. mirabilis* flagella (arrowheads) and fimbriae with enterocyte microvilli; d: *E. coli* C25 adherent to the enterocyte microvillous surface, demonstrating bacterial adherence in the absence of surface appendages (note contact of individual microvilli [arrowheads] with bacterial surface).

flammatory bowel disease, reactive arthritis, endotoxaemia, and sepsis. Experiments using humans and laboratory animals have provided much information concerning the clinical conditions associated with bacterial translocation, but mechanistic information is limited by the complex physiology of the intestinal tract. Cultured enterocytes appear to be a relevant model that may be used to apply the techniques of basic cell biology to the *in vivo* phenomenon of bacterial translocation. Correlation of results from *in vivo* and *in vitro* models should aid in clarifying the mechanisms involved in bacterial translocation. These results may have broad implications because current evidence indicates that the process of bacterial translocation may not be limited to the intestinal tract, but may occur at other mucosal surfaces such as those of the reproductive and respiratory tracts.

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GRONINGEN REDUCTION OF IMAGE DATA: A MICROBIOLOGICAL IMAGE PROCESSING SYSTEM WITH APPLICATIONS IN IMMUNOFLUORESCENCE AND MORPHOMETRY

MICHAEL H.F. WILKINSON, GIJSBERT J. JANSEN,
and DIRK VAN DER WAAIJ

Laboratory for Medical Microbiology, University of Groningen,
Groningen, The Netherlands

SUMMARY

The interaction between the intestinal flora and the immune system is complex, and as yet little understood. The Groningen Reduction of Image Data (GRID) image processing system is a relatively new tool in the investigation of this interaction. The image processing approach allows measurement of morphological and immunological characteristics of faecal bacteria, which have not been cultured, and should therefore represent the flora in the intestinal lumen well. In this review, the main application programs of GRID in the field of bacterial morphology and (immuno-)fluorescence detection are presented. Its low cost hardware set-up, based on ordinary personal computers is described. Examples of the research done and data acquired with the system are given. Future plans include multi-colour fluorescence measurement. The system allows rapid quantification of morphology and immunofluorescence, and can combine both types of data in "fluoro-morphometry": quantifying patterns of fluorescence as a function of shape. These patterns could lead to new insights into the interaction between intestinal flora and immune system, though the interpretation is as yet not simple.

INTRODUCTION

It has been pointed out by numerous authors (e.g. *Taylor* and *Heimer*, 1975; *Hiraoka* et al., 1987; *Aikens* et al., 1989; *Tanke*, 1989), that one of the major problems when viewing microscopic slides of any kind is the lack of quantitative methods of describing what is seen. Without such quantitative information, it becomes very difficult to compare results obtained by different observers, even when viewing the same slide, on the same microscope. Things invariably become worse when different microscopes and different slides are used. One particular area where quantitative measurements are useful is in immunofluorescence. Usually the titre of a serum is judged visually from immunofluorescence slides. The observer must estimate which of a series of wells, containing a dilution sequence of serum, has a positive fluorescence. The highest dilution still judged to be "positive" is designated the titre. Each observer will tend to use his or her own criteria when estimating the level of

fluorescence, and these criteria will not even remain constant with one observer. The adaptation of the eye to darkness, eye-fatigue, changes in ambient light conditions, and many other factors will tend to change a human observer's idea of what is positive or negative fluorescence. It is difficult to estimate the number or percentage of bacteria, which are positive, and impossible to distinguish autofluorescence from immunofluorescence. It has been shown by *Apperloo-Renkema* et al. (1990) that with a single observer there may be as much as a factor of four difference *on average* between duplicate measurements (i.e. 2 titre steps on a ^{2}log dilution sequence). It was also shown that a computerised system was at least twice as accurate. When estimating the morphological characteristics in a similar subjective manner, the classification problems are equally bad. Distinguishing "coccoid rods", from "slightly rod-like cocci" is just one example of a distinction that is impossible to make reliably and reproducibly with the naked eye (*Bacquero* et al., 1988). Visual assessments do of course have a solid place within microbiology, and the fact that they can be useful is not contested. Their main drawback is the unknown, but in any case large, amount of noise, or random error, inherent in such a subjective approach. The main advantage of automating the assessment of microscopic slides is the acquisition of quantitative data, which should be more accurate, for which error margins can be properly estimated, and which can be reproduced elsewhere. We can conclude that, though it is by no means useless to make visual assessments of bacteriological (and other) slides, deriving quantitative data from such slides can provide a much more solid scientific base of observations to build our theories on.

Many others have pointed this out (for a review see *Tanke*, 1989), and a lot has been done in the field of image processing of slides of eukaryote cells and tissues (e.g. *Gross* and *Loew*, 1989; *Swanson*, 1989; *Lamaziere* et al. 1993), but only recently has much work been done on the (smaller) prokaryotes, and much of that either using electron micrographs (e.g. *Vardi* and *Grover*, 1992), or bacterial colonies or cultures (e.g. *Waterhouse* et al., 1993) or limit the image analysis to a simple counting (*Singh* et al., 1989; *Evans-Hurrell* et al., 1993). Many of these image processing efforts are not very much automated, as in the case of *Vardi* and *Grover* (1992), who measured bacterial lengths by pointing at start and end point on the screen (for thousands of bacteria), or *Evans-Hurrell* et al. (1993) who counted the bacteria manually, from the video screen.

This situation lead to the design of the GRID image processing system. It was designed first of all to measure bacterial shapes from ordinary (light) microscopic slides quantitatively (*Meijer* et al., 1990). This found applications in the assessment of changes in the gut flora, both in healthy volunteers and during antibiotic treatment (*Meijer* et al., 1991a, 1991b). Extensions to the system, for use in immunofluorescence work, have been made by *Apperloo-Renkema* et al. (1990), and this work is being continued by the authors. In this paper, the set-up of the GRID system, its hardware, its software, its design goals and its applications, will be reviewed.

THE GRID SYSTEM

Hardware

When the GRID system was first envisaged in about 1983, most image processing was done on large computers (mini-computers and upwards, e.g. PDP 11-70 for the GIPSY system [*Allen* and *Terlouw*, 1981]). At that time, personal computers were generally thought of as completely inadequate for the computing speed and data storage requirements of image processing. Partly because of lack of funds, the GRID system was first conceived on an Apple II compatible computer (Unicom), with a video capture board, which could only distinguish two shades of grey (black and white), and an expansion board with a Motorola 68000 processor. The system ran under the CP/M68K operating system. On this minimal hardware set-up, all the basic functions of morphology were first implemented and tested. Much of this code is still used in our bacterial morphology measurements today. As faster platforms became more and more affordable this first system was abandoned and we moved to IBM PC-AT compatible machines. It is a fairly recent development that the most powerful IBM PC-AT compatible computers are regularly being used for image processing (e.g. *Groen* et al., 1988, *Froehling*, 1990, *Hewison* et al., 1993, *Lamaziere* et al., 1993).

At this moment 5 different computer systems are used to run GRID software in our laboratory, and a sixth has recently been installed at the Department of Dermatology, University Hospital, Groningen. Though the system could be ported to many different computer environments, our hardware consists of a IBM PC-AT compatible computers based on Intel 80286, 80386 or 80486 main processors, preferably supported by a mathematical co-processor, running Microsoft MS-DOS versions 3.30 and upwards (2.0 should work as well). The memory of the smallest (and oldest system) is 640 kilobytes (kB) but 2 megabytes (MB) is recommended as a minimum. Hard disk in our computers range from a mere 20 MB to 120 MB. Considering the data bulk gathered on the faster systems each day (60 MB of raw data!), more is better, when it comes to disk sizes. A tape-streamer is incorporated in two systems, as backup and permanent mass storage device. The key element in each computer system is an expansion board, which allows the computer to accept input from standard video cameras. Two different types of these so-called frame-grabbers are used in our system. The earlier type is the PIP-1024(A or B) frame grabber (MATROX Ltd., Dorval, Quebec, Canada). It can accept either European or American black and white video signals, and can hold up to 4 images with a resolution of 512 by 512 pixels and 256 grey levels. This board does not perform many extra image processing functions itself. The main burden of the image processing falls on the main processor of the computer. The second type is the MVP-AT board (also by MATROX). This can do everything the PIP-1024 can, but has powerful image processing functions implemented in the hardware of the board. This greatly enhances the performance of the image processing functions in the system. As an example, subtracting two images from each other takes 4 to 7 seconds on the PIP-1024 systems, but only 1/30th of a second on a MVP-AT. The MVP-AT also supports full colour (RGB) video signals, at a resolution of 512 by 512 pixels and 16.7 million colours per pixel. Quite obviously, the latter board is the preferred one. The cameras connected to the systems are almost exclu-

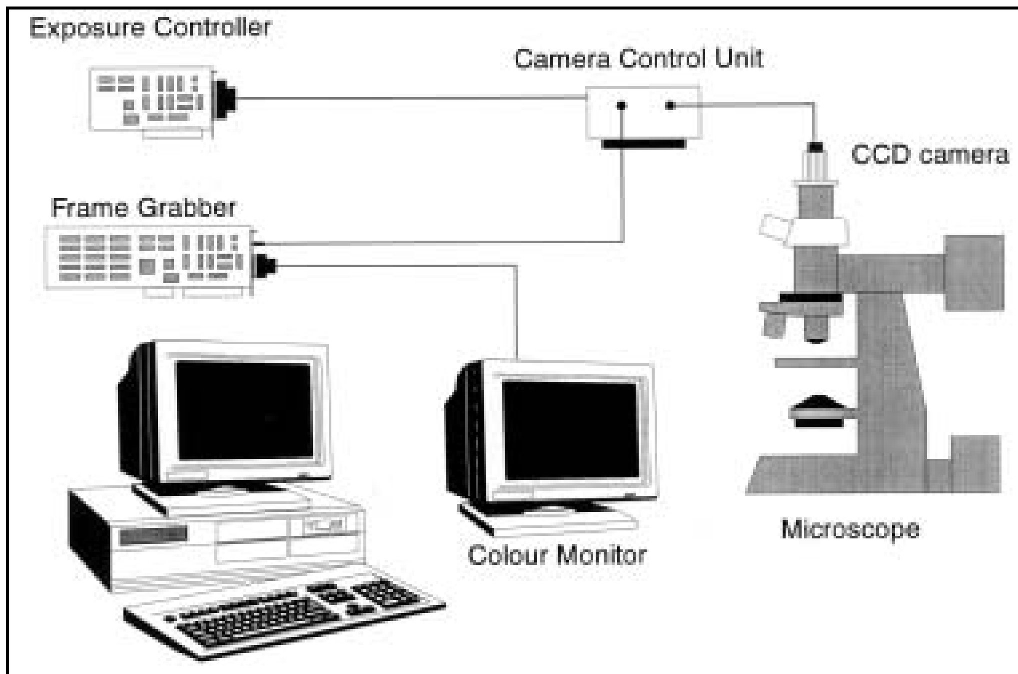

Figure 1: Hardware set-up of the GRID system. The two expansion boards shown are placed inside the personal computer. The video camera is normally attached to a microscope.

sively Loral Fairchild CCD-5000/1 cameras (Loral Fairchild, Sunnyvale, CA, USA). This is an industrial charge coupled device (CCD) camera, with the CCD chip cooled to 20 K below ambient temperature by a Peltier element. These cameras have a special purpose connector (user I/O connector) which allows access to the camera's video timing circuitry. A special expansion board designed by M.H.F. Wilkinson (*Wilkinson* et al., 1992) is connected to this connector, and allows software exposure time control. The exposure time of the camera can be increased from a single video frame (=33 ms) to any integer number of frames. This feature is used extensively in faint light detection (e.g. fluorescence). Each system has two video monitors: one is connected to the standard video output of the computer and is used for the normal dialogue, and one is connected to the frame grabber, to display the current video image, the progress of the image processing, etc. Each video camera is connected to a (fluorescence) microscope. Several types of microscopes are in use: a Leitz Orthoplan, two Olympus BH2s and a Zeiss fluorescence microscope. The Leitz Orthoplan microscope has electronically controlled shutters in front of both standard and ultra-violet light sources. These can be software controlled using an extension of the exposure control board. A diagram of the hardware is shown in Figure 1.

Software

General design considerations

Many image processing packages, such as GIPSY and AIPS in astronomy (*Allen* and *Terlouw*, 1981), and a plethora of commercial packages (e.g. *Groen* et al., 1988), require quite a bit of specific image processing knowledge from the end user. Usually, they are menu driven, or use an interpreter of

some kind to access image processing functions. Though these packages are extremely flexible and easy to use for image processing and computer specialists, they leave much to be desired for medics, biologists, and many other scientist who frequently use image processing systems. Most of these systems do allow the user to store a sequence of commands as a "macro", so it can be played back using a single command, and other systems are fully programmable, but the knowledge needed to customise the systems this way is often underestimated. It was decided that GRID should be designed as a set of programs, each capable of performing a fairly limited set of tasks, capable of running with as little user involvement as possible, and asking only those questions which all those not fully versed in the inner workings of image processing could make sense of. Making each program perform a limited set of tasks greatly increases user friendliness, by not offering too many options at once, at the expense of some flexibility. Vital in this approach was the continuous involvement of one or more programmers, with experience in image processing. The task of these programmers was to analyse the requirements of the microbiologists, and then to write programs that perform precisely the tasks required. In our view, it is a tribute to this design philosophy, that many scientists and students with little or no previous experience with computers, have collected and processed many thousands of images on our systems. Usually they could work with the system and get their data on paper within a few days.

Apart from user-friendliness, the system should be easily portable to other platforms, as hardware obsolescence proceeds at an exponential rate. Therefore, the software has been written in a layered fashion, the bottom layer shielding the hardware specific functions from the top. In this way only a relatively small set of procedures need be rewritten when moving to different hardware. The last port of the system to different hardware took only about three weeks, for a single programmer.

To ensure software durability, documentation of the inner workings is essential, and guidelines for documentation have been drawn up, though when writing quick patches for problems they are not always adhered to. This is often a problem in a single programmer environment. Too often, the short-term operation of the system takes precedence over long-term objectives, and writing documentation gets shifted to a low priority.

Application programs

As has been described, the GRID system consists of a number of programs, which perform specific image processing tasks. These programs fall into three broad categories. The first category consists of interactive image acquisition programs. Each such program is geared to a specific type of experiment. They are highly automated, and do not require much (if any) knowledge of image processing to operate them. The oldest, simply dubbed RECORD, acquires grey-scale images of bacterial slides, stained in a variety of ways, segments them automatically into objects (bacteria) and background, and stores the resulting binary images for further analysis. Alternatively, it can store grey-level information as well to allow analysis programs to measure the density of the stain in each object. The automated detection of object boundaries is the key feature of the program, which allows objective measurement of the morphology of the bacteria. The method, due to *Kittler* et al. (1985), uses the gradients in the image to determine which grey levels lie precisely

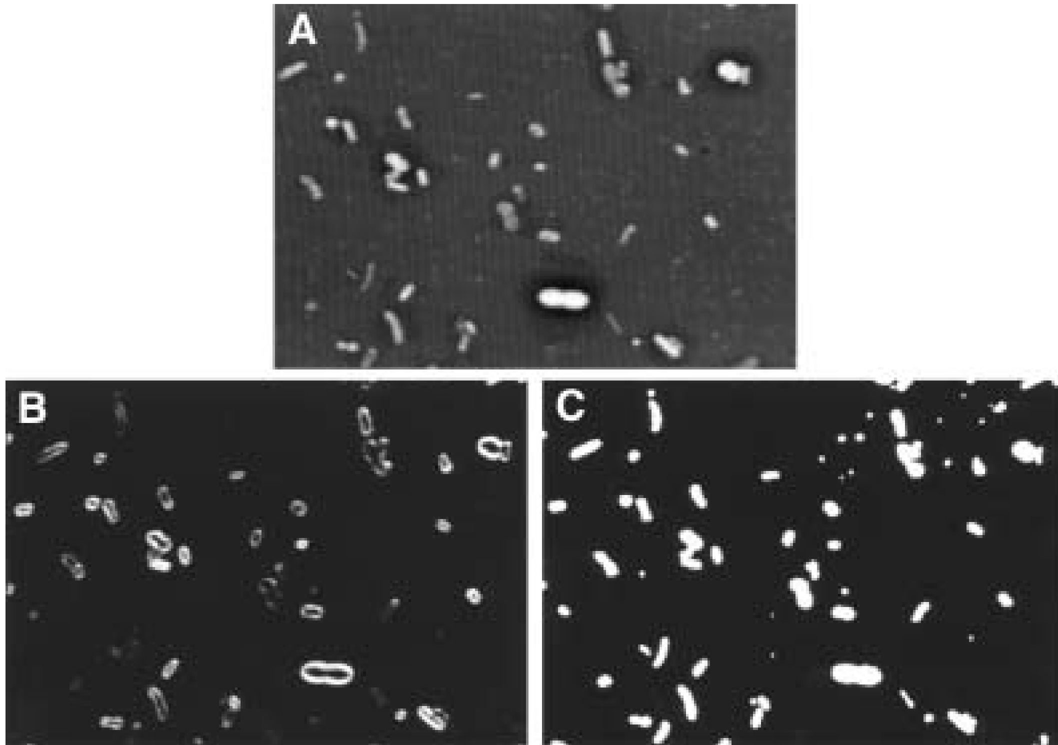

Figure 2: Nigrosin counterstained bacteria acquired by GRID system program MEDEYES. Background noise has been subtracted. Neither background nor bacteria have a uniform brightness (A).
Gradient of image "A", showing edges of bacteria. Note that certain edges are much brighter than others, indicating differences in the brightness of the bacteria (B).
Image "A" segmented into objects (white) and background (black), using local thresholds determined from image "A" and "B". Note that bacteria of different brightness have all been segmented properly (C).

on object boundaries in each region of the image. Pixels darker than these local thresholds (or lighter, depending on the stain) are considered part of an object, others are part of the background. Figure 2 shows the grey scale image, gradient image, and resulting binary image, with white representing objects, and black background.

A somewhat more complicated program, called PHASECON, geared to phase contrast combined with immunofluorescence acquisition also exists. It allows the acquisition of phase contrast and immunofluorescence images of a single field of view, at user selectable exposure times. The phase contrast image is segmented into objects and background in the same way as in RECORD. Only parts of the fluorescence image which coincide with an object in the phase contrast image, or lie within a certain distance from an object, are stored for analysis.

A second group of programs is rather simpler. They perform analysis on the images acquired by the first set of programs in a batch-oriented way. All the user must do is specify which image files must be analysed, and the programs will analyse the entire batch. This analysis is generally carried out at night. A typical system can routinely analyse 40,000 to 60,000 bacteria per day. The fastest system has done more than 100,000 in a single batch at night.

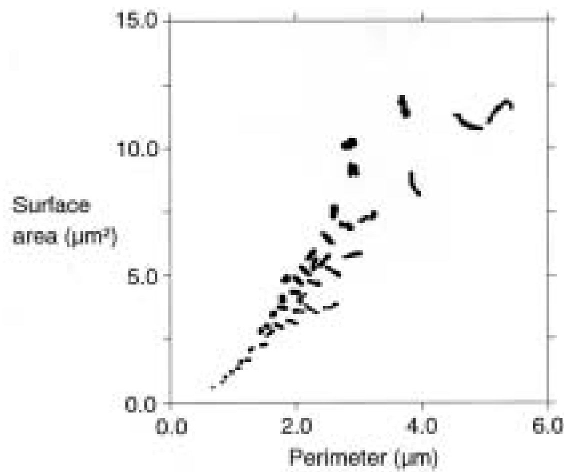

Figure 3: Modified scatterplot (Bart-O-Gram) of surface area vs. perimeter of nigrosin counter-stained faecal bacteria. An approximately quadratic relationship between the two parameters is visible.

The analysis can be broken down into three phases. In the first phase, simple morphological parameters such as width, surface area, and perimeter are measured, with or without fluorescence brightness or stain density. In second phase the raw morphological data are converted to three more or less independent shape factors dubbed F1, F2 and F3, or principle components. This method was pioneered by *Meijer* et al. (1990) in our laboratory. It has been shown that these shape factors, which are weighted averages of the logarithms of the raw data, explain more than 99% of the variability of bacterial shapes within human faecal flora, and are not highly correlated, as the raw data are (*Meijer* et al., 1991b). The difference is clearly shown in Figures 3 and 4,

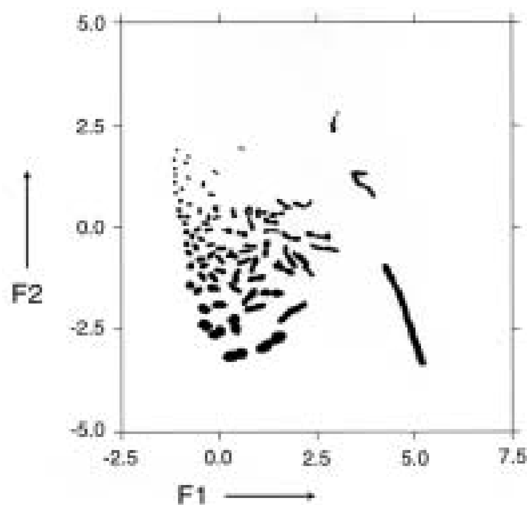

Figure 4: Bart-O-Gram of optimum shape factors F1 vs. F2. Interdependence has clearly decreased. Not all bacteria are plotted to prevent cluttering of image.

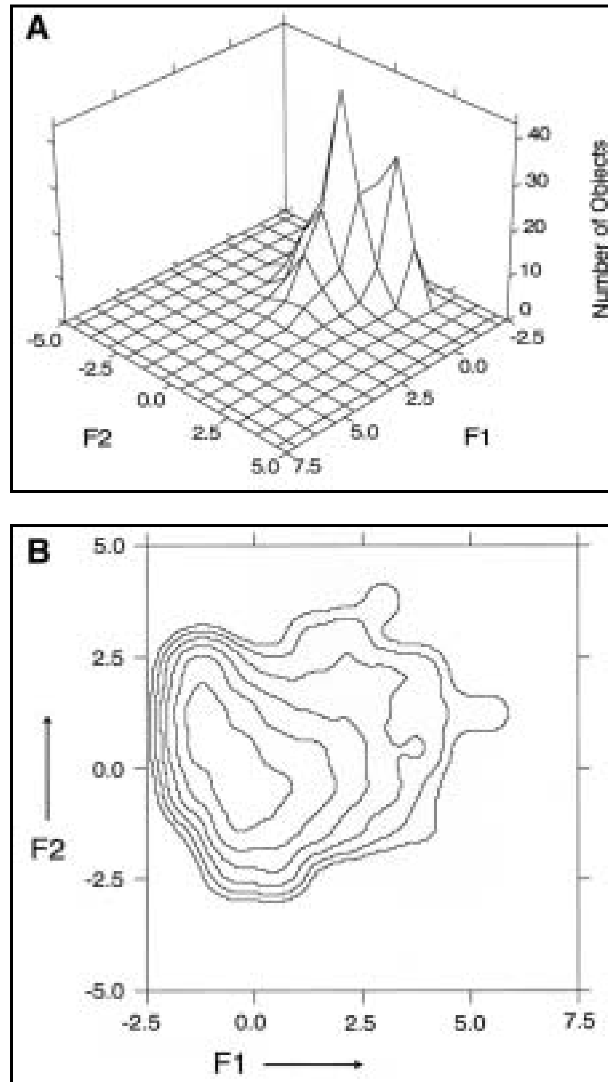

Figure 5: Surface plot of number of bacteria as a function of both F1 and F2. Omission of bacterial shapes allows more quantitative information to be shown (A).
Contour plot showing number of bacteria as a function of F1 and F2. Contours represent iso-density lines at ^{2}log intervals (B).

which show plots of perimeter vs. surface area, and F1 vs. F2. In the third phase of analysis, the processed morphological data are used to compute statistics of either the distribution of the number of bacteria or their fluorescence as a function of the shape factors. This allows quick assessment of changes in the bacterial population as a whole, either purely morphological, or in fluorescence distribution. Surface and contour plots of both number of bacteria and mean fluorescence as a function of F1 and F2 are shown in Figures 5 and 6.

The last category currently contains just one program: DATAPLOT. It is an interactive graphical data representation program, specially adapted to our image processing needs. Though our pro-

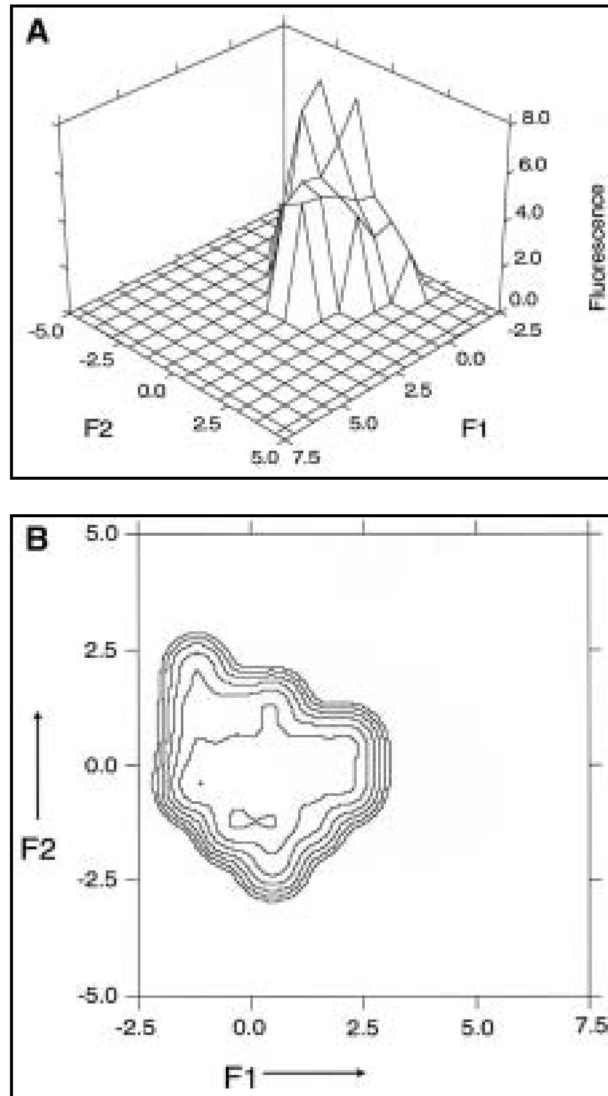

Figure 6: Surface plot of mean fluorescence (in arbitrary units) as a function of both F1 and F2 (A). Contour plot showing mean fluorescence (in arbitrary units) as a function of F1 and F2. Contours show iso-fluorescence lines at 1/2 ^{2}log intervals (B).

grams generate files which could be read by any graphical package available commercially, the number of display options found in these packages was found inadequate. DATAPLOT allows users to create a multitude of different graphs, displaying the relationships between and distributions of any two or three parameters in the data set. The simplest form of plot is the scatter plot, which can be made by most graphical packages. A modified version of these plots is also available in DATAPLOT. Instead of printing a star or other symbol at the appropriate location in the graph, the actual bacterial shapes are plotted. This type of graph (dubbed Bart-O-Gram, after the inventor *Bart*

Meijer [1990]) is shown in Figures 3 and 4. DATAPLOT can also compute and plot the distributions as shown in Figures 5 and 6, optionally adding a Bart-O-Gram as an overlay, to get an idea of the bacterial shapes.

The GRID system contains one more program: MEDEYES (*Meijer*, 1991c). It is radically different from all others in the system in that it does require image processing knowledge to utilise it to its full capability. It is an easily expandable interpreter, which is used mainly by the programmers of the system to experiment with new image processing procedures. It uses a Pascal-like language with full-blown flow control with IF-THEN-ELSE constructs, REPEAT-UNTIL, WHILE-DO and FOR loops. Any new image processing function or procedure can be added to the program, and can be tested thoroughly, in a variety of conditions, without the need for a special test program. MEDEYES is also useful when one simply wants to take a couple of snapshots of a slide, and wants to see what various image processing functions do to it.

All programs in the system have been developed in our lab, using mainly Microsoft Pascal 4.0, C 5.1, and occasionally assembly language (Microsoft Macro Assembler) as programming languages. The frame grabber functions are accessed through libraries supplied by the manufacturer (MATROX). To improve portability, these functions are never called directly, but are reached through an interface library. Two interface libraries exist at this moment: One for the MVP-AT and one for the PIP-1024. These interface libraries shield the main program modules from differences between the frame grabber boards, allowing easy upgrading of each PIP-1024 to MVP-AT systems. Frame grabbers supplied by other manufacturers could also be linked to the GRID system in this way.

The user interface

All interactive programs, except for MEDEYES, have the same user interface. This improves the user friendliness of the system, since all programs have the same "look-and-feel". The user interface, dubbed SCREENIO, is basically keyword driven, and only slightly more sophisticated than the glass teletype interface supported by standard Pascal and C. It is, however, far more robust than the standard interface of these languages, and it provides a standard screen layout. The robustness of the interface stems from the fact that the input of the user is considered to be a simple stream of characters, which is parsed for meaningful information. If the string is not considered meaningful to the program, it alerts the user to this with an error message, and re-states the question. By contrast, if the Pascal READLN statement were to be used to get a number from the user, and the user entered a character other than a digit, the program would crash immediately, and the user would, justifiably, be upset. Whenever possible, SCREENIO offers default values, which are chosen when the user only presses "enter". These values are chosen in such a way that the user can almost always just press "enter" to proceed. SCREENIO also supports menus, function keys, and special commands, and "hidden" questions. Hidden questions are questions that are never put on the screen. They are more or less internal (or advanced) features of programs, the settings of which should not be altered by inexperienced users, but which should be accessible by certain experienced users.

The screen layout is fairly basic. The screen is divided into five areas. At the top of the screen there is the INFO area, a sort of status bar. It is a single line of information on the general status of the program. The second and largest area

by far, is the display area, which is used by all programs to display any kind of data or instructions to the user. Below this is the user-type-in (UTI) area. This is the area in which all questions appear, and in which the user types his answer. Error messages appear below this in a two-line error message area. At the very bottom of the display there is a function key display.

In the most recent additions to the programs some window-oriented features have been added, to improve user friendliness even further. These windows are simple forms, which pop up on the display, and let the user edit the value of each item.

Future extensions of the system are bound to be even more window oriented, as users place higher demands on the ease of use of programs, but for relatively simple programs such as RECORD and PHASECON, the simple SCREENIO system is quite sufficient.

Applications and results

The GRID-system has been operational as a research tool for about 3-4 years now, and a growing number of publications using results obtained by it have been produced. During the first stage of its use, the system had to be validated, and its strengths and weaknesses evaluated. *Meijer* et al. (1990) first showed that the system could distinguish pure cultures of bacteria purely on the basis of the morphological distribution of the constituent bacteria. Single bacteria could not be assigned to a particular species on the basis of shape. It was also shown (*Meijer* et al., 1991b) that subtle changes in time in morphological composition of the faecal flora healthy volunteers could be detected by the system. These same fluctuations pose a lower limit to the magnitude of changes in morphology as a result of treatment or disease that can be detected. Later the system was compared to classical microbiological methods, in a study of the effect of ceftriaxone on the intestinal flora (*Meijer* et al. 1991a; *de Vries-Hospers* et al., 1991). The results of the image processing were obtained much faster than anaerobic culturing, and correlated well with the other methods. At this time the statistical processing (phase 3 of the analysis) was standardised for the morphometric part of the GRID system.

In 1989 the fluorescence package (PHASECON) became operational, and it too had to be validated first. It was first shown by *Apperloo-Renkema* et al. (1991) that the system could measure titres of circulating antibodies directed against *Enterobacteriaceae* with greater accuracy than the human observer. Later this computerised immunofluorescence technique was combined with the morphometry (*Apperloo-Renkema* et al. 1992), assessing antibody titres as a function of shape in healthy volunteers (*Apperloo-Renkema* et al. 1990a, 1990b). Though the interpretation of the results was (and is) not straightforward, the system proved to be a good tool in investigating the interaction between immune system and intestinal flora, and allowed rapid monitoring of changes in that interaction.

Both hardware and software have since been improved, and each improvement had to be validated in its turn. The addition of exposure control has boosted the sensitivity dramatically (more than a hundred fold) (*Wilkinson* et al., 1992). It has been shown that the accuracy of titre measurement has been improved (*Jansen* et al., 1993a), and titres were found to be reproducible to within 10% on a linear scale. This corresponds to an accuracy of better than 0.15 titre step on a ^{2}log dilution sequence. Impressive though this may seem, a fluorescence activated cell sorter (FACS) could probably do the same, and faster. The addition of mor-

phological information is in fact the most interesting option offered by this system, and this is the field we are currently working on. As ever, the first stage of such work is the validation of the system on healthy volunteers, and this has recently been done (*Jansen* et al., 1993b).

DISCUSSION

The current status of GRID

Image processing has proved a useful technique in many fields of science, and the GRID system is just one of many image processing systems allowing scientists to quantify what they see. The main difference with many other systems is that it has been designed for use by medics and biologists, not computer scientists. This 'push-button', rather than menu oriented, approach means that the scientists can work with programs tailor made for specific problems. They can concentrate on biological or medical issues, not computer science. The disadvantage of our approach is that it takes rather longer to get started in the first place. A commercially available package can simply be ordered, plugged in, and run. Adapting the existing system to new problems requires more work too, though we are currently streamlining our software to cope with this. The modular approach we (and many others) use means that we now have a large library of ready-for-use building blocks, that can be assembled in a variety of ways to produce different programs. The major problem in this approach is the need for a certain critical mass of programmers and other computer scientists to keep the system viable in the long run. Until now, the development has largely been a single programmer effort. In practice this means that it is difficult to keep up with new developments, and keep the software well documented and readable to others. Each programmer has his own style of programming, and what seems perfectly simple and readable code to one programmer, who has grown into a specific field of work, may be completely unclear to others. To alleviate this problem we have now contacted the department of computing science of the University of Groningen, to assist us in our work.

Scientific results and prospects

GRID has been used successfully as a tool in the research of the intestinal flora, both purely morphologically (*Meijer* et al., 1990, 1991a, 1991b), and in its interaction with the immune system (*Apperloo-Renkema*, 1991, 1990a, 1990b). It has been shown that shifts in the morphological distribution of bacteria in a population can be correlated with classical microbiological measurements. Others (*Bacquero* et al., 1988) have argued that the morphological diversity of bacteria is a measure of the biological diversity and therefore the health of the ecosystem. This morphological diversity has been quantified using the GRID system (*Meijer*, 1991a, 1991b) in a fast (within one day), reliable, and reproducible way. What morphometry cannot do is identify bacterial species.

Immunofluorescence measurement using this system is both faster and more accurate than naked eye observation, with an added advantage that the measurement need not be done in a dark room. Using the system purely for immunofluorescence does not utilise the system fully. Though more expensive, a FACS is probably a better tool, if fluorescence information is all that is required.

Adding (immuno-)fluorescence to the morphological technique is probably the most powerful option of our system. The interpretation of the results does still pose something of a problem, but inter-individual differences, or shifts in time, in the interaction between immune system and gut flora can be detected and quantified.

Future developments

At this moment we are working on multicolour fluorescence measurement, allowing such measurements as simultaneous IgA, IgG and IgM measurement on bacteria. Aside from immunofluorescence, any other fluorescent probe or monoclonal antibody could be used in this technique. This, and the simultaneous measurement of Ig and any combination of probes could open up new areas of research, though the interpretation of the data would certainly require some thought.

Work is also in progress on the file and project management. In future it should be possible to compile image databases, which the scientists could browse through at will. All data pertaining to slide, patient, and experiment should be available to the scientist immediately. Such sets of data and images could also allow computer programs to perform systematic searches for those features, which most accurately distinguish between different diseases.

CONCLUDING REMARKS

Using the GRID system, we have started to measure a number of interesting effects, which could probably not have been detected in any other way. As ever, new data pose at least as many questions as they answer, so more work, both on the theoretical and experimental side, is needed to interpret what is seen. Whatever the problems in the interpretation, image processing has greatly increased the value of our microscopes.

GRID is an effective research tool, but as with many tools it must be honed to proper sharpness to maximise its usefulness. A system developed in a laboratory requires on-site knowledge of computer programming techniques to keep it operational. A single programmer is not enough in the long run: Three or four computer experts is probably a minimum "critical mass" necessary. This is not always affordable. To ensure continuity, it is best to collaborate with a department of computing science, and such collaborations can be very fruitful.

ACKNOWLEDGEMENTS

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CHARACTERISATION OF BACTERIAL SPECIES BY IMAGE ANALYSIS

B.C. MEIJER and G.J. KOOTSTRA

Laboratory for Medical Microbiology, University of Groningen, Groningen, the Netherlands

SUMMARY

Microscopic images of pure cultures of eight different bacterial species were measured using digital image analysis. The morphometrical characteristics of cells from the same culture turned out to be varied, but consistent differences were shown to exist between species. Those differences can be used for rapid automatic detection of variations in the composition of gut microflora.

INTRODUCTION

The resident flora of the human gut is very complex: It consists of around 400 species, most of which are anaerobic (*Moore* and *Holdeman*, 1974). Culturing and identifying all of them is extremely time-consuming and therefore impractical and too expensive for routine use. Other methods for obtaining information about gut flora are needed.

Digital image analysis is a relatively new research method, which has not yet been much used in microbiology (e.g. *Sager* et al., 1988, *Reid* and *Hawthorn*, 1988, *Fernandes* et al., 1988). Similar work in industrial mycology using a Coulter counter instead of an image analyser has been reported (*van Huyns* et al., 1988). In our laboratory we intend to apply digital image analysis to the analysis of faecal flora. In very general terms our strategy is as follows: A large number (around 500) of bacterial cells are measured, yielding measurement values (e.g. its length, projected surface area, perimeter) for each cell. Every bacterium will thus be represented by a vector in the space of possible measurements. We shall call the distribution of those vectors for a population of bacteria its morphometrical distribution. From the morphometrical distribution we shall try to obtain information about the flora.

Before dealing with the complex gut flora we first study pure cultures, which are much simpler and better characterised. This approach may provide us with realistic expectations of the kind of information we may expect from image analysis when we study gut flora.

Even between cells in a single pure culture of a common species such as *E. coli* a great morphological diversity exists. *E. coli* cells are not all "Gram-negative rods": Some are coccoid, some are filamentous. Nevertheless a Gram-stained culture of *E. coli* has a characteristic microscopic appearance, to a large extent due to the morphologic diversity itself. We infer that it may be possible to characterise and distinguish pure cultures from different species by the morphometrical distributions of their bacterial cells. It is probably difficult, if not impossible, to deduce the species of one bacterium from its morphology, or

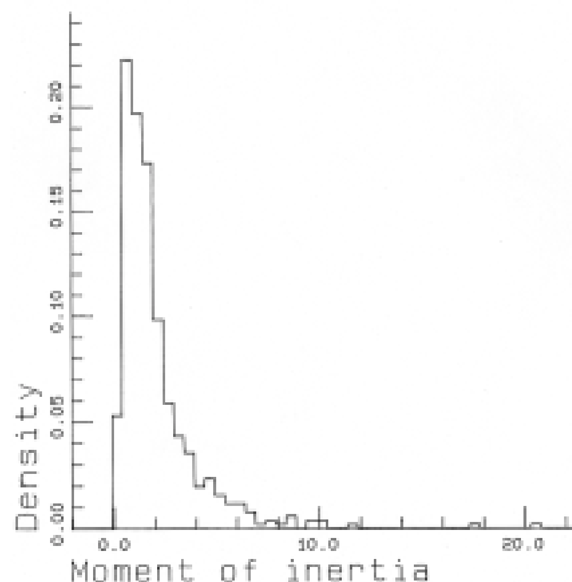

Figure 1: Distribution of the moment of inertia, I, for *K. pneumoniae*.

to disentangle the flora of the gut into it's many constituent species by morphometry.

Our present study is an attempt to characterise bacterial pure cultures of various species and culture ages. We chose aerobic species for two reasons. Firstly, they are easier to culture than anaerobes. Secondly, the questions we try to resolve by this experiment are very general: What do the morphometrical distributions for the various species look like; are they different between one species and another; do they depend on culture age; and how much information about a mixed culture can be obtained from its morphometrical distribution? Results and conclusions obtained for aerobes will guide further studies on the complex anaerobic faecal flora.

MATERIALS AND METHODS

Eight pure cultures were obtained from clinical material. They were of the following species: *Streptococcus pyogenes*, *Escherichia coli*, *Streptococcus* group D (Enterococcus), *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The identifications were made by standard laboratory methods. We subcultured the strains in BHI broth and grew them for four nights; with 24 h intervals we made nigrosin stained slides by a method modified from Fleming's (*Cruickshank* et al., 1975): We prepared staining fluid by adding 10 g of water-soluble nigrosin (GT Gurr) to 100 ml of a 0.25% solution of Tween-80. Equal volumes of culture broth and staining fluid were then mixed. A drop of the suspension was spread on a clean, fat-free microscopic slide as if making a blood smear. In such smears, bacteria show clear against a dark background. We made four slides for each strain. We recorded the time needed for the various steps in preparing the slides.

For image analysis we used a PC/AT

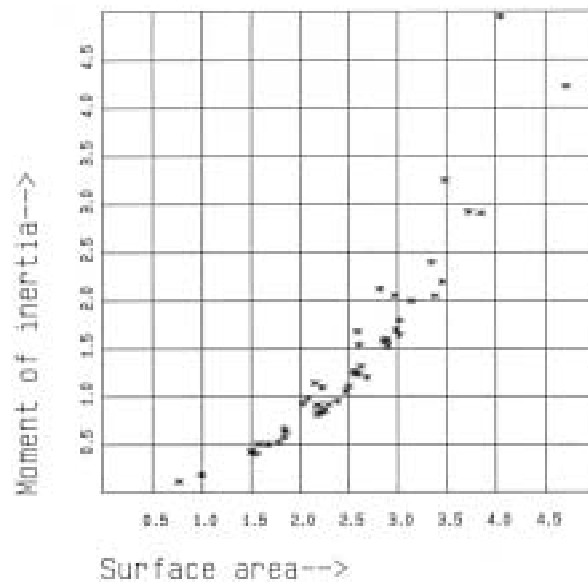

Figure 2: Scatter plot of surface area [(µm)^2] vs. moment of inertia [(µm)^4] for *K. pneumoniae*.

compatible computer equipped with a Matrox PIP 1024 video digitiser board (Matrox, Quebec, Canada). Except for the manufacturer's library, we developed the software ourselves.

For about 500 bacteria on each of the slides we measured the surface area (A), the perimeter (P), the moment of inertia (I) and the area of the convex hull (H).

DATA ANALYSIS

Linearisation and elimination of theoretical dependencies

The raw data from the measurements have undesirable properties from a statistical point of view. Firstly, the parameters have skewed distributions, as illustrated in Figure 1 for the moment of inertia of *Klebsiella pneumoniae*. Secondly, all variables depend on the size of the bacterium and so on one another. Finally, those relationships are not linear (Figure 2). New parameters were computed and logarithmic transformation was employed to overcome those problems. The process yielded four new variables: The logarithm of the area (a), the first and second form factors (f_1 and f_2), and the concavity index (c). These were used for subsequent analysis. Figures 3 and 4 show the improvements in distribution characteristics, independence on size and linearity. For a technical discussion we refer to Appendix A.

Mahalanobis transformation

For direct comparisons among species, representation of the measurements in terms of the new variables still carries some drawbacks. In particular, the measurements for a given species will scatter elliptically around the species mean, not spherically. Figure 5 illustrates this for *E. coli*, *K. pneumoniae* and *S. aureus*. We need to transform the whole plane so that the ellipses turn

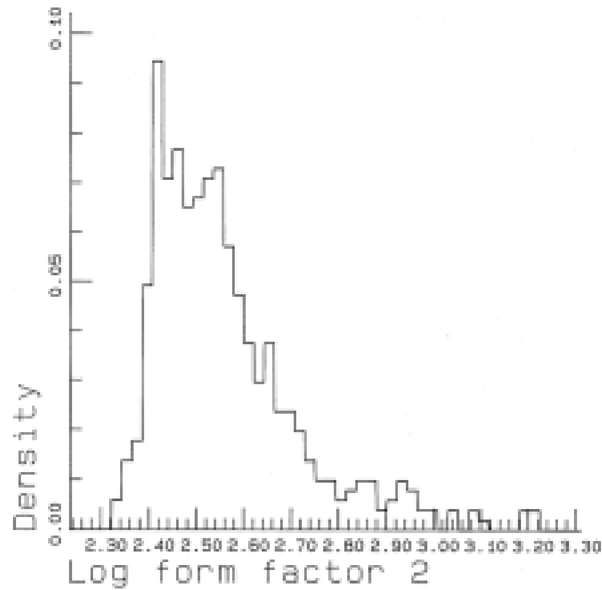

Figure 3: Distribution of the natural logarithm of the second form factor for *K. pneumoniae*.

into circles; the position of their centres will then be such that the distances among them are true measures of dissimilarity (Mahalanobis transformation; Figure 6). In the transformed space, comparison between any pair of species reduces to a comparison of the one-dimensional distributions along the axis connecting the two centres.

Secondly, we cannot be sure that keeping four variables to describe the data is ideal in terms of the ratio of sig-

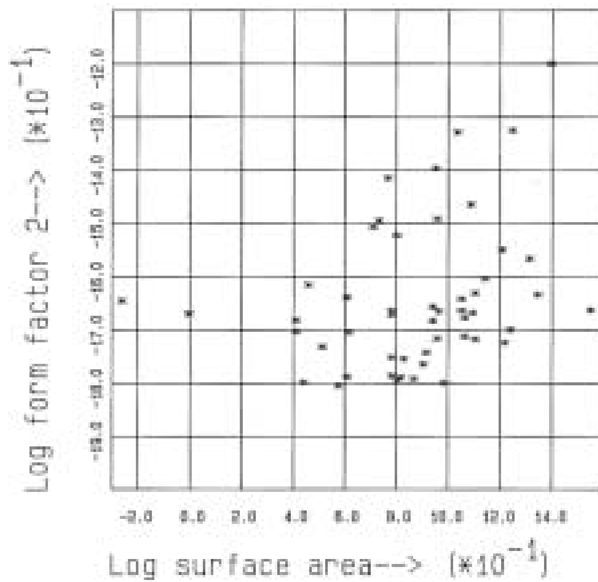

Figure 4: Scatter plot: Log surface area vs. log second form factor for *K. pneumoniae*.

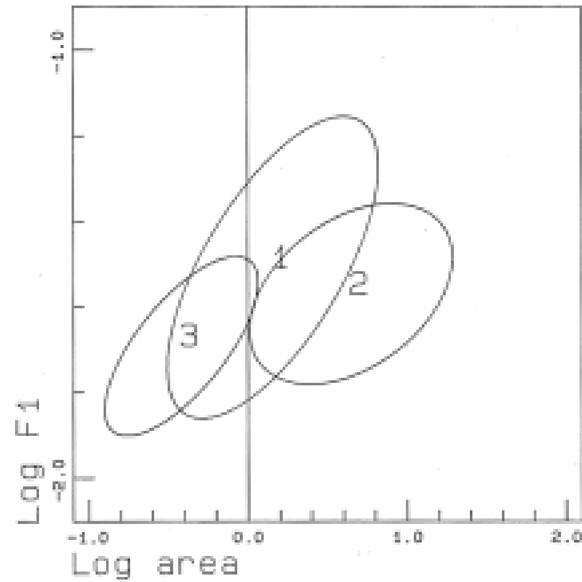

Figure 5: Scattering ellipses for *E. coli* (1), *K. pneumoniae* (2) and *S. aureus* (3) in the plane determined by area and form factor 2.

nal to noise. Besides, the analysis of mixtures, described below, requires economy in the use of variables. In Appendix B a technical method is described that combines Mahalanobis transformation and dimension reduc tion. Application of this method to our measurements yielded three new vari-

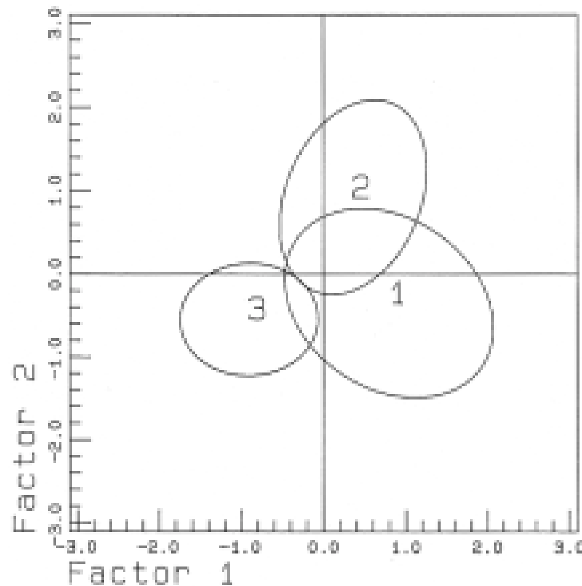

Figure 6: Scattering ellipses for *E. coli* (1), *K. pneumoniae* (2) and *S. aureus* (3) for the principal components derived from area and form factor 2.

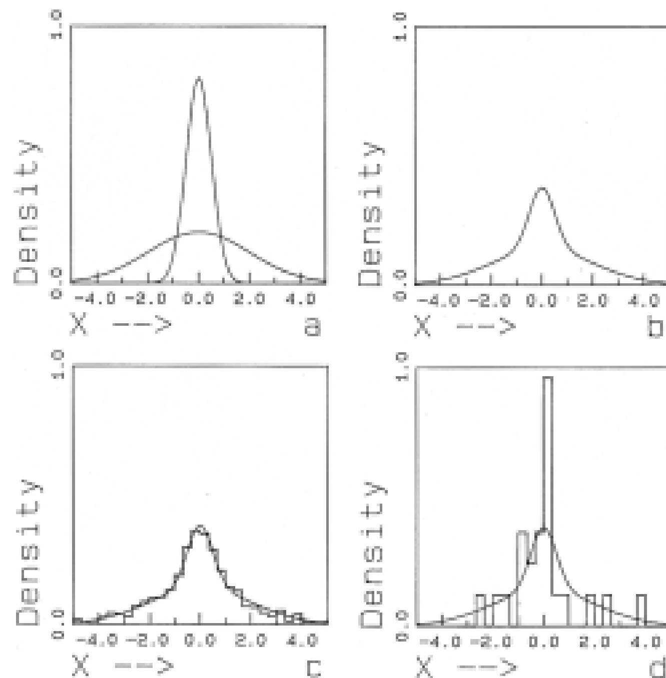

Figure 7: Simulated decomposition experiment with two easily distinguishable parent distributions. a) The two parent distributions; b) The composite distribution; c) Sample density for a sample of 1000; d) Sample density for a sample of 25.

ables: Factors 1, 2 and 3. Factor scores were computed for all the bacteria; the rest of the analysis was performed using only these factor scores.

Multidimensional scaling

At this stage, the dissimilarities among species were given as distances in three-dimensional space. For a more visual representation than a table of numbers can provide, a two-dimensional drawing was constructed by multidimensional scaling. In such drawings the distance relationships are preserved as closely as possible. For technical details we refer to *Mardia* et al., 1979.

Multivariate analysis of variance

A problem not yet addressed is the identification and quantitative description of the sources of variation in the data. How much variation can be ascribed to difference among species, to culture age and to the combination of culture age and species? Are the effects from those sources statistically significant and quantitatively important? Multivariate analysis of variance (MANOVA) was used to answer these questions. This technique is described in detail by *Mardia* et al (1979).

Decomposition of mixtures

A mixture containing many bacteria of more than one species will give rise to a morphometrical distribution, which is a weighted sum of the distributions of the constituent species. Mixtures with fewer bacteria in the same proportions will yield approximations to this sum. Figure 7 illustrates this for a simulated hypothetical pair of species; in this case it would be rather easy to determine the proportions from the larger sample. The

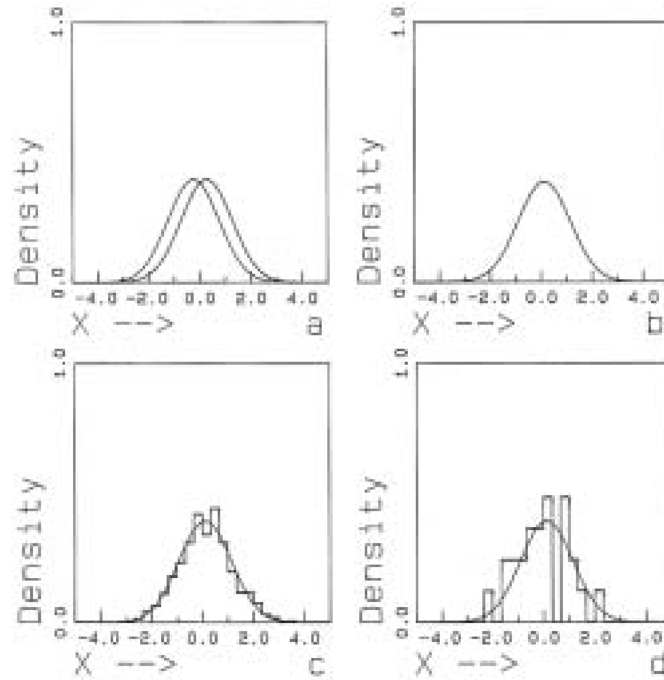

Figure 8: Simulated decomposition experiment with two similar parent distributions.
a: The two parent distributions; b) The composite distribution; c) Sample density for a sample of 1000; d) Sample density for a sample of 25.

smaller sample does not permit accurate reconstruction. Note that both distributions have the same mean: This does not prevent us from decomposing the mixture. In Figure 8 the differences between the species distributions are subtler, and decomposition is more difficult. The results of both simulation experiments are given in Table 1.

A statistical approach to this problem makes use of the maximum likelihood principle. The proportions from the various species assumed to be present in the mixture are adjusted so that the likelihood for the measurements to be as they are is maximal. For a technical description we refer to Appendix C.

Table 1: Results of one-dimensional simulation

Experiment	Mean	Variance	True proportions	Estimated proportions (n=1000)	Estimated proportions (n=25)
1	0.0	0.25	0.7	0.690 (0.03)	0.47 (0.15)
	0.0	4.0	0.3	0.310 (0.03)	0.53 (0.15)
2	-0.25	1.0	0.4	0.345 (0.04)	0.6 (0.4)
	0.25	1.0	0.6	0.655 (0.04)	0.4 (0.4)

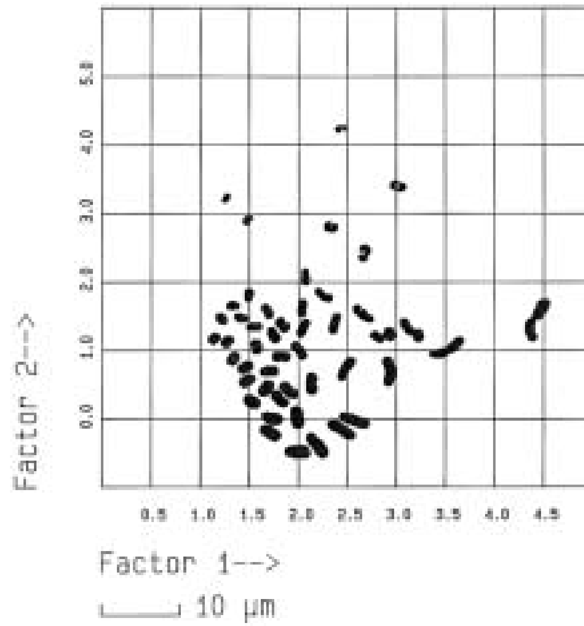

Figure 9: Modified scatter plot of *Klebsiella pneumoniae*: The bacteria have been drawn on the spots corresponding to their factor scores.

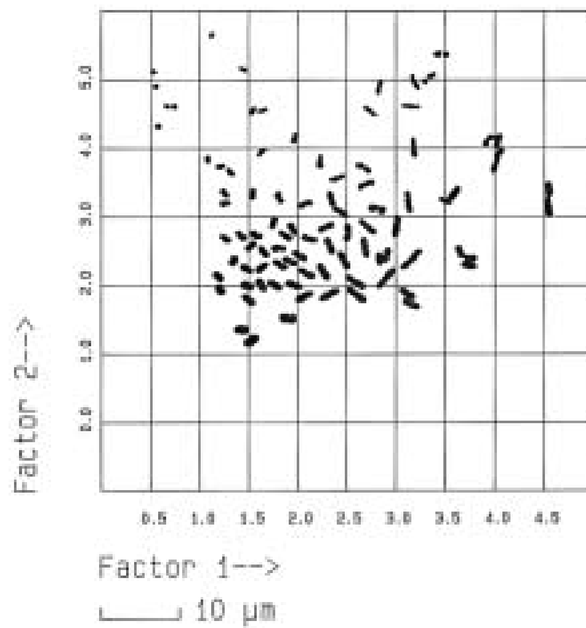

Figure 10: Modified scatter plot of *Pseudomonas aeruginosa*: The bacteria have been drawn on the spots corresponding to their factor scores.

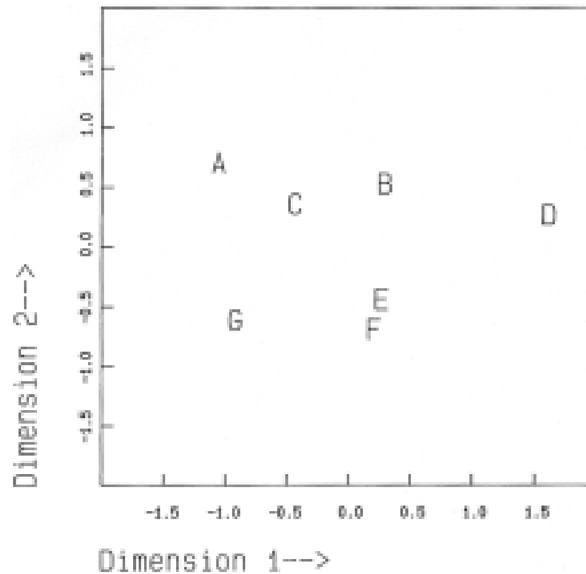

Figure 11: Distances between species centroids, drawn in two dimensions by multidimensional scaling. Meaning of the letters: A: *S. pyoqenes*. B: *E. coli*. C: *Enterococcus* sp. D: *K. pneumoniae*. E: *P. mirabilis*. F: *P. aeruginosa*. G: *S. aureus*.

To test the method, we prepared two random samples from the data files of the seven species, containing known proportions from each, and then computed maximum likelihood estimates for the proportions. The first sample contained 140 bacteria from each of the seven species. In the second only *Streptococcus pyogenes*, *E. coli* and *S. aureus* were present: 330 of each.

RESULTS

Twenty slides could be made in 6 h. Recording and measuring the images from those twenty slides took 26 additional hours, 11 of which required the presence of a human operator.

In Figures 9 and 10 we give two examples of the distribution of forms from pure cultures. Both figures are modified scatter plots in which the bacteria are drawn on the spots corresponding to their first two principal component scores.

Basic statistics of our measurements are given in Table 2. The centroids in principal component space for the species were all significantly different from one another ($p<0.1\%$). Table 3 records the standardised distances between species centroids: The unit of distance on each component is the pooled within-species standard deviation for that component. We derived Figure 11 from the distance table by multidimensional scaling.

The results of variance analysis are summarised in Table 4. We see that the effects of species, culture age and the combination of both are all significant, but the species effect dominates the others by a factor of at least 10.

Among all species distribution moments of up to third order were compared. Of the 399 comparisons made, only 39 yielded no significant difference at the 5% level (Student's t test).

Table 2 : basic statistics for the slides

Species	Day	N	Area (μm^2)	First form factor (-)	Second form factor (-)	Concavity factor (-)
S. pyogenes	1	631	1.50 (0.04)	16.7 (0.30)	0.25 (0.004)	0.15 (0.009)
	2	719	1.41 (0.04)	16.7 (0.30)	0.26 (0.005)	0.14 (0.007)
	3	615	1.47 (0.05)	16.8 (0.30)	0.26 (0.006)	0.15 (0.010)
	4	680	1.91 (0.06)	17.9 (0.30)	0.27 (0.005)	0.17 (0.009)
E. coli	1	380	2.25 (0.05)	16.4 (0.20)	0.26 (0.004)	0.08 (0.004)
	2	473	1.94 (0.06)	17.2 (0.30)	0.29 (0.006)	0.09 (0.005)
	3	493	2.07 (0.06)	18.6 (0.30)	0.31 (0.007)	0.14 (0.007)
	4	572	2.06 (0.05)	17.3 (0.20)	0.28 (0.005)	0.10 (0.005)
Enterococcus	1	418	1.97 (0.04)	18.1 (0.30)	0.30 (0.006)	0.17 (0.010)
	2	549	1.64 (0.04)	16.7 (0.30)	0.26 (0.004)	0.13 (0.007)
	3	498	1.61 (0.04)	16.4 (0.30)	0.26 (0.005)	0.13 (0.007)
	4	692	1.39 (0.04)	14.8 (0.20)	0.22 (0.003)	0.09 (0.005)
K. pneumoniae	1	508	2.93 (0.04)	13.1 (0.10)	0.20 (0.002)	0.04 (0.003)
	2	673	2.04 (0.04)	14.6 (0.20)	0.22 (0.002)	0.08 (0.003)
	3	811	2.05 (0.04)	14.1 (0.10)	0.21 (0.002)	0.06 (0.003)
	4	293	2.18 (0.07)	13.6 (0.20)	0.21 (0.003)	0.05 (0.004)
P. mirabilis	1	463	1.50 (0.03)	14.7 (0.20)	0.23 (0.003)	0.06 (0.003)
	2	634	1.50 (0.03)	14.9 (0.20)	0.23 (0.002)	0.07 (0.0031
	3	800	1.21 (0.02)	15.0 (0.20)	0.24 (0.003)	0.07 (0.003)
	4	1000	1.41 (0.03)	15.6 (0.20)	0.24 (0.003)	0.08 (0.004)
P. aeruginosa	1	721	1.41 (0.02)	15.5 (0.10)	0.25 (0.003)	0.07 (0.003)
	2	970	1.17 (0.02)	14.7 (0.10)	0.23 (0.002)	0.05 (0.003)
	3	1442	1.07 (0.01)	14.3 (0.07)	0.22 (0.001)	0.05 (0.002)
	4	1732	0.94 (0.01)	13.3 (0.05)	0.20 (0.001)	0.04 (0.001)
S. aureus	1	626	0.44 (0.01)	12.5 (0.10)	0.18 (0.002)	0.03 (0.003)
	2	865	0.88 (0.01)	13.6 (0.10)	0.20 (0.002)	0.07 (0.003)
	3	565	0.77 (0.02)	13.1 (0.10)	0.19 (0.002)	0.05 (0.004)
	4	542	0.85 (0.02)	13.1 (0.10)	0.19 (0.002)	0.05 (0.004)

The numbers between parentheses are the standard errors for the estimated means.

The results of our analysis of mixed data files are summarised in Table 5.

Determination of the most likely species for the 28 slides yielded correct classifications for all slides.

DISCUSSION

From the modified scatter plots we see that the variables measured reflect differences in shape and size. The morphometrical distribution for *Klebsiella* is, for example, easily distinguished from that for *Pseudomonas*. On the other hand, there is a substantial area in which both distributions overlap.

Table 3 : Distances between centroids for species in principal components

	1	2	3	4	5	6
1 S. pyoqenes	0.000					
2 E. coli	1.382	0.000				
3 Enterococcus sp.	0.642	0.865	0.000			
4 K. pneumoniae	2.145	1.593	1.595	0.000		
5 P. mirabilis	1.563	0.839	0.962	1.382	0.000	
6 P. aeruqinosa	1.696	1.053	1.148	1.614	0.291	0.000
7 S. aureus	1.172	1.607	1.020	2.094	1.145	1.xxx

The simplest mixed data file, containing only three dissimilar species, is dealt with satisfactorily (see Table 5, columns II and III). The estimates for the proportions are correct within their error margins, both when all seven species are used, and when only the three species actually present are employed to explain the moments of the resultant morphometrical distribution. The mixture of all seven species however is not resolved by our method. Note especially the obviously incorrect results for *Proteus* and *Pseudomonas*. When species are morphologically similar, there is not enough information in the mixture to distinguish between them. Obviously this problem depends on the number of species used: The similarity between a given species and the one most resembling rises with the size of the group to choose from.

Normal gut flora may contain up to about 400 different species. Even when many bacteria are measured, we cannot expect accurate estimates for the num-

Table 4: Analysis of variance

Source of variance	Degrees of freedom	Covariance matrix			F	Wilks' lambda
Species	6	372.4			414.54	
		-280.0	926.5		1018.31	
		88.2	-23.0	475.5	520.88	
						0.583
Age	3	10.5			11.72	
		0.2	70.0		76.98	
		15.9	7.1	25.0	27.39	
						0.982
Species and age	18	30.1			33.47	
combined		-21.4	78.4		86.15	
		6.4	12.5	21.0	23.03	
						0.880
Residual	19337	0.898				
		0.001	0.910			
		-0.007	0.012	0.913		

Table 5: Analysis of mixed data files

Species	I*			II			III		
	True	Comp	(SE)**	True	Comp	(SE)	True	Comp	(SE)
S. pyogenes	0.14	0.12	(0.02)	0.00	0.03	(0.03)	0.00	-	
E. coli	0.14	0.29	(0.04)	0.33	0.32	(0.02)	0.33	0.32	(0.02)
Enterococcus sp.	0.14	0.03	(0.04)	0.00	0.01	(0.03)	0.00	-	
K. pneumoniae	0.14	0.21	(0.03)	0.33	0.35	(0.02)	0.33	0.32	(0.02)
P. mirabilis	0.14	-0.37	(0.08)	0.00	0.00	(0.20)	0.00	-	
P. aerigunosa	0.14	0.54	(0.10)	0.00	0.00	(0.20)	0.00	-	
S. aureus	0.14	0.16	(0.02)	0.33	0.30	(0.03)	0.33	0.36	(0.02)

*: I = equal numbers of all species were used for the mixture.
II = species 2, 4 and 7 were present in equal numbers; data from all species were used to resolve the mixture.
III = The same mixture as in II, now resolved using only data from the species present.
**: True: true proportions.
Comp: computed proportions.
SE: estimated standard error for the computed proportions.

bers of all those species present in a specimen. On the other hand, we have shown the species to differ with regard to the means and higher moments of their morphometrical distributions. Those measures will therefore be sensitive to the balance between species composing the microflora.

A distinguishing and biologically important property of normal gut flora is its morphological diversity (*Baquero* et al., 1988). Loss of diversity will result in a narrower distribution. Digital image analysis may then provide a fast and reliable indication of diversity loss, such as occurs in overgrowth or with the therapeutic use of antibiotics.

The modified scatter plot is useful as an aid to the human viewer. It provides a systematical survey of large numbers of bacteria, grouped automatically according to their size and shape. Moreover, when viewed from a larger distance, it gives an indication of the form of the morphometrical distribution of all the bacteria together. Plots of this kind may be the most useful products of morphometrical analysis of mixed floras.

The discrimination between species by image analysis, though rather successful in our small group of species, is not practically useful. When more species are considered, morphometrical space would quickly get too crowded for accurate identification to be possible. In our view the great effort is needed to build a database and to chart the effects of environmental influences such as antibiotic use on bacterial shapes is not worthwhile.

What counts in practice is a simple and rapid method to determine a significant change in the composition of the intestinal microflora, for instance during antibiotic therapy. Some antibiotics, such as the β-lactams, influence the shape of bacteria directly, and so could disturb the morphometrical distribution of a strain, and its analysis. For the analysis of mixed flora this is problem is not as serious as it seems. If a certain strain in the flora is morphologically changed by the antibiotic, it is to be expected that the strain will not reproduce as effectively as it did before the therapy. It will therefore be overgrown by more resistant strains, whose morphol-

ogy remains unchanged. For this reason, we may disregard the direct effect of antibiotics on morphology.

Pure bacterial cultures display consistent differences in morphometric characteristics, as determined by image analysis. These differences are useful for the automatic detection of variations in gut flora. The method is fast enough for experimental use in a clinical setting. With more efficient image processing machines practical applicability is likely to improve.

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APPENDIX A:
DERIVED VARIABLES; LOGARITHMIC TRANSFORMATION

The following formulae were used to find the transformed variables a, f_1, f_2 and c:

$a = \ln A$, the logarithm of the area ; {A.1}

$f_1 = 2 \ln P - \ln A$, the first form factor; {A.2}

$f_2 = \ln I - 2 \ln A$, the second form factor; {A.3}

$c = \ln H - \ln A$, the concavity factor. {A.4}

The first variable (a), measures size. f_1 indicates the irregularity of the contour; f_2 reflects circularity of the overall form of the bacterium, and c will be large if the bacterium is curved or otherwise concave.

APPENDIX B:

MAHALANOBIS TRANSFORMATION AND SPECTRAL DECOMPOSITION

We postulate that the measurements from our seven species are distributed according to N(μi, Σ): The covariances are assumed equal. The Mahalanobis distances between groups are given by

$$D_{ij}^2 = (\mu_i - \mu_j)' \Sigma^{-1} (\mu_i - \mu_j) \qquad \{B.1\}$$

We wish to transform the original variables so that the Euclidean distance expressed in the new variables equals the Mahalanobis distance. An example of such a transformation, transforming old x to new y, would be

$$y = \Sigma^{-1/2} x$$

but, since the orientation of the new axes does not matter, the general form is

$$y = \Sigma^{-1/2} R x \qquad \{B.2\}$$

with R an arbitrary orthogonal (rotation or reflection) matrix. Now, the covariance matrix Σ can be written

$$\Sigma = \Gamma D \Gamma'$$

where Γ is the matrix of eigenvectors (being orthogonal, it effects a rotation or reflection), and D the diagonal matrix of eigenvalues. We have

$$\Sigma^{-1} = \Gamma D^{-1} \Gamma'$$

and

$$\Sigma^{-1/2} = \Gamma D\Sigma^{-1/2} \Gamma' \qquad \{B.3\}$$

Now, if we right-multiply both sides of {B.3} by Γ we get

$$\Sigma^{-1/2} \Gamma = \Gamma D^{-1/2} \qquad \{B.4\}$$

which can be fitted in {B.2}to yield

$$y = \Sigma^{-1/2} \Gamma x = \Gamma D^{-1/2} x \qquad \{B.5\}$$

as one of the transformations having the desired effect on distances. This form has the advantage that it is equivalent to transforming to principal components and scaling the components' variances to unity; this can be done using a multivariate statistics package. Note that it is necessary to subtract the group means before estimating the pooled within-group co-variance. The transformation should be performed without subtracting group means, however.

Nothing can be said in advance about the discriminatory value of separate principal components; although a component has a low associated eigenvalue, it may distinguish well between groups. If reduction of dimensionality is desired, one must first transform to canonical variates, discard one or more of them, and then apply the transformation described above. This is what we did for the present study, keeping three of the four dimensions.

APPENDIX C: DECOMPOSITION OF MIXTURES

Given a mixture containing bacteria from n different species in unknown proportions, we wish to compute the proportions from the morphometrical distribution of the mixture. We assume that the morphometrical distributions of the species themselves are known with complete accuracy.

Let the distribution of measurements x for species k be given by the probability density function (p.d.f.) $f_k(x)$; the p.d.f. for a mixture will then be

$$f(x;\theta) = \sum_{k=1}^{n-1} \theta_k f_k(x) + \left(1 - \sum_{k=1}^{n-1} \theta_k\right) f_n(x) \qquad \{C.1\}$$

in which the θ_k are the proportions. Note that $f(x;\theta)$ is a bona fide p.d.f. only when all the weights θ_k are between 0 and 1, and the value of the first k-1 weights does not exceed 1. The log likelihood for the complete data matrix X (containing m measurements), given θ, will be

$$l(X;\theta) = \sum_{i=1}^{m} \ln f(x_i;\theta) \qquad \{C.2\}$$

Numerically, a value of θ can be found to maximise $l(X;\theta)$; statistical theory (see e.g. *Mardia* et al. 1979) even gives an expression for the covariance of θ. The derivation of this expression involves the expectation operator and differentiation under the integral sign; it is valid only under certain regularity conditions. In our case, these conditions are met only when all the weights are between 0 and 1.

FLUOROMORPHOMETRICS: A NEW APPROACH IN CHARACTERISING FAECAL FLORA

GIJSBERT J. JANSEN and MICHAEL H.F. WILKINSON

Laboratory for Medical Microbiology, University of Groningen, Groningen, The Netherlands

SUMMARY

Using image analysis and quantitative immunofluorescence, three new methods in characterising faecal microflora are presented. These methods are: Morphological analysis of faecal objects, measurement of titres of circulating Ig against faecal antigens and the combination. Morphological analysis is the least sensitive approach, which is only applicable when large fluctuations in the morphological composition of the faecal microflora have to be monitored. Biological fluctuations in titres of circulating Ig against faecal antigens can be performed at a statistically significant level for IgM and IgG and the distribution of titres of circulating IgG over 144 morphological distinct subsets of faecal bacteria yields a unique pattern in a group of ten volunteers.

INTRODUCTION

Research concerned with the interaction between gutflora and the immune system of the host is a rapidly evolving field since the importance of the gutflora in the aetiology of rheumatoid arthritis (*Hazenberg* et al., 1992), nosocomial infections (*Cerra* et al., 1992), Crohn's disease (*Giaffer*, 1991) and colonic carcinogenesis (*Lidbeck* et al., 1992) is recognised. Thereupon, detailed knowledge of the immunological aspects of the host-gutflora interaction may also explain the decrease in infection risk after the oral intake of viable microorganisms ('probiotics') which is observed on several occasions in different species of vertebrates, including humans (*Metchnikoff*, 1907, *Gorbach*, 1987, *Pollman*, 1986, *Rusch*, 1986).

Earlier research by Perdigon and coworkers in mice has revealed that a change in the bacteriological composition of the gutflora results in a significant response of different immunological systems. For example, the oral intake of viable lactic acid bacteria results in activation of macrophages (*Perdigon*, 1986), a NK-cell response (*Kato*, 1984) and elevation of titres of circulating IgG directed against the latter (*Perdigon*, 1988).

In these experiments, pure cultures of lactic acid bacteria were used as antigenic substrate. As a result of this experimental design, the *in situ* processing of surface antigenic epitopes on the lactic acid bacteria by bacterial enzymes present in the digestive tract, is not taken into account.

In order to overcome this problem a method was developed which enables *in situ* quantification of the total amount of circulating (or mucosal) antibodies bound to faecal bacteria (*Apperloo-Renkema* et al., 1991a). In addition, the morphology of the faecal objects (pre-

dominantly bacteria) can also be quantified (*Meijer*, 1989). Synthesis of these two strategies has - on a semi-quantitative level - been accomplished by *Apperloo-Renkema* and co-workers (1992).

Further quantification of the combined fluorimetrical and morphometrical method, or 'fluoromorphometrical' method, has been performed by *Jansen* and co-workers (1993a).

The morphometrical, the fluorimetrical and the fluoromorphometrical method have been applied in projects concerned with the interaction between host organism and gutflora. Using the morphometrical method, *Meijer* and co-workers (1992) observed a significant decrease in the diversity of shape of faecal bacteria after the host received antimicrobial chemotherapy. Using the fluorimetrical method, *Jansen* and co-workers (1993b) were able to monitor longitudinal fluctuations in titres of circulating Ig against gutflora at a significant level. Using the fluoromorphometrical method, *Apperloo-Renkema* and co-workers (1992) found that the titres of serum Ig directed against 28 morphologically distinct groups of faecal bacteria could be quantified reproducibly and that the pattern of 28 titres is unique per pair of serum and gutflora.

In this paper the morphometrical, fluorimetrical and the fluoromorphometrical method will be presented simultaneously. Furthermore, an overview of previously obtained results using the three methods mentioned above will be given.

METHODS

Hardware

Although both hardware and software have been described previously (*Jansen* et al., 1993b), they will be mentioned here briefly. An ultra-violet microscope equipped with a phase contrast condensor, a 100-watt mercury lamp and a CCD video camera were used. Furthermore, the system comprises of a Compaq deskpro 80486 microcomputer with 8 Mb RAM, a MATROX MVP/AT image-processing board and an exposure-control expansion board interfacing the computer to the CCD. With this board, which was developed in our laboratory (*Wilkinson*, 1993), the integration time of the camera can be increased from one video frame (= 1/30 s) to any integer number of video frames. Analysis of the phasecontrast image of a microscopic slide results in a binary image of 512x512 pixels, containing only background and faecal objects. In addition, under ultra violet illumination, a fluorescence image is generated containing the fluorescence intensities belonging to the objects in the accompanying binary image.

Morphometrical analysis

Description of the faecal objects in the binary image which result from the phase contrast image after digital image processing, requires quantitative morphometrical parameters. We have used four morphometrical parameters derived (*Meijer*, 1991a) which are based upon the area (A), perimeter (P), moment of inertia (I) and area of convex hull (H) of the faecal objects. The moment of inertia is defined as:

$$I = \sum_i \left[(x_i - x)^2 + (y_i - y)^2 \right] \qquad (1)$$

In which the x_i are the x co-ordinates of the pixels belonging to the object, and x is their mean; likewise for y_i and y. Based on these four measurements, the morphological parameters derived were:

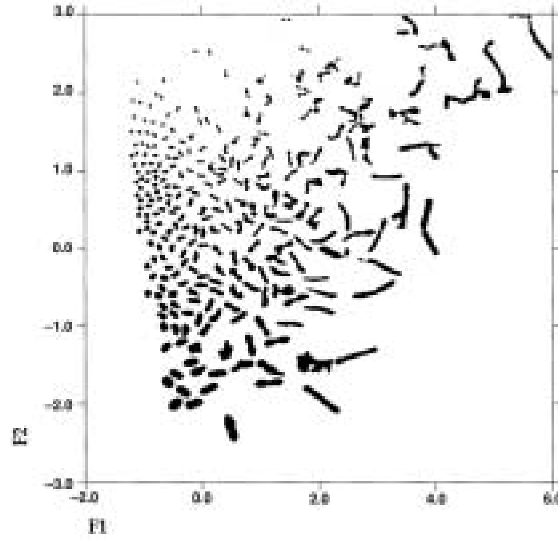

Figure 1: Morphometrical plot. Objects in the morphometrical region are represented as bacteria instead of symbols. For reasons of clarity some bacteria are omitted.

$$a = \log A \tag{2}$$

$$f1 = 2\log P - \log A \tag{3}$$

$$f2 = \log I - 2\log A \tag{4}$$

$$c = \log H - \log A \tag{5}$$

Four principal component scores (F1, F2, F3 and F4) were calculated from these morphological parameters. The matrix of transformation coefficients applicable on the entire dataset was created previously by Meijer et al. using 58 samples of faecal bacteria from nine healthy volunteers. It was decided to use two principal components (F1 and F2) because these two already explain most (i.e. 93.5%) of the original variance. Because principal components F1 and F2 are linearly unrelated they define a morphometrical plane. Although the morphometrical plane is infinite by definition, we focus attention only on the morphometrical region defined by $-2<F1<8$ and $-5<F2<5$ because over 99% of the faecal objects fall into this part of the morphometrical plane. The morphology of objects present in a representative faecal sample and their localisation in the morphometrical region is depicted in Figure 1. Then - in order to measure the variety of bacterial form in the morphometrical plane - the morphometrical entropy (S) was calculated. The entropy is computed by dividing each axis of the morphometrical region in sixteen equally sized intervals, so that the morphometrical plane becomes divided into 16x16 fields. For each field we compute:

$$p_i = n_i/N \tag{6}$$

This p-value comprises the probability that a bacterium from the specimen will yield a datapoint falling into morphometrical field i (n_i is the number of bacteria in cell i; N is the total number of bacteria in the dataset). The morphometrical entropy is now estimated by:

$$S = -\sum_i p_i \log p_i \tag{7}$$

When the distribution of datapoints over the morphometrical region is homoge-

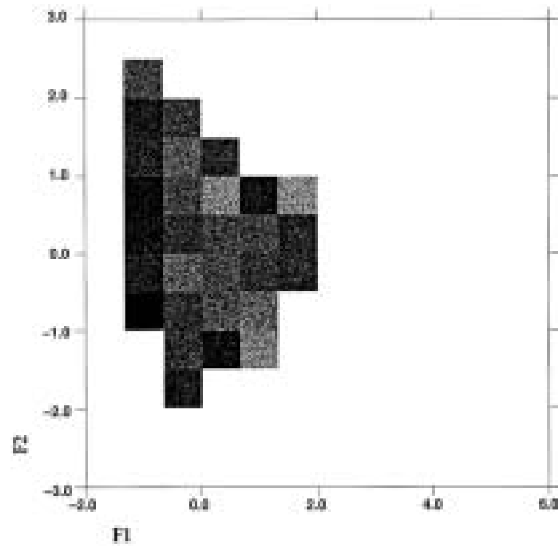

Figure 2: Density plot. Morphometrical region divided into 144 discrete fields or morphotypes. The titre of circulating IgG bound to the bacteria within a morphotype is proportional to the intensity of grey-dithering per field. Grey-levels are relative i.e. the morphotype containing the objects which bind circulating IgG to the most becomes black. Morphotypes containing objects which bind no circulating IgG are white.

nous the value of S is large and when the location of the datapoints is confined to a small area of the morphometrical region, the value of S is small.

Fluorimetrical analysis

For each faecal object in the phasecontrast image, the average grey-value of the corresponding pixels in the fluorescence image is calculated. Then the average grey-level of the pixels in the area which surrounds the objects to a distance of eight pixels is computed, excluding any pixels belonging to other objects. These two averages are then subtracted and the difference, multiplied with the serum dilution factor, is called the fluorescence level. With this method noise due to local differences in background fluorescence is eliminated. For each measurement the median fluorescence level of at least 1000 faecal objects is determined. The median instead of the mean was employed to deal with occasional outliners in the fluorescence distribution.

The performance of this method - using autochthonous polyclonal circulating antibodies directed against faecal flora - has recently been validated (*Jansen* et al., 1993b).

In serological procedures it is common practice to express antibody concentrations as titre. Titre is defined as the inverse of the dilution which is needed to satisfy some arbitrary condition. This arbitrary condition is also designated as the threshold value. In our case, titre may be defined as an arbitrary fluorescence level divided by the actual fluorescence level although we prefer to use the median grey-level because this is a continuous measure of antibody concentration, while the titre depends on a threshold-value.

Fluoromorphometrical analysis

Combining both the morphometrical and the fluorimetrical analysis of faecal flora has been done by *Apperloo-*

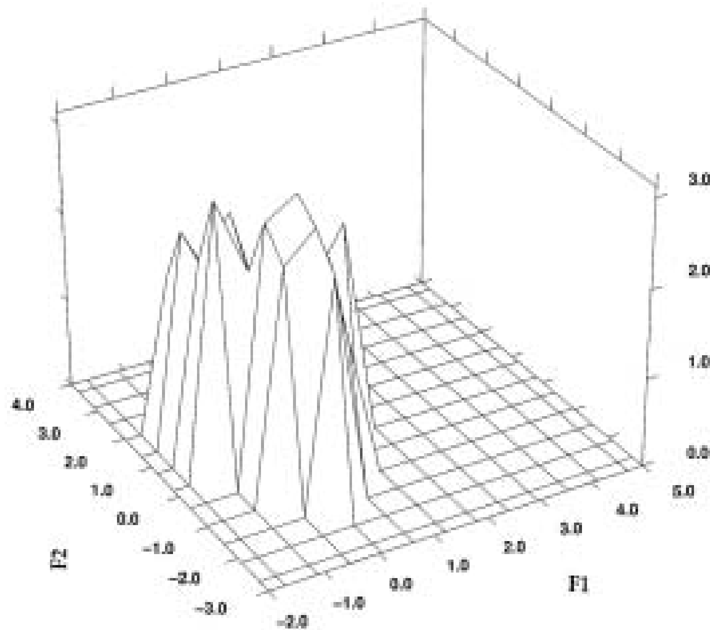

Figure 3: Hiddenline plot. Titres of circulating IgM over bacteria in the morphometrical region represented by the peak height in the third dimension.

Renkema and co-workers (1992). In their study the binding of molecules of circulating Ig over 28 morphologically distinct subsets of faecal bacteria - defined by 28 different, non- overlapping, F1- and F2-ranges - was measured at a semi-quantitative level. *Jansen* and co-workers (1993a) further optimised this fluoromorphometrical method. In their approach, the morphometrical region (i.e. -2<F1<8 and -5<F2<5) is arbitrarily divided into 12x12 equally sized squares. Each square is then called a 'morphotype' because objects within one morphotype appear uniform to the observer. Per morphotype the mean fluorescence of the objects it contains minus the mean fluorescence of the objects in the corresponding morphotype of the negative control is computed, yielding a set of 144 fluorescence intensities. The distribution of circulating IgG over a sample of gutflora is depicted by means of a density plot in Figure 2. Furthermore, using the same faecal sample, the distribution of circulating IgM over the faecal objects present in this gutflora is depicted by means of a hiddenline plot in Figure 3. In Figure 4 the distribution of circulating IgA is depicted by means of a combined contour- and morphometrical plot.

Assuming that these fluorescence intensities - which may be transformed to titre - are independent, they define a 144-dimensional datavector. In 144-dimensional space, the length of a datavector reflects the overall fluorescence intensity and the direction of the datavector reflects the distribution of the fluorescence intensities over 144 morphotypes.

Differences in the binding of molecules of circulating Ig over the 144 morphotypes between two subjects (say: A and B) are reflected by the angle between their corresponding datavectors. Using euclidian metric, the angle (ß) between the datavectors derived from volunteer A and B can be com

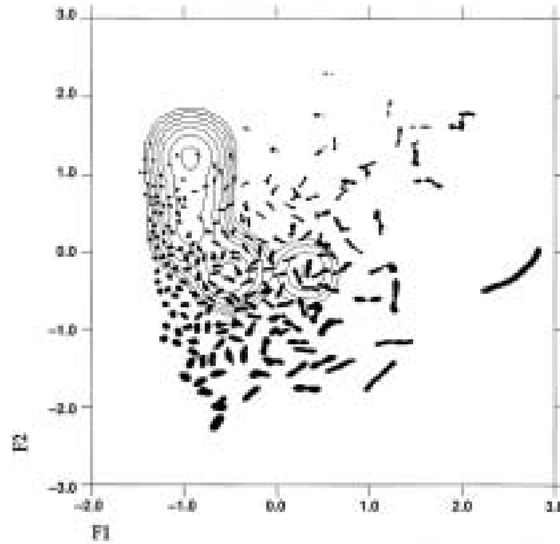

Figure 4: Combined morphometrical and contourplot. Bacteria in the morphometrical regions represented as bacteria instead of symbols. For reasons of clarity some bacteria have been omitted. The contours represent titres of circulating IgA directed against the underlying morphotypes. The outer contour represents objects with a fluorescence level of 2.00. Subsequent contours indicate the location of objects which exhibit a titre twice as large as the contour by which it is enclosed.

puted by means of formula 8.

$$\cos \beta = \frac{AB}{|A| \times |B|} \qquad (8)$$

In this formula, A B do represent the inner product of datavectors A and B, while |A| and |B| represent the euclidian length of datavector A and the euclidian length of datavector B respectively.
The cosine of the angle between two datavectors represents a measure of similarity because when A and B are equidirectional, the cosine of the angle they enclose is one and when A and B are fully independent the cosine of the angle they enclose is zero.

DISCUSSION

The host organism is in direct contact with the mucosal part of the indigenous gutflora. The bacteriological composition of the lower mucosal flora of the gut correlates well with the bacteriological composition of colorectal part of the luminal flora which is present in faeces (*Johansson* et al., 1992). Therefore, the use of faeces as representing the lower mucosal gutflora seems correct.

However, faecal flora may contain as many as 400 species of mainly anaerobic bacteria (*Moore*, 1974). Thus, when the composition of the gutflora is under consideration, culturing and identification techniques are very laborious and time consuming. Also, culturing of bacteria on other media than pure gut mucus may alter the expression of antigenic epitopes on the bacterial cell wall and, furthermore, the absence of intestinal glutamases and proteases (which modificate antigenic epitopes under *in vivo* conditions) adds to the unreliability

of culturing when the interaction with the host's immune system is assessed under *in vitro* conditions (*Osagawara* et al., 1985).

Alternative approaches have been developed e.g. measurement of the activity of bacterial enzymes present in the faeces (*Welling* et al., 1990) or analysis of gutflora-associated metabolites like short chain fatty acids (*Cummings*, 1991). Though the activity of bacterial enzymes can be assessed rapidly and accurately, their value in characterising faecal flora is limited because different species of bacteria may produce the same enzyme. Using the analysis of short chain fatty acids, both genus and species name of a pure culture can be elucidated. However, in a complex ecosystem like faecal flora this method lacks sufficient discriminating power.

Semi-quantitative morphological analysis of - Gram-stained - faecal flora has been performed by *Baquero* and co-workers (1988). In this study faecal objects were classified by eye into 40 morphologically distinct categories. Apart from the error which results from the inherent subjectivity of this method, *Meijer* and co-workers (1991b) have demonstrated that morphological subsets of faecal objects actually do not exist. Instead of that, the distribution of morphometrically defined shapes of faecal objects is a continuum.

The system of Meijer and co-workers has been applied in a hospital environment to quantify the influence of cefalosporins on the morphological composition of faecal microflora. In a group of eleven healthy volunteers a significant decrease of the morphometrical entropy (S) was observed after intramuscular administration of 1 g of ceftriaxon (*Meijer* et al., 1992).

Because the contact between gut flora and host organism is believed to occur predominantly at the mucosal membrane of the digestive tract, research in the field of host-gutflora interaction has so far mainly concerned the mucosal immune system (*McGhee* et al., 1992). In addition to the mucosal immune system, antigens originating from the gut flora may occasionally also be presented to the systemic immune system. Mechanisms responsible for this transfer of antigen comprise bacterial translocation to other lymphoid organs than gut associated lymphoid tissue (*Debure*, 1987; *Wells*, 1987a, 1987b).

The presence and titre of circulating Ig directed against indigenous gut flora has firstly been demonstrated by *Apperloo-Renkema* and co-workers (1991b) using a quantitative immune fluorescence method. With an improved quantitative immune fluorescence method, Jansen and co- workers were able to detect fluctuations in the capacity of faecal bacteria to bind circulating Ig at a statistically significant level. The quantitative immune fluorescence - or fluorimetrical - method has the obvious advantage that objects which are clearly not of bacterial origin can be excluded from the analysis. When measuring levels of circulating Ig directed against intestinal bacteria fluorimetrically, coefficients of variation of 6.1%, 6.4% and 9.8% were obtained for IgG, IgM and IgA respectively. Therefore, the fluorimetrical method can be used when the interaction between a polyclonal antiserum and a complex antigenic substrate like intestinal bacteria is studied.

When the fluoromorphometrical method is employed, the titre of circulating antibodies directed against morphologically identical faecal bacteria can quantified. Morphologically identical bacteria do not necessary belong to the same species, as Meijer and co-workers have demonstrated. Conversely, within a population of bacteria belonging to one species large morphological deviations do occur. Therefore, the division

of the morphometrical region (-2<F1<8 and -5<F2<5) into 144 equally sized fields or morphotypes has no relation with conventional bacteriological nomenclature. Results of this fluoromorphometrical analysis have to be interpreted in terms of humoral reactivity against morphologically identical faecal bacteria.

Despite this restriction, Apperloo-Renkema and co-workers have demonstrated - at a semi-quantitative level - that the distribution of circulating Ig over 28 arbitrarily chosen morphotypes yields a pattern which is unique per gutflora-serum combination. This finding was confirmed by Jansen and co-workers using the previously described method. Performance analysis of the fluoromorphometrical method (unpublished data) reveals that the error of this method is largely due to inaccuracies in the process of slide manufacturing (i.e. the assay error), while the error due to longitudinal fluctuations in the antibody repertoire of the serum and the antigenic composition of the faecal flora is only half the magnitude of the assay error.

The fluoromorphometrical method may be useful when small changes in the composition of the gutflora are to be expected. An especially interesting field of research could be the influence of live bacterial food additives, or probiotics (*Fuller*, 1988) on the composition of the gutflora and the influence of this alteration on the repertoire of circulating Ig. Such a study, using *Enterococcus faecalis* as a probiotic is currently being undertaken.

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FLOW CYTOMETRY ANALYSIS OF FAECAL BACTERIA: INDICATION FOR MUCOSAL IMMUNOLOGICAL HYPOREACTIVITY AGAINST INDIGENOUS ANAEROBES

L.A. VAN DER WAAIJ[1], G. MESANDER[2], P.C. LIMBURG[2], and D. VAN DER WAAIJ[1]

[1]Department of Medical Microbiology, and [2]Department of Internal Medicine, University Hospital Groningen, Groningen, The Netherlands

SUMMARY

In the present study we describe a flow cytometry method for analysis of non-cultured anaerobic bacteria present in human faecal suspensions. Non-bacterial faecal compounds, bacterial fragments and large aggregates could be discriminated from bacteria by staining with propidium iodide (PI), setting a discriminator on PI-fluorescence and by exclusion of events with large forward scatter. Since anaerobic bacteria, which comprise over 99.9% of all faecal bacteria, die during sample preparation, a fixation step was not necessary. By staining with FITC-labelled monoclonal antibodies a rapid evaluation of faecal flora is possible without culturing with this new flow cytometry method.

Secretory IgA is the major effector system of the mucosal immune system. A second aim of this study was to analyse the *in vivo* IgA-coating of anaerobic bacteria present in faecal samples. The fluorescence distribution of IgA-coated bacteria labelled with FITC-anti-Hu-IgA had overlap with non-coated bacteria. However, with match region subtraction, detection of low levels of specific FITC-fluorescence on IgA-coated bacteria was achieved. Flow cytometry analysis of faecal samples of 22 healthy human volunteers shows that with this sensitive method on average only 45% of all bacteria present in faecal suspensions are coated with IgA. The absence of coating with IgA of the other 55% may not be due to lack of stimulation of the mucosal immune system, since intact bacteria and bacterial antigens continuously non-specifically penetrate the colonic mucosa and, secondly, most anaerobic species continuously colonise the colonic mucosa as part of a stable ecosystem. Therefore, the absence of IgA-coating suggests immunological tolerance of the mucosal immune system for the non-coated bacteria. This tolerance could be important for prevention of inflammation in the colonic mucosa. Furthermore, the presence of IgA on faecal anaerobes suggests that sIgA may be not very important in prevention of colonisation of these anaerobic species.

INTRODUCTION

In the colonic mucosa many predominantly IgA secreting plasmacells, lymphocytes and phagocytes are present. These cells are just separated from

the intestinal lumen by a single layer of epithelial cells. However, despite the luminal presence and passive penetration into the mucosa of enormous amounts of antigens both of dietary and bacterial origin, normal intestinal histology is maintained.

In the colon bacterial antigens predominate since there are as many as 10^{11} bacteria per gram contents and most dietary antigens are degraded. It is important to realise that over 99.9% of the colonic microflora consists of a stable ecosystem of possibly as many as 400 different species of anaerobic bacteria in an individually characteristic composition (*Holdeman* and *Moore*, 1974, *Simon* and *Gorbach*, 1984). These bacteria are relatively seldom infectious, colonise the mucuslayer (mucus flora) and seed into the colonic luminal contents (luminal flora). Potentially pathogenic aerobic *Enterobacteriaceae* spp. (like *Escherichia coli*) comprise less than 0.1% of the colonic flora.

The human immune system consists of two, more or less independent, parts: The systemic immune system and the mucosal immune system. One of the main effector mechanisms of the mucosal immune system is secretory IgA (sIgA) which is secreted in large amounts into the intestinal lumen (*Conley* and *Delacroix*, 1987). The main function of sIgA is presumably immune exclusion, i.e. prevention of penetration of soluble antigens and microorganisms by agglutination and by countering bacterial adherence. Furthermore, sIgA can possibly prevent activation of complement by inhibition of IgG and IgM binding. Finally, sIgA can mediate bacterial killing by cell mediated mechanisms via synergism with non-specific anti-microbial factors as lactoferrin and lactoperoxidase. For these reasons, sIgA is assumed to play an important role in the prevention of mucosal inflammation (*Childers* et al., 1989).

A second mechanism to prevent colonic inflammation is specific mucosal non-responsiveness, i.e. absence of an IgA response (*van der Waaij* and *Heidt*, 1986). Chronic peroral immunisation with *Streptococcus mutans* leads to stable acquired suppression of the specific mucosal sIgA response (*Riviere* et al., 1992). Even a few oral doses bacteria can sometimes lead to suppression of the specific sIgA response (*Hahn-Zoric* et al., 1989). Furthermore, most proteins do not elicit secretory IgA responses after oral feeding (*Elson*, 1985).

Little is known about the mucosal humoral response against human anaerobic bacteria. *Monteiro* et al. (1971) incubated some cultured anaerobic bacterial strains derived from human faeces with homogenates of human colonic mucosa. Binding of mucosal IgA was not observed to any of the anaerobic species detected by immunofluorescence microscopy. However, culturing of bacteria may change their surface antigens and only some of the anaerobic bacteria are easily cultivable (*Ogasawara* et al., 1985). Furthermore, evaluation of immunofluorescence staining by eye is very difficult. In order to determine the *in vivo* coating with IgA of faecal anaerobes, we developed a new rapid and sensitive flow cytometry method to analyse non-cultured anaerobic bacteria present in faecal suspensions (*van der Waaij*, 1994). Flow cytometry offers a rapid method for the characterisation of individual cells in mixed populations by physical and biochemical aspects. While major attention has been paid to measurement of eukaryotic cells, only some attempts have been made to analyse bacterial populations. In this field, the focus has been on bacterial pure cultures (*van Dilla* et al., 1983), though in some studies non-cultured mixed bacterial populations like aquatic bacteria were characterised (*Robertson* and *Button*,

1989). No reports, however, on flow cytometry of faecal anaerobes have been published yet. In the present study we analysed the *in vivo* IgA-coating of anaerobic bacteria present in faecal samples of 22 healthy human volunteers. To validate flow cytometry data, all samples were analysed by a formerly developed computer image analysis system as well. We show that not all faecal anaerobic bacteria are coated with IgA.

MATERIALS AND METHODS

Volunteers and sampling

Twenty-two healthy volunteers, 13 male and 9 female, aged 21-61 years (median 32 years), provided a faecal sample. Exclusion criteria were: Immunocompromised conditions (corticosteroids, diabetes, etc.), antibiotic use less than two weeks before sampling, diarrhoea, and pregnancy. Each faecal sample was divided into 12 portions of 0.5 gram, frozen within three hours after defecation and stored until use at -20°C.

Pure cultures

Pure cultures of human faeces derived *Bacteroides fragilis*, *Fusobacterium* spp., and *Clostridium difficile* were grown under anaerobic circumstances in chopped meat carbohydrate. Pure cultures of human derived *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* spp., and *Pseudomonas* spp. were incubated in Brain Heart Infusion broth (BHI, Oxoid Hampshire, England). All strains were stored at -20°C until use.

Reagents

Affinity-purified polyclonal fluorescein isothiocyanate (FITC)-labelled goat $F(ab')_2$ anti-human IgA (F/P ratio = 2.0) from Kallestad (Austin, TX), FITC-labelled goat $F(ab')_2$ anti-mouse IgM (F/P ratio = 3.2) from Protos immunoresearch (San Francisco, CAL), BSA (fraction V) from Boehringer Mannheim (Mannheim, Germany), and propidium iodide (PI) from Sigma (St. Louis, MO) were used.

Study design

Faecal samples and suspensions of different pure cultured bacteria were analysed by flow cytometry as well as by image analysis. *In vivo* IgA-coating of anaerobic bacteria was determined by staining faecal suspensions with FITC-$F(ab')_2$ anti-human IgA and subtraction of background-fluorescence as measured in non-stained suspensions. In order to determine the intra-assay variation, one faecal sample was included in each series of measurements.

Flow cytometry

Instrumentation

Flow cytometry was performed with an EPICS-ELITE (Coulter-Electronics, Hialeah, FL). Filtersettings were 525 BP for FITC, 550 LP and 630 BP for measurement of PI. Acquisition and analysis were done with standard ELITE software comprising the Immuno-4 program to determine the percentage of stained events. Sample excitation was done by an Argon laser operating at 15 mW and 488 nm.

Calibrations and discriminator

The flow cytometer was calibrated with Fluoresbrite plain microspheres (Polysciences, Inc., Warrington, PA) 0.72 µm in diameter, on forward scatter (FSC), side scatter (SSC) and FITC-fluorescence. Fluorescence quantification was done with fluorescein quantification kits (Quantum™ 24 and Quan-

tum™ 25, from Flow Cytometry Standards Corp., NC, USA). To determine the level of background noise we used plain microspheres (Polysciences, Inc., Warrington, PA) 0.79 μm in diameter, which were assumed to have no fluorescence. For bacterial measurements the discriminator was set on propidium iodide (PI) fluorescence as a specific probe for bacteria. The discriminator value was determined by a filtered (0.22 μm Millipore, Molsheim, France) bacteria free solution of PBS/PI (4 mg/l) and set at a level with minimal background noise. For some pure cultures a discriminator set on FSC was used and its value was determined with filtered PBS/PI at a level with minimal background noise.

Actual analysis

Of each sample a portion incubated with PBS (background fluorescence) and a portion incubated with FITC-labelled goat $F(ab')_2$ anti-human IgA were analysed. Both measurements were performed with 10,000 events, at a flow rate of 1000-1500 events/sec. Data were stored in listmode on disc. The fluorescence was recorded logarithmically, FSC linearly. The mean fluorescence (logarithmic scale) was the fluorescence value corresponding with the calculated mean channel-number (linear scale) of all events and was therefore not a true mean. Percentages of stained bacteria were determined with immuno-4 software (Coulter).

A sorting experiment was performed with several gates on FSC, SSC, PI and FITC fluorescence. Sorted bacteria were collected on a slide and further evaluated by microscopy and computer image analysis.

Isolation and preparation of faecal bacteria for flowcytometry

Half a gram of faeces was suspended in 4.5 ml filtered (0.22 μm) PBS, homogenised on a Vortex mixer during 1 min. and centrifuged at low speed (35 g, 20 min.) to separate larger faecal particles from bacteria. Of each supernatant 20 μl (containing about 10^8 bacteria) was washed once in 1 ml of filtered PBS and centrifuged at 8000 g for 10 min. to remove non-bound faecal IgA. The pellet was resuspended in 60 μl of PBS/BSA (1% v/w) or in FITC-labelled goat $F(ab')_2$ anti-human IgA (1:100 in PBS/BSA; 1% v/w). Suspensions were incubated for 30 min. at room temperature. One ml of PBS was added and mixed prior to centrifugation (8000 g, 10 min.). This wash procedure was repeated once. Finally, the bacterial pellet was resuspended in 500 μl PBS, added to 20 μl PI (final concentration: 4 mg/l), stored on ice in the dark and analysed within two hours.

In order to determine the specificity of the FITC-$F(ab')_2$ goat anti-Hu-IgA, a FITC-$F(ab')_2$ goat anti-mouse-IgM was added to human faecal bacterial suspensions. Further processing was identical to the procedure described above.

Preparation of pure cultured bacteria for flowcytometry

Pure cultured bacteria were washed once with PBS, adjusted to the optimal bacterial concentration (10^8 bacteria/ml) in 500 μl, added to 20 μl PI (final concentration: 4 mg/l), stored on ice in the dark and analysed within two hours. Special precautions were taken to prevent environmental contamination by these bacteria.

Computer image analysis

Instrumentation

The instrumentation has been described in detail elsewhere (*Apperloo-Renkema* et al., 1992). Briefly, we used an Olympus BH-2 microscope equipped with a phase-contrast and 100 watt high-pressure mercury vapour lamp and a BP490 filter block (transmission of

490-750 nm). A high resolution CCD videocamara was mounted on top of the microscope. This was connected to an 80286 based AT compatible computer with a MVP-AT image processor board and a monitor. An exposure-control expansion board enabled us to record long-exposure images (4 sec.). The image acquisition software was developed specifically for this application in our laboratory. Fluorescence measured by each pixel of the video camera was calibrated using a uniformly fluorescent uranyl glass slide (Schott, Mainz, Germany) and was therefore expressed in uranyl units (uU). Fluorescence quantification was done with fluorescein quantification kits (Quantum™ 24 and Quantum™ 25). A 2D-surface area threshold was set on 0.15 μm^2, i.e. small objects not believed to be bacteria.

Actual recording

To record the fluorescence of each object in the microscopic field of view, it is necessary to acquire two images: (a) with morphological information (phase-contrast), and (b) with fluorescence information. For each object, the average level of fluorescence within the object is computed, as well as the exact morphology. All measurements were performed with at least 1000 objects per sample and the median and third quartile of fluorescence as well as the median bacterial surface area were determined.

Isolation and preparation of faecal bacteria for image processing

The procedure is in part analogous to the sample preparation procedure used for flow cytometry (*Apperloo-Renkema* et al., 1992). Half a gram of faeces was suspended in 4.5 ml of demineralised water, homogenised on a Vortex mixer during 1 min. and centrifuged at low speed (35 g, 20 min.). The bacteria in the supernatant were diluted to a 2% suspension in demineralised water with 0.5% Tween 80 (Merck, Germany) and 10 µl was pipetted into a well of three different degreased twelve well slides (Immunocor, France) which previously had been coated with a 10% poly-l-lysine solution (Sigma Diagnostics, St. Louis, USA) in order to ensure optimal adhesion of the faecal bacteria to the slide. After drying and fixation in acetone during 10 min., gentle washing (PBS, 5 min.) and again drying, slides were stored at -20°C before use. After thawing, 20 µl of FITC-labelled goat $F(ab')_2$ anti-human IgA (1% (v/v) in PBS/BSA (1% v/w)) or 20 µl of PBS/BSA (1% v/w), was pipetted into each well. After incubation for 30 min. in a moist chamber at 20°C in the dark, slides were washed gently three times in PBS, embedded in mounting fluid (glycerol/Tris buffer v/v 1:1), covered with a cover slide and stored in a moist chamber at 4°C in the dark for at least 2 h and max. 72 h. A third slide was stained with PI (20 µl, 100 mg/l PBS) and measured within two hours. Pure cultured bacteria were processed as for flow cytometry and image analysis recording was identical to the procedure for faecal suspensions.

Mucus flocks

Half a gram of faeces was suspended in 4.5 ml of PBS, homogenised on a Vortex mixer during 1 min., diluted to a 10% suspension in demineralised water with 0.5% Tween 80 (Merck) and finally 10 µl was pipetted into a well of four different degreased twelve well slides. One of each slides was stained with FITC-labelled goat $F(ab')_2$ anti-human IgA, FITC-labelled mouse anti-human IgA1 and FITC-labelled mouse anti-human IgA2 as described above, however, wash steps were as brief as possible. The other slides were stained with the mucus stain alcian blue by Dr. W. Timens (Groningen), as is routinely performed in his laboratory.

Statistical analysis

Spearman rank correlation coefficients were computed to determine the relations between variables of fluorescence, FSC and surface area. Two tailed probabilities are presented. Simple linear regression analysis was performed in order to determine the best fitted line between variables. The coefficient of variation of six separate analyses of one faecal sample was used to describe the intra-assay variation of flow cytometry.

RESULTS

Bacterial size calibration

In flow cytometry, forward scatter (FSC) corresponds to cell-volume. However, with image analysis the 2 dimensional (2D) surface area of recorded bacteria is determined. Therefore, we determined the relation between FSC and 2D-bacterial surface area by measurement of seven pure cultures of human faeces derived aerobic and anaerobic bacteria with flow cytometry and image analysis. There was a considerable variation in FSC within a pure culture, however, FSC-mean and median 2D-surface area were correlated ($p<0.01$). The smallest bacterial species measured, a *Pseudomonas* spp., had a median 2D-surface area of 0.55 μm^2 and a mean FSC of 23.

Elimination of large aggregates

Faecal suspensions contain large particles, apart from single bacteria. Despite low speed centrifugation during sample preparation, however, large particles were still observed during flow cytometry measurement. In order to determine the FSC above which no single bacteria were present, the 2D-surface area of faecal objects present in samples of 22 healthy human volunteers were measured with computer image analysis. Objects with a 2D-surface area larger than 5 μm^2 were nearly all composed of aggregated bacteria. Extrapolation of the relation between surface area and FSC results in a corresponding FSC = 1000. Therefore, all further flow cytometry analysis was performed with a gate set on FSC <1000. Therewith 6% of the events were excluded. In order to check whether events with FSC >1000 were aggregates, a sorting experiment was performed. Sorted objects visually evaluated by phase contrast microscopy, all appeared to be aggregates. However, 8% of all objects with a surface area <5 μm^2 appeared to be aggregates of smaller bacteria and could therefore not be excluded by criteria based on bacterial size.

Only propidium iodide positive events were analysed by flow cytometry

For flow cytometry analysis of eukaryotic cells, a discriminator is usually set on FSC. However, the FSC of a proportion of faecal bacteria is too low to be analysed with a discriminator set on low FSC. We therefore stained all faecal bacterial suspensions with propidium iodide (PI), a fluorescent stain for double stranded DNA/RNA. Since anaerobic bacteria probably died during our sample preparation procedure, they have become permeable for PI. With a discriminator set on PI-fluorescence it was possible to analyse events with very low FSC. A second advantage of a discriminator set on PI-fluorescence is exclusion of PI-non-bacterial compounds (e.g. cellular debris, mucus) and bacterial fragments present in washed faecal bacterial suspensions. To make sure that the ex-

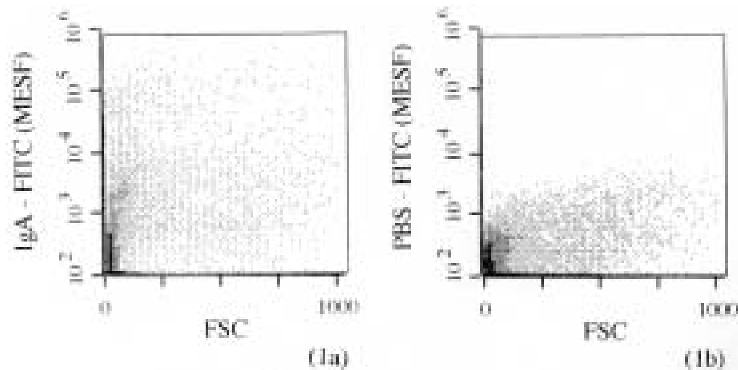

Figure 1: Forward scatter (FSC) versus fluorescence scatterplots of a sample stained for IgA (1a) or without staining (1b).

cluded PI-events were indeed fragments or objects of non-bacterial origin, their morphology was determined by flow cytometry sorting (with a discriminator set on FSC). Only a small proportion (20%) of all events was PI^-. Visual evaluation of the sorted PI^- events by phase contrast microscopy was performed. Besides large amounts of small objects, presumably bacterial fragments, irregular clearly non-bacterial objects (presumably mucus fragments and undigested dietary compounds) were seen. A second experiment to determine the morphology of PI^- objects was performed by image analysis of PI stained slides with bacterial suspensions of all faecal samples. The PI fluorescence distribution showed a distinct non-stained peak (PI^-). The median percentage PI^- objects was 40% (range 22-65%) with a median 2D-surface area of 0.46 μm^2. Morphologically, PI^- objects were largely concentrated in the region of small coccoid objects which was scarcely populated by PI^+ objects. With our image analysis system it is not yet possible to measure FITC at the same time as PI. In order to eliminate many PI^- objects from further analysis by image analysis software, we excluded all small coccoid objects by morphological criteria.

Size of faecal anaerobic bacteria

Faecal bacteria form a morphological heterogeneous population. In order to determine the normal variation in distribution of faecal bacterial size, we analysed faecal bacterial suspensions of 22 healthy human volunteers both by flow cytometry and image analysis. There was a considerable variation of FSC within samples as expressed by a CV of 110-159%. The median FSC of all samples was 129 (range 79-183) corresponding with a 2D-surface area of 1.0 μm^2. Most (80%) events had a FSC <200, corresponding with a 2D surface area of <1.5 μm^2. For determination of the median bacterial 2D-surface area, PI stained slides were measured by image analysis and PI^+ objects were evaluated. The median 2D-surface area of all faecal samples was 1.07 μm^2. Of each sample about 80% of the PI^+ objects had a surface area <1.5 μm^2. There was a good correlation between median bacterial 2D-surface area and mean FSC ($p<0.01$), which was comparable to pure cultures.

In vivo IgA-coating of faecal anaerobic bacteria

We analysed by flow cytometry faecal bacterial suspensions stained with affinity purified FITC-$F(ab')_2$ anti-Hu-

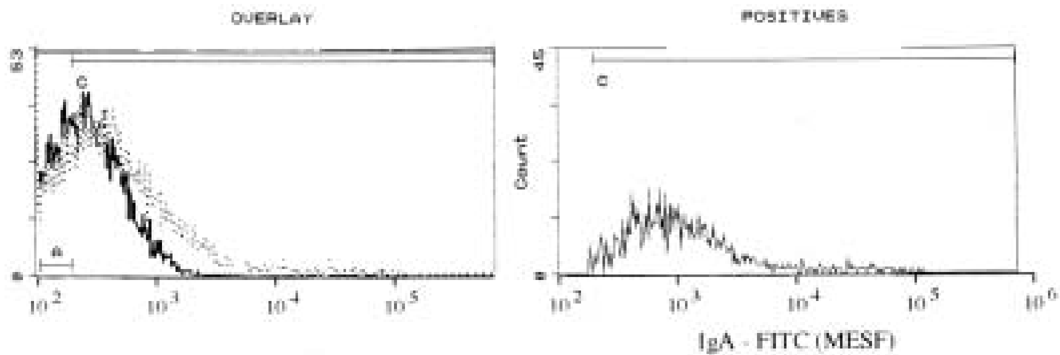

Figure 2: Typical example of fluorescence distributions of non-stained bacteria (overlay, continuous line) and bacteria labelled with FITC-anti-IgA (dotted line) as recorded by flow cytometry. The fluorescence histogram of non-stained bacteria was subtracted from that of stained bacteria by match region subtraction. The matched region (A), the region in which the percentage of stained bacteria is calculated (C) and the resulting fluorescence histogram (overlay) are shown.

IgA as well as non-stained samples. Fluorescence of non-stained bacteria as measured with FITC-filtersettings, was only present in the lower channels (Figure 1). The fluorescence distributions of all non-stained samples were similar with a median value of 0.24 corresponding with $2x10^2$ MESF. To determine the contribution of photomultiplier background noise to these extremely low fluorescence values, we analysed non-fluorescent latex particles of bacterial size (0.79 μm). Their mean fluorescence value was 0.142 and their fluorescence distribution was thinner than that of all non-stained faecal suspensions. To determine the specificity of the measurement of *'in vivo'* IgA-coating of faecal bacteria, faecal samples were stained with a nonsense FITC-$F(ab')_2$-anti-mouse IgM. No non-specific staining was detected.

All samples were found to contain IgA-coated bacteria and there was not a clear discrimination between fluorescence values of stained and non-stained bacteria (Figure 1). In order to estimate the percentage of faecal bacteria coated with IgA and their levels of fluorescence we performed match region subtraction by immuno-4 software. With this method, fluorescence histograms of non-stained samples are matched over a region with histograms of corresponding stained samples and subtracted (Figure 2). Herewith, a much more accurate approximation of the real percentage stained bacteria can be achieved than by simple subtraction (sladek). Figure 3 shows that the median percentage of stained bacteria was 45% (range 24-74). Their median fluorescence value was 2.33 corresponding with 1300 MESF. This implicates that the median faecal anaerobe is coated with 650 molecules of IgA (F/P ratio = 2). Within the samples there was not a clear relation between FSC and fluorescence of bacteria labelled with FITC-anti-IgA (Figure 1).

Validation with image analysis

In order to validate flow cytometry data, all faecal samples were analysed by image analysis as well. Since total numbers of bacteria recorded by image analysis were low and no histogram subtraction software was available, only the median and third quartile (Q3) of fluorescence were calculated. In order to estimate the specific fluorescence (i.e. fluorescence due to labelling with

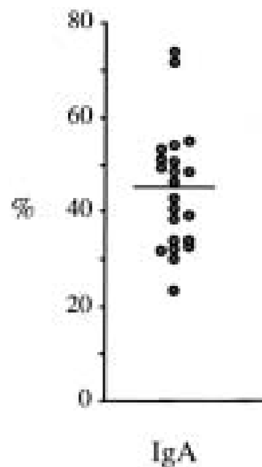

Figure 3: Percentage of faecal bacteria coated with IgA as determined by match region subtraction. The median percentage is shown.

FITC-anti-IgA) of each sample the median (and Q3 of) fluorescence of non-stained bacteria was subtracted from the median (and Q3 of) fluorescence of stained bacteria. Also with image analysis all samples were found to contain IgA-coated objects and there was not a clear discrimination between fluorescence distributions of stained and non-stained objects. Fluorescence data of bacteria labelled with FITC-anti-IgA obtained by both methods were correlated. The mean fluorescence of stained bacteria as determined by flow cytometry correlated significantly with the Q3 of specific fluorescence obtained by image analysis ($p<0.05$). In contrast with the Q3, the median specific fluorescence (image analysis) did not correlate with flow cytometry data. Strongly fluorescent bacteria within a mixed population like faecal flora should be recognised by both systems. Fortunately, one faecal sample contained a separate small population (2%) of strongly FITC-anti-IgA labelled bacteria with a mean FSC of 180 (Figure 4). Morphology of these bacteria was determined by image analysis as well as by flow cytometry sorting. The most fluorescent bacteria recorded by image analysis were coccoid rods with a median surface area of 1.3 μm^2. Flow cytometry sorted bacteria were visually evaluated and were morphologically identical to the coccoid rods recorded by image analysis.

Morphology of the most strongly IgA-coated bacteria

We wondered whether, within each sample and between different samples, there was a bacterial species which was preferentially strongly coated with IgA. Since al anaerobic bacteria will have died due to oxygen during sample preparation, determination by culturing was not possible. However, with an in our group formerly developed image analysis system, it is possible to analyse and describe bacterial morphology by morphological parameters and record bacterial fluorescence values as well (*Apperloo-Renkema* et al., 1992). However, in contrast with flow cytometry, determination of specific fluorescence values with this image analysis system is time consuming and is less sensitive. Here we used the morphological parameters to arbitrarily define six morphological populations com-

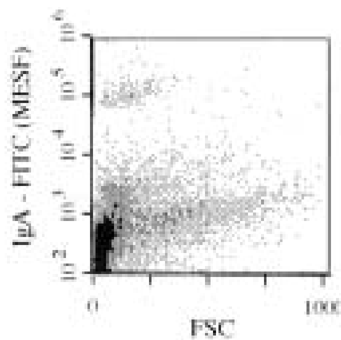

Figure 4: FSC versus FITC-fluorescence distribution of a faecal bacterial suspension stained for IgA. This sample contained a separate strongly IgA-coated population which was sorted by flow cytometry.

prising most non-aggregated bacteria. In each sample the 3% most strongly FITC-anti-Hu-IgA labelled bacteria were in at least three morphological populations. However, in most samples one population predominated.

IgA-coated bacteria in small faecal mucus flocks

In all non-centrifuged faecal suspensions, small irregular flocks were present consisting of packed bacteria embedded in an amorphous substance as seen by phase contrast microscopy. Most flocks had a diameter of 15 μm, although some exceeded 300 μm and flocks were present in a concentration of about 10^7/gram faeces. All flocks stained with the mucus stain alcian blue. Bacteria within the flocks were visually evaluated by phase contrast microscopy. They were found to be a very heterogeneous bacterial population that resembled the population present as single bacteria in the corresponding faecal suspensions. Bacteria divide continuously. One would expect that if a strongly sIgA-coated anaerobe divides, it results in two strongly sIgA-coated anaerobes which immediately agglutinate due to the sIgA. Although we realise it is not a sensitive method, we looked at the FITC-anti-Hu-IgA staining pattern of anaerobes within mucus-flocks. They were visually evaluated by fluorescent microscopy with dark-adapted eye. Flocks in all 22 samples contained visibly fluorescent single bacteria, however, the majority of the bacteria had no strong fluorescence. Agglutination of fluorescent bacteria with other fluorescent bacteria was not seen. The most strongly fluorescent bacteria within each flock had identical morphology and were present in about the same concentrations compared to those present as single bacteria in the corresponding bacterial suspensions. In conclusion, mucus flocks are presumably composed of the same bacteria as found as single bacteria within faecal suspensions. Furthermore, within mucus flocks strongly IgA-coated bacteria are not agglutinated.

DISCUSSION

Flow cytometry analysis of non-cultured faecal anaerobic bacteria

In the present study we describe a new rapid flow cytometry based method to analyse non-cultured anaerobic bacteria present in human faecal suspensions.

Major problems in our approach appeared to be the small size of some faecal bacteria, the presence of non-bacterial objects and bacterial fragments as well as the presence larger objects formed by bacterial aggregates. Since aerobic bacteria comprise less than 0.1% of the faecal flora, their contribution to the data will be negligible (*Meijer* and *van Santen*, 1986). A discriminator set on FSC, as is commonly used for eukaryotic analysis, is sufficient to analyse suspensions of larger bacteria without interference of background noise (*Evans* et al., 1990). Our faecal bacterial suspensions, however, appeared to contain small bacteria as well with a FSC at a level at which background noise is present. As was described by others for aerobic bacteria (*Tyndall* et al., 1985), we show here that with a trigger set on propidium iodide (PI) staining, small intact anaerobic bacteria can be selectively analysed without interference of background noise. In contrast to aerobic bacteria, which need some kind of fixation to become killed and permeable for the fluorescent dsDNA/RNA stain PI, anaerobic faecal bacteria stain with PI without other treatment than exposure to oxygen during sample preparation. A second advantage of a discriminator set on PI-fluorescence is exclusion of PI^- non-bacterial objects. A characteristic of most small non-bacterial faecal compounds (e.g. cellular debris, mucus) and bacterial fragments is that they do not contain dsDNA or RNA and therefore will not stain with PI. In order to make sure that the discarded PI^- events were indeed non-bacterial compounds and bacterial fragments, we performed a sorting experiment by flow cytometry as well as analysis of PI-stained slides by image analysis. Both control experiments confirmed that most PI^- objects comprised small objects, presumably bacterial fragments. Furthermore, irregular clearly non-bacterial objects were seen. Faecal bacterial suspensions contain large particles, apart from single bacteria. However, despite low speed centrifugation during sample preparation, large particles were still observed during flow cytometry measurement. For this reason we used bacterial size as an additional selection criterion. In order to objectively determine the maximal FSC of single faecal bacteria, the 2D-surface area of the largest single human faecal bacteria was determined by image analysis, as well as the relation between FSC and bacterial 2D-surface area. This resulted in a FSC <1000 for single bacteria. Flow cytometry sorting and subsequent visual evaluation with a phase contrast microscope showed that events with a FSC >1000 were indeed aggregates. However, aggregates of small bacteria (approximately 8 % of the PI^+ objects) were smaller than single large bacteria and could therefore not be excluded from evaluation. In conclusion, with a discriminator set on PI-fluorescence and exclusion of events with high FSC, most if not all analysed events are intact predominantly single faecal anaerobic bacteria.

Size of anaerobic faecal bacteria

To our knowledge, no data on the size of bacteria present in faecal suspensions have been published yet. Therefore, faecal samples of 22 healthy volunteers were analysed by flow cytometry and data were validated by analysis of the same samples by computer image analysis as well. Faecal flora consists of a heterogeneous population of possibly as many as 400 different species of anaerobic bacteria (*Holdeman* and *Moore*, 1974). In faecal suspensions each species is presumably present in low concentrations. This heterogeneity was reflected in the large coefficient of variation (CV) of the FSC distributions within a sample, compared to pure cul-

tures. The majority of the measured faecal bacteria were small, with a FSC corresponding with a 2D-surface area <1.5 μm^2. This surface area is smaller than that of pure cultures studied of *E. coli* or *Klebsiella* spp., but larger than that of *Pseudomonas* spp.

Comparison of flow cytometry with image analysis

In order to compare flow cytometry with our image analysis system, analogous parameters of bacterial size and fluorescence were measured of pure cultures and all 22 human faecal samples. In contrast with flow cytometry, with our image analysis system it is not yet possible to measure two different fluorescent stains at the same time, i.e. FITC together with PI. Consequently, PI^- objects could only be partially eliminated from further evaluation by morphological criteria. We found a linear relation between FSC and bacterial 2D-surface area as was determined with analysis of several pure cultures by flow cytometry as well as image analysis. In contrast with flow cytometry, image analysis records morphology in addition to bacterial size. Furthermore, the threshold for accurate measurement of bacterial size is lower for image analysis (0.15 μm^2) than for flow cytometry (0.5 μm^2). However, flow cytometry acquisition rate is 1,000x higher compared to image analysis. Flow cytometry, furthermore, was more effective in the reduction of autofluorescence, could effectively eliminate PI^- events and offered match region subtraction software. For these reasons, flow cytometry was more sensitive for measurement of low levels of FITC-fluorescence than image analysis. Nevertheless, as discussed above, there was a significant correlation between specific fluorescence values as determined by both methods. In conclusion, in comparison with image analysis, flow cytometry is more sensitive for measurement of low levels of specific fluorescence, has the ability of multiple-colour-fluorescence and has a very high acquisition rate. However, image analysis records bacterial morphology and has a lower 2D-surface area threshold. Despite these differences, parameters of bacterial size and specific fluorescence as measured by both methods were correlated. This validates both methods.

Not all bacteria are coated with IgA

Here we show that on average only 45% of all bacteria present in faecal suspensions are coated with IgA. Before discussing the immunological implications, several possible technical explanations for this absence of coating of part of the faecal flora should be considered.

Our flow cytometry method in combination with match region subtraction is very sensitive for measurement of specific fluorescence on small objects like bacteria. Specific fluorescence due to labelling with only a few hundred molecules FITC anti-IgA could be detected (*Sladek* and *Jacobberger*, 1993) and non-specific staining, as determined with a nonsense polyclonal antibody was very low (*van der Waaij*, 1994). However, sensitivity can be further increased by amplification of the staining signal. Therefore we cannot rule out the possibility that the median percentage of bacteria coated with IgA is higher than 45%. Secondly, since bacteria are regarded as propidium iodide positive objects with a FSC <1000, analysed events are anaerobic bacteria and most of them are single bacteria. Third, over 99.9% of all colonic bacteria form a stable ecosystem of presumably as many as 400 different, predominantly non-pathogenic, anaerobic bacterial species (*Moore* and *Holdeman*, 1974; *Simon* and *Gorbach*, 1984). It is there-

fore very unlikely that all non-coated bacteria are new species just arrived in the colon. Fourth, on morphological grounds we make plausible in this article that the composition of the mucus-flora, which is in close contact to the mucosal immune system, is similar to the composition of the lumen flora, i.e. the bacteria analysed for IgA-coating. A fifth possible explanation for the absence of IgA-coating is that IgA-coated bacteria were agglutinated and therefore selectively eliminated by centrifugation during sample processing. However, in non-centrifuged suspensions IgA-coated agglutinated bacteria were not seen. Even proteolytic activity in intestinal fluid cannot explain the absence of IgA-coating since faeces was found to contain only very little proteolytic activity (*Vos* and *Dick*, 1991). Furthermore, with FITC conjugated monoclonal antibodies specific for an antigenic determinant on the Fc portion of IgA (i.e. IgA1 and IgA2), we could detect large amounts of intact IgA present on non-agglutinated faecal bacteria (*van der Waaij*, 1996). Finally, it is unlikely that most anaerobe-specific IgA has got low avidity since slides with mucus flocks were washed as little as possible and still non-IgA-coated bacteria could be observed within these flocks.

In conclusion, fluorescence due to labelling of luminal anaerobic bacteria with FITC anti-IgA as determined by flow cytometry is representative for the IgA-coating as present on bacteria colonising the intestinal mucus. About half af all anaerobic bacteria present in faecal suspensions is not coated with IgA as measured by this technique.

Mucosal non-responsiveness for part of the host's anaerobic colonic flora

Faecal dry wet weight consists for 75% of anaerobic bacteria and therefore, the faecal flora comprises an enormous amount and variety of antigens (*Stephen* and *Cummings*, 1980). There is a continuous non-specific uptake of particulate (i.e. bacteria) and soluble antigens from the gut lumen by specialised epithelial cells, i.e. M cells as well as through normal epithelial cells and tight junctions (*Owen* et al., 1986; *Wells* et al., 1988). It is therefore plausible that antigens of anaerobic species will all be continuously located in the gut wall. The major effector system of the specific mucosal immune system is secretory IgA. Large amounts of IgA (2.5 g/day) are secreted into the lumina of the digestive tract (*Conley* and *Delacroix*, 1987). This is about 10^6 IgA molecules per faecal bacterium. However, despite this enormous amount of IgA, apparently not all bacteria are *in vivo* coated with IgA. At least two possible mechanisms can explain this lack of IgA-coating. First, bacteria may use camouflage techniques by expression of host-identical epitopes on their surface. It is plausible that bacteria adapt to their host's immune system during years of colonisation (*Duval* et al., 1981). Moreover, gutflora is presumably derived after birth from the parents and therefore the result of ages of adaptation. However, only relatively few bacterial antigens cross-reacting with host antigens are known (*Feretti* et al., 1980; *Foo* and *Lee*, 1974; *Yu* et al., 1991). Furthermore, there is no direct evidence yet that bacterial expression of cross-reacting antigens helps them to evade the immune system. A second mechanism to explain the lack of IgA-coating of anaerobic bacteria is acquired mucosal non-responsiveness, resulting in suppression of the specific mucosal IgA response (*van der Waaij* and *Heidt*, 1986). This may be induced by the continuous presence of large stable amounts of antigens in the digestive tract. There is some evidence that absence of a specific mucosal IgA re-

sponse after feeding of bacterial antigen (*Riviere* et al., 1992) or other antigens (*Elson*, 1985) may occur. However, most experiments do show a specific mucosal IgA response after intra-gastral intubation or feeding of antigens. In combination with a specific hyporeactivity in the systemic compartment this is called oral tolerance (*Tomasi*, 1980).

We conclude that the lack of *in vivo* IgA-coating of part of the faecal anaerobic bacteria is presumably largely due to an acquired mucosal non-responsiveness. However, evasion of the immune system by antigenic adaptation of anaerobic bacteria is possibly present as well.

Function of IgA in relation to the anaerobic gutflora

Our findings challenge the current dogma that bacterial agglutination by IgA plays an important role in resistance to colonisation. Despite 45% of the bacteria are coated with IgA, the colonic anaerobic flora comprises a stable ecosystem of a heterogeneous mucus colonising bacterial population. Several antibacterial functions are attributed to IgA, like synergism with non-specific antibacterial factors (i.e. lactoferrin), immune exclusion (bacterial agglutination, prevention of epithelial attachment and prevention of epithelial invasion) and inactivation of bacterial enzymes (*Childers* et al., 1989; *Williams* and *Gibbons*, 1972). However, coating with IgA obviously does not result in removal of the species from the flora. This implicates that IgA-coating of these colonic anaerobic bacteria does not hamper them from mucosal adherence and colonisation and does not result in bacterial killing. Furthermore, agglutination of strongly IgA-coated bacteria with other strongly IgA-coated bacteria was neither seen in mucus flocks (mucusflora) were IgA concentrations are high and peristaltic force low, nor in faecal suspensions.

It is important to realise that IgA-coating is present on bacteria that are part of a stable ecosystem, which is the result of years of adaptation. Therefore, these bacteria must have developed yet unknown strategies, other than cleavage of IgA, to evade the potential harmful effects of IgA. Furthermore, killing of commensal bacteria may not be the goal of the mucosal immune system. As was suggested by *Childers* et al. (1989) the function of the intestinal secretory immune system is not sterility, but rather, symbiosis with the commensal microorganisms by regulation of the mucosal microbial ecosystem.

In conclusion, it is not clear what the effect of IgA-coating is on bacteria.

Prevention of intestinal inflammation

The colon harbours an enormous amount and variety of antigens of which most are of anaerobic bacterial origin. All these antigens are just separated from the mucosa by a single layer of epithelial cells. However, in the colonic mucosa only a moderate chronic inflammation is present. As discussed above, apart from non-immunological mechanisms, reduction or prevention of inflammation by commensal intestinal anaerobic bacteria may be largely mediated by two mechanisms. The most powerful mechanism is specific mucosal tolerance for anaerobic bacterial antigens. Commensal bacterial antigens penetrating into the colonic mucosa will be degraded by phagocytes that are abundantly present in the mucosa without eliciting an inflammatory response. However, if tolerance is an acquired state of the mucosal immune system, for instance due to suppression, it harbours the danger of derailment with consequent loss of tolerance and mucosal inflammation. This mechanism could play a role in the pathogenesis of

the idiopathic inflammatory bowel diseases colitis ulcerosa and Crohn's disease. Mucosal tolerance due to bacterial camouflage by antigens cross-reacting with host antigens will be optimal for harmless bacteria, as are presumably most commensal anaerobes. A second mechanism for prevention of mucosal inflammation by antigens of anaerobic bacteria may be coating with IgA. IgA can prevent uptake of luminal antigens into the mucosa (immune exclusion), it can hamper binding of IgG and IgM to other bacterial surface antigens (*McLeod-Griffiss* et al., 1975) and finally, it presumably prevents activation of the complement cascade (*Childers* et al., 1989). Therefore, IgA-coating will strongly reduce intestinal inflammation but cannot prevent it. We may only speculate why some of the anaerobic bacteria are strongly coated with IgA, others are coated with only little immunoglobulins and many not coated at all. It is plausible that potentially pathogenic anaerobic bacteria, i.e. those who are able to invade the mucosa, are coated with IgA to prevent them entering the mucosa. However, most anaerobes are not potentially pathogenic. Since the epithelial lining is only one cell-layer thin, it may sometimes be discontinuous due to sloughing of epithelial cells at the villus tips (*Wells* et al., 1988) and due to local cell death (local viral/bacterial infection, local ischaemia, local trauma, etc). Furthermore, peristaltic forces can be enormous. We speculate that it will often happen that anaerobic bacteria are forcedly pushed into the damaged mucosa. The presence of anaerobes in an environment of tissue damage may overcome the specific mucosal non-responsiveness and results in an immune response comprising secretory IgA. However, after a few weeks or months this specific IgA response may again slowly disappear.

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NITRIC OXIDE IN THE PATHOGENESIS OF ULCERATIVE COLITIS AND THE POSSIBLE ROLE OF GUT BACTERIA IN ITS SYNTHESIS

STEPHEN J. MIDDLETON, MARIA SHORTHOUSE, and JOHN O. HUNTER

Department of Gastroenterology, Addenbrooke's Hospital, Cambridge, England

SUMMARY

Nitric oxide is an important biological mediator of muscle relaxation in the cardiovascular system. Following our discovery that nitric oxide was also responsible for tonic relaxation of colonic muscle by the enteric nervous system, we investigated the possibility that its synthesis was increased in ulcerative colitis, which may explain the impaired contractility of colonic muscle associated with this condition.

The effects of human leukocytes on precontracted colonic circular muscle from male Wistar rats was investigated. Muscle strips were mounted in organ baths and mechanical activity measured with isometric force transducers.

Amino acids were measured by high performance liquid chromatography and an amino acid analyser in rectal biopsies from patients with active ulcerative colitis (AUC) and compared with quiescent (QUC) and healthy controls (HC). Citrulline concentration was taken as an indirect measure of nitric oxide synthesis. Nitric oxide synthase (NOS) activity was measured in rectal biopsies from patients with AUC and HCs. The production of ^{14}C-citrulline from ^{14}C-arginine was taken as an index of nitric oxide synthase activity.

Human polymorphonuclear leukocytes and mononuclear cells relaxed precontracted colonic circular muscle strips and this was reduced in the presence of NO antagonists. Citrulline concentrations were significantly greater in rectal biopsies from patients with AUC than QUC or HCs. Incubation of biopsies from AUC with L-NMMA reduced citrulline levels. Constitutive NOS and inducible NOS activities were increased in AUC but were undetectable in HCs. Faeces from AUC but not HCs had constitutive NOS activity.

We conclude that nitric oxide synthesis is increased in ulcerative colitis. Mucosal inducible NOS activity is increased in AUC and probably originates in leukocytes which produce sufficient nitric oxide to relax colonic muscle *in vitro* and may contribute to the motility disturbance in AUC. Faecal constitutive NOS activity is increased in AUC and may arise from an abnormal colonic microflora present as a pathogenic factor in this condition.

INTRODUCTION

Spontaneous mechanical activity of colonic circular smooth muscle is under non-adrenergic, non-cholinergic (NANC) tonic neural inhibition (*Crema*

et al., 1968; *Koch* et al., 1988). Following identification of nitric oxide (NO) as an endothelial derived relaxing factor (*Palmer* et al., 1987), NO has been proposed as a neurotransmitter in both the gastrointestinal tract and the brain (*Bult* et al., 1990; *Knowles* et al., 1990). The enzyme NO synthase (NOS) has been identified in the myenteric nerve plexus (*Bredt* et al., 1990). Synthesis of NO from L-arginine is inhibited by certain L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) (*Rees* et al., 1989). Inhibitory L-arginine analogues block certain NANC mediated motor events, such as muscle relaxation produced by electrical field stimulation of the canine ileo-colonic region (*Boeckxstaens* et al., 1990), and NANC relaxation of the internal anal sphincter (*Tottrup* et al., 1992). NO elevates intracellular cyclic GMP levels by increasing the activity of soluble guanylate cyclase (*Knowles* et al., 1990). Cyclic GMP causes muscle relaxation (*Nakatsu* and *Diamond*, 1987).

In previous work (*Middleton* et al., 1993), we have shown that NO biosynthesis mediates NANC tonic neural inhibition of spontaneous mechanical activity in distal colonic circular smooth muscle. The colonic smooth muscle adjacent to the inflamed mucosa of patients with ulcerative colitis (UC) has diminished spontaneous activity. This leads to a reduction of muscle tone and a loss of colonic segmentation which are associated with diarrhoea (*Kern* et al., 1951; *Garrett* et al., 1967; *Connell*, 1962; *Spriggs* et al 1951). In 1980 Snape et al. showed that the gastrocolonic reflex of patients with UC to be attenuated. This was thought to result from electromechanical disassociation of colonic smooth muscle (*Snape* et al., 1980; *Snape* and *Kao*, 1988). The mediator of these mechanical abnormalities was not however identified.

In this paper we report the results of investigations to elucidate the synthesis of NO by human leukocytes in patients with UC. The activity of NO synthase in human rectal biopsies has been studied and the nature of this enzyme examined in both biopsy material and faeces from both patients suffering from UC and from normal controls.

METHODS

Investigations of the effects of nitric oxide derived from human leukocytes

Materials

Drugs and solutions were prepared on the day of use. The following were obtained from Sigma Chemicals Ltd. and where necessary dissolved in distilled water immediately before use: Acetylcholine, superoxide dismutase, N^G-methyl-L-arginine, N^Gmethyl-D-arginine, methylene blue, tetrodotoxin, FMet-Leu-Phe, and hypaque 1017 and 1119. Indomethacin was initially dissolved in 10mM Na_2CO_3 and further diluted in Krebs-Henseleit solution. Oxyhaemoglobin was prepared from bovine haemoglobin (75% methaemoglobin, Sigma Chemicals Ltd.) and its purity was assessed spectrophotometrically according to the method described by *Martin* et al (1986). Preparations were accepted if their concentrations of oxyhaemoglobin were greater than 90%.

Preparation of Tissue

Male Wistar rats weighing 250-400g were killed and strips of colonic circular smooth muscle were attached to isotonic transducers (Harvard, Kent, England) in 2 ml organ baths and perfused by

oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution, composition: (mM/l) NaCl 118; KCl 4.69; $MgSO_4$ 1.13; $CaCl_2$ 2.56; $NaHCO_3$ 25; $NaHPO_4$ 1.15; glucose 5.5. This had a pH of 7.4 to 7.6 at 37°C. Muscle strips were mounted with the longitudinal axis parallel to the direction of the circular muscle bundles. Temperature was regulated and pH monitored intermittently. Muscle strips were maintained under a tension of 3 g, which produced near optimal contraction and experiments were commenced after a stabilisation period of two hours, which was found necessary in preliminary studies to ensure consistent muscle performance. Muscle strips were precontracted by 10 μM acetycholine and the mean amplitude of steady state contractions was measured for two minutes before and after the addition of leukocytes. Differences between the effects of paired leukocyte samples were tested with student's 't' test for paired data.

Cell Preparation

Venous blood from healthy human volunteers aged between 18 and 70 years was collected with EDTA or glass shot beads (Scientific Furnishings, Macclesfield, England) to remove platelets by defibrination. Blood (5 ml) was layered above an 8 ml bilayer of hypaque 1017 and 1119 in equal volumes and centrifuged at 700 g for 25 minutes. Mononuclear cells (macrophages and lymphocytes with or without platelets) and granulocytes (neutrophils, basophils, and eosinophils) were aspirated from two distinct layers. Leukocytes were washed twice in l0 ml Krebs-Henseleit solution (37°C) containing l00 nM/l indomethacin (KHI) to inhibit prostaglandin synthesis, centrifuged at 200 g for 10 minutes and resuspended in 5ml KHI. Leukocyte suspensions were accepted if red cell corpuscles were <5% of total and viability was >95% as judged by a tryphan blue exclusion test (Sigma Chemicals Ltd., England). This test was used to ensure a 100% death rate of cells frozen in liquid nitrogen. Cell suspensions were centrifuged at 200g for 10 minutes and the pellet of cells added to the organ bath of which the bathing fluid was used to resuspend them for transfer.

Experimental controls consisted of a similar procedure without the presence of cells.

Nitric oxide synthesis in human rectal mucosa

Patients were selected if, at sigmoidoscopy, they had macroscopically active or quiescent UC or normal rectal mucosa. Rectal biopsy specimens were frozen in liquid nitrogen or placed in short term tissue culture within 30 seconds of collection.

Concentrations of 40 amino acids were measured in biopsy samples from 6 patients with histologically active UC and 7 with normal histology by high performance liquid chromatography after derivitisation with 9-fluorenylmethyl chloroformate (*Einarsson* et al., 1983). Forty-one amino acids were measured with an amino acid analyser (LKB, Biochrom) in specimens from 5 patients with histologically active disease and 5 with histologically quiescent disease. In the short term tissue culture experiment, paired rectal biopsy samples from 8 patients with active UC were incubated for 2 hours in oxygenated modified Krebs-Henseleit at 37°C with either L-NMMA or the inactive isomer D-NMMA (200 μmol/l). After incubation specimens were immediately frozen in liquid nitrogen and amino acids measured by HPLC after derivitisation with 9-fluorenylmethyl chloroformate. Amino acid concentrations in inflamed and non-inflamed mucosa were compared by the Wilcoxon rank-sum test and, after incubation with L-NMMA or D-NMMA, by

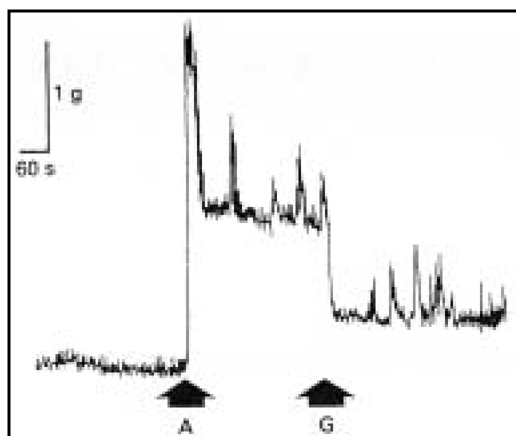

Figure 1: Typical chart recording. Distal colonic circular smooth muscle contracted by acetylcholine (l0 μM) (A) and relaxed to granulocytes (5x 10^8 cells/l) (G).
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the Wilcoxon matched pairs signed-ranks test.

Measurement of mucosal nitric oxide synthase activity

Rectal mucosal biopsies were taken at routine sigmoidoscopy from 11 randomly selected patients (5 distal colitis, 4 left sided colitis, 2 total colitis, mean [SD] age 42 ± 9.8, 2 female, 9 male) attending the gastroenterology clinic at Addenbrooke's Hospital. All had macroscopically active UC, subsequently confirmed histologically. Control biopsies were taken from 10 patients (mean age 38 ± 10.5, 4 female, 6 male) with minor symptoms who had histologically normal mucosa and did not subsequently develop inflammatory bowel disease.

NO is produced by NOS from L-arginine with the liberation of equimolar amounts of citrulline (*Hibbs* et al., 1987a). The inhibitory effects of

Table 1: Substances with known effects on the NO-cGMP pathway affected relaxation of precontracted distal colonic circular smooth muscle by granulocytes

	Cells 1^{-1}	Mean muscle relaxation (SEM) (%)	No of samples (pairs)	p-value
Viable	5 x 10^8	78 (11.7)		
Non-viable	5 x 10^8	0.37 (3.3)	8	<0.005
Control	1 x 10^8	50 (9.5)		
Oxyhaemoglobin	1 x 10^8	25 (5.9)	8	<0.005
N^G-monomethyl-D-arginine	1 x 10^8	53 (11.8)		
N^G-monomethyl-L-arginine	1 x 10^8	1.8 (3.9)	9	<0.02
Control	5 x 10^8	73 (13.5)		
Methylene blue	5 x 10^8	24 (6.1)	7	<0.01
Control	7.5 x 10^7	37 (11.6)		
Superoxide dismutase	7.5 x 10^7	96 (3.6)	7	<0.005

Mean relaxations are compared with controls by Student's t test for paired data.
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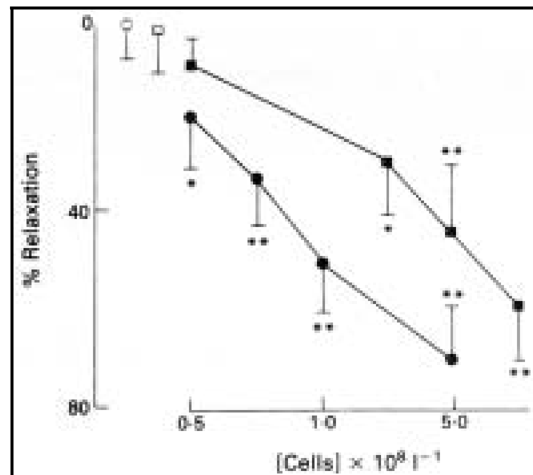

Figure 2: Granulocytes (●) and mononuclear cells (■) relaxed precontracted distal colonic circular smooth muscle in a concentration dependent manner. Mean percentage relaxations of muscle (±SEM) are shown. Collective means and standard errors of controls for both groups of cells are indicated by open symbols. Data were analysed with Student's t test for paired data (*$p<0.05$; **$p=0.01$). This figure is reproduced with permission from: Gut 34: 814-817 (1993).

N^G-methyl-L-arginine (L-NMMA), the specific inhibitor of both NOS enzymes (*Palmer* and *Moncada*, 1989) on the conversion of ^{14}C-arginine to ^{14}C-citrulline in the presence of calcium (*Marletta* et al., 1988), was used as an index of total (TNOS) activity and in the absence of calcium, as an index of inducible (INOS) activity (*Busse* and *Mulech*, 1990). The ^{14}C-citrulline was separated by using thin layer chromatography (TLC) using a method modified from *Hibbs* et al. (1987a).

Measurement of faecal nitric oxide synthase activity

Faecal samples were collected from 11 patients with macroscopically active UC (3 distal, 5 left sided, 3 total, 5 female, 6 male, mean age [SD] 41 ± 9.4) who had histologically active disease on a rectal biopsy taken within the previous month. Control samples of faeces were collected from 9 healthy volunteers (mean age [SD] 45 ± 11, 4 female, 5 male) with no history of gastrointestinal disturbance. Measurements of NOS activity were performed by the same method used for mucosal specimens. The investigation of faecal NOS activity was qualitative.

Differences in ^{14}C-citrulline production between paired incubations of mucosal biopsies and faeces in the presence of L- or D-NMMA were tested by students t-test for paired data and the Wilcoxon matched-pairs signed-ranks test respectively and significance taken as $p<0.05$.

RESULTS

Investigation of the effects of nitric oxide derived from human leukocytes

Granulocytes and mononuclear cells produced concentration dependent relaxations of circular smooth muscle precontracted with 10 μM/l acetylcholine (Figures 1 and 2). Non-viable leuko-

Table 2: Substances with known effects on the No-cGMP pathway affected relaxation of precontracted distal colonic circular smooth muscle by mononuclear cells

	Cells l^{-1}	Mean muscle relaxation (SEM) (%)	No of samples (pairs)	p-value
Viable	5 x 10^8	46 (13.8)		
Non-viable	5 x 10^8	(23% contraction) (12)	7	<0.005
Control	8 x 10^8	63 (8.3)		
Oxyhaemoglobin	8 x 10^8	25 (7)	9	<0.005
NG-monomethyl-D- arginine	2 x 10^8	33 (12.1)		
NG-monomethyl-L-arginine	2 x 10^8	7.7 (8.7)	12	<0.05
Control	5 x 10^8	43 (20)		
Methylene blue	5 x 10^8	(3.5% contraction) (15)	7	<0.01
Control	5 x 10^7	12 (7.6)		
Superoxide dismutase	5 x 10^7	54 (11.7)	15	<0.02

Mean relaxations are compared with controls by Student's t test for paired data.
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cytes did not relax muscle. Removal of platelets did not alter relaxation of precontracted muscle by $1x10^8$/l mononuclear cells which was 22 (7%) with platelets and 26 (10%) without (p>0.1, n=7 pairs). Therefore platelets were not removed from mononuclear cell suspensions in the subsequent experiments as this process reduced cell yield. Substances known to affect the NO-cGMP pathway affected muscle relaxation by leukocytes (Tables 1 and 2). Addition of 200 nM oxyhaemoglobin and 10 μM methylene blue to the organ bath, one and 10 minutes before cells respectively, reduced muscle relaxation. Incubation of cells for 45 minutes with 100 μM N^G-monomethyl-L-arginine reduced muscle relaxation, but 100 μM N^G-monomethyl-D-arginine had no effect. Superoxide dismutase (60 units/ml) added one minute before leukocytes produced an increase in muscle relaxation. Tetrodotoxin (100 nM) did not affect muscle relaxation by leukocytes (n = 5, not shown). Leukocytes were activated by incubation for one hour with FMet-Leu-Phe 100 nM. Activated mononuclear cells (5 x 10^7/l) caused a mean muscle relaxation of 43.6 (15%) compared with 8.3 (4%) by paired non-activated cells (n = 10 pairs, p<0.05). Activation of granulocytes did not increase muscle relaxation (mean relaxation by activated granulocytes 21.3 (10%) compared with 18.4 (6%) by non-activated cells (n = 12 pairs, p=0.7).

Nitric oxide synthesis in human rectal mucosa

Citrulline, arginine and cystine were the only amino acids whose concentrations differed significantly between groups. Citrulline was higher in active than in quiescent colitis (p<0.05) or normal mucosa (p<0.05). Significantly higher concentration of arginine and cystine were found in patients with active colitis. Citrulline concentrations were significantly lower in biopsy specimens incubated with L-NMMA than in those incubated with D-NMMA (Figure 3, Table 3).

Measurement of mucosal nitric oxide synthase activity

NOS activity was not detected in histologically normal mucosa. Inflamed mucosa produced ^{14}C-citrulline from ^{14}C-arginine at a mean rate (±SE) of 2.1±0.75 nM/mg/min in the presence of

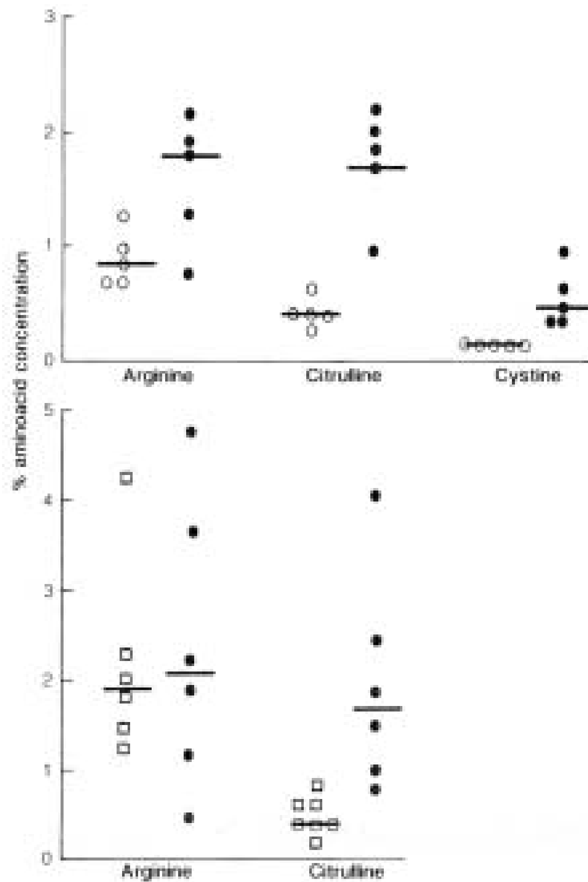

Figure 3: Aminoacid concentrations in rectal biopsy specimens. Concentrations shown as percentage of total aminoacids measured. Concentrations in patients with active ulcerative colitis (●) were compared with those in patients with quiescent disease (O) (upper) or histologically normal mucosa (□) (lower). Bars = medians.
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control (D-NMMA) which was reduced to 1.55±0.4 nM/mg/min by L-NMMA. In the absence of calcium mean ^{14}C-citrulline production was 1.5±0.3 nM/mg/min with D-NMMA falling to 1.28±0.3 nM/mg/min with L-NMMA. The difference between TNOS and INOS activity was taken as constitutive (CNOS) activity.

Removal of calcium reduced mean ^{14}C-citrulline production from 2.2±0.75 nM/mg/min to 1.5±3 nM/mg/min which corresponds to calcium dependent ^{14}C-citrulline product of 0.7±0.08 nM/mg/min.

Production of ^{14}C-urea was not affected by either L-NMMA or removal of calcium.

Measurement of faecal nitric oxide synthase activity

L-NMMA reduced ^{14}C-citrulline production by faeces from patients with UC but did not affect faeces of healthy subjects in this manner. In the absence of calcium L-NMMA did not reduce ^{14}C-citrulline production. Production of ^{14}C-urea by faeces was not affected by L-NMMA or removal of calcium.

Table 3: Aminoacids* in paired rectal biopsy specimens after incubation with L-NMMA or D-NMMA (200 μmol/l)

Patients	With D-NMMA Arginine	Citrulline	With L-NMMA Arginine	Citrulline
1	3.8	1.7	5.6	1.1
2	1.8	5.0	0.6	0.3
3	6.7	3.1	4.7	2.7
4	8.4	1.9	0.5	0.5
5	5.4	6.2	2.1	1.0
6	1.4	6.4	0.9	3.7
7	..	12.8	..	7.0
8	7.2	1.7	..	1.1

*Values are percentages of total aminoacids measured.
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DISCUSSION

Granulocyte and mononuclear cells relax colonic circular smooth muscle strips, precontracted by acetylcholine. The mediator of muscle relaxation is unlikely to be a prostanoid as leukocytes were incubated with 100 nM indomethacin. Leukotrienes produce contraction of this tissue and thromboxane has no effect (*Middleton* and *Hunter*, 1992). Muscle relaxation was increased by superoxide dismutase and reduced by oxyhaemoglobin and pre-incubation with methylene blue. Incubation of effector phagocytes with N^G-monomethyl-L-arginine reduced muscle relaxation, whereas incubation with N^G-monomethyl-D-arginine had no effect. Only viable mononuclear cells and granulocytes caused muscle relaxation, suggesting that the relaxing factor is not stored by these cells. Muscle relaxation was not affected by tetrodotoxin and therefore unlikely to be mediated by neural elements. These findings strongly support the suggestion that effector phagocytes relax circular smooth muscle by the release of NO.

Our results are in agreement with those of others who found that activated and non-activated macrophages (*Salvemini* et al., 1989; *Stuehr* et al., 1989; *Marletta* et al., 1988) and granulocytes (*McCall* et al., 1989; *Schmidt* et al., 1989) relax vascular smooth muscle by release of NO that is synthesised from L-arginine by a stereo-specific enzyme, NO synthase. Release of NO is increased by activation of macrophages (*Iyengar* et al., 1987) but not granulocytes (*Wright* et al., 1989), possibly because of the simultaneous increase in production of superoxide anions that react with NO.

Leukocytes, forming part of the inflammatory infiltrate of ulcerative colitis and at other sites of inflammation in the gastrointestinal tract, may produce smooth muscle relaxation via release of NO. Diffusion of NO through the submucosa might be facilitated by the formation of a stabilising adduct with a carrier molecule such as cysteine, or a thiol containing protein such as albumin (*McCall* and *Vallance*, 1992; *Thornbury* et al., 1991; *Ignarro*, 1990). Formation of these S-nitrosothiol compounds has been shown to increase the biological half life of NO in physiological solutions from three to five seconds (*Palmer* et al., 1987; *Harbison* et al., 1986) to

about 40 minutes (*Stamler* et al., 1992). Pacemaker cells are located on the submucosal surface of the circular muscle (*Smith* et al., 1987). These cells not only produce electrical slow wave pacemaker activity responsible for the spontaneous mechanical activity of circular smooth muscle but also form a regenerative surface that propagates this activity. Damage to these cells reduces electrical pacemaker activity and impedes its propagation (*Sanders* et al., 1990). NO or its adduct may inhibit this pacemaker activity or the response of myocytes to it, thus reducing spontaneous mechanical activity and causing a reduction in smooth muscle tone. In severe inflammation where the muscularis propria is infiltrated by leukocytes, profound dilatation may occur such as that seen in toxic megacolon (*Heppell* et al., 1986). It has recently been shown that NO relaxes the human internal anal sphincter (*Burleigh*, 1992). This may contribute to the urgency to stool, often associated with ulcerative colitis, if inflammatory cells release NO in sufficient amounts to affect sphinteric function.

Smooth muscle relaxation by NO is mediated by raising intracellular cGMP concentration, which inhibits the release of calcium from intracellular stores (*Nakatsu* and *Diamond*, 1989; *Arnold* et al., 1977; *Middleton* et al., 1992) and may produce mechanical changes without alterations in membrane potential (*Ito* et al., 1980). This may explain the electromechanical disassociation found by *Snape* et al. (1980) as the cause of reduced gastrocolonic reflex in patients with ulcerative colitis.

It is possible that the increased NO synthesis in our patients with UC was mediated by infiltration of the mucosa by leukocytes. Because citrulline concentrations in the rectal mucosa differed in all the experiments during the measurement of mucosal nitric oxide synthase activity, it is most unlikely that the increase in citrulline occurred by chance. Since L-NMMA has no effect on arginase or arginine decarboxylase activity (*Moncada* et al., 1991), increase citrulline biosynthesis must be a consequence of NO synthase activity which simultaneously results in the production of equimolar amounts of NO (*Hibbs* et al., 1987b). The increased concentrations of cysteine in active UC (Figure 3) can be explained because NO is known to combine with cysteine to form S-nitrosocysteine which liberates NO yielding cystine (*Yeates* et al., 1985).

The inhibitory effect of L-NMMA on the formation of ^{14}C-citrulline suggests that inflamed rectal mucosa in UC has increased NOS activity. This was reduced by approximately 60% on removal of calcium implying that 60% of total mucosal NOS activity is due to the constitutive enzyme. Removal of calcium had a greater inhibitory effect on ^{14}C-citrulline production than could be explained by CNOS inhibition alone implying that calcium restriction may also inhibit enzymes other than CNOS.

Increased mucosal INOS activity in ulcerative colitis can be explained by the presence of leukocytes, which do not contain the constitutive enzyme whose increased activity is not therefore easily explained. Enteric bacteria were seen contaminating mucosal biopsy specimens under light microscopy, and samples of faeces from patients with active UC had CNOS activity whereas those from healthy controls did not. Leukocytes shed into the lumen in ulcerative colitis are present in faeces but do not contain CNOS. This must therefore have another origin, and may be present in as yet unidentified microorganisms. Sulphate reducing bacteria are more common in faeces from patients with UC (*Florin* et al., 1990) and it is possible that CNOS is present in these or other microorganisms. Several enteric

bacteria have been shown to produce NO in micromolar concentrations in anaerobic culture at pH similar to that of the colonic lumen in UC (*Raimundo* et al., 1992).

Low concentrations of NO cause relaxation of colonic circular smooth muscle (*Middleton* and *Hunter*, 1992) and increased NO synthesis in UC may contribute to the associated smooth muscle dysfunction (*Spriggs* et al., 1951; *Kern* et al., 1951) which produces an attenuated gastrocolonic reflex (*Snape* et al., 1980), impaired contraction of colonic smooth muscle (*Cohen* et al., 1986) and the profound dilatation seen in toxic megacolon (*Heppell* et al., 1986). NO is directly toxic to host and target cells (*Billiar* et al., 1989) and, together with the superoxide anion, may under certain circumstances give rise to the formation of the peroxynitrite radical which may lead to the generation of highly toxic hydroxyl radicals (*Beckman* et al., 1989)

In certain experimental models of inflammation, NO may be cytoprotective, possibly by limiting microvascular damage (*Boughton-Smith* et al., 1992). It may also be involved in defence against microorganisms (*Granger* et al., 1986), although many bacteria are resistant to it (*Saito* et al., 1991). However, in ulcerative colitis production of NO may also lead to host tissue damage.

The formation of carcinogenic nitrosamines by leukocytes is NO dependent (*Grisham* et al., 1992) and is favoured by the low pH of the colonic lumen in UC (*Marletta* 1988). This may contribute to the associated risk of colonic neoplasia (*Collins* et al., 1987, *Korelitz* 1983).

Thus, NO biosynthesis has been shown to mediate NANC tonic neural inhibition of spontaneous mechanical activity in distal colonic smooth muscle. NO may be important in producing many of the clinical effects known to be associated with UC, such as diarrhoea, smooth muscle dysfunction, toxic megacolon or neoplasia. NO has been shown to be produced by human leukocytes and infiltration of inflammatory cells may be a factor in increasing NO production in inflamed colonic mucosa. However, the increased activity of NO synthase that we have demonstrated in the mucosa involves not only the inducible but also the constitutive form of the enzyme. As constitutive NO synthase is not found in leukocytes, it seems likely that some faecal CNOS is derived from other sources such as colonic bacteria. NO synthase activity was not demonstrated in faeces from healthy human volunteers and it seems possible that an abnormal colonic microflora, which produces excessive amounts of NO, may contribute to the disease process in ulcerative colitis. Further studies on the role of the intestinal microflora in the production of NO are currently underway.

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OLD HERBORN UNIVERSITY SEMINAR ON IMMUNE SYSTEM AND MICROFLORA: MINUTES AND OVERVIEW OF THE DISCUSSIONS

DIRK VAN DER WAAIJ

Laboratory for Medical Microbiology, University of Groningen, Groningen, The Netherlands

DISCUSSION PARTICIPANTS (in alphabetical order):
Herma Apperloo-Renkema, Joseph Beuth, John Cebra, Peter J. Heidt, John Hunter, Gijs Jansen, Jaap Kampinga, Tore Midtvedt, Masanobu Nanno, Carl-Eric Nord, Volker Rusch, Dirk van der Waaij, Laurens van der Waaij, Carol Wells, and Michael Wilkinson

INTRODUCTION

Type of patients, type of infections in hospitals since the introduction of antimicrobial drugs

As outlined by Maxwell Finland in 1959 (*Finland* et al., 1959), both the spectrum of bacteria involved in serious infections in hospitalised patients and the type of patients have changed significantly since the introduction of antimicrobial drugs in 1935. In the pre-antimicrobial era, infections by pathogens dominated the scene. Since antimicrobial drugs became available, potentially pathogenic (opportunistic) bacteria and later yeasts caused most infections. In these years, also the more compromised type of patients gradually replaced the conventional type of infected patient with an infection by a pathogen.

Resistance to antimicrobial drugs

Since the introduction of antimicrobial drugs, it also became evident that, regardless many repeated attempts of the pharmaceutical industry, it has not been possible to master the resistance problem. It is for this and other reasons not to be expected that antibiotics will provide the ultimate answer to the problem of opportunistic infections in the increasing number of compromised patients in our hospitals.

Effect of antimicrobial drugs on intestinal microflora

Many antibiotics have appeared to affect the indigenous microflora of the digestive tract. Suppression of the microflora is an important factor in the transmission of resistance to other bacteria in the tract as well as in the spread of resistant bacteria to other patients.

The Old Herborn University Seminars

It was decided in 1985 to organise annually a meeting with more time for discussion than for presentations on the various aspects involved in the interactions between host (patient) and his microflora. This kind of meeting has become known as the Old Herborn University Seminar. It was hoped that, by collecting information around this theme by inviting experts in the various subsets of this issue, we might learn step by step in which manner healthy individuals interact with and withstand opportunistic microorganisms.

Recently, during the Third South Pacific Congress on Chemotherapy in Infectious Diseases, data have been reported about the incidence of multiply resistance. In a number of hospitals in some countries, antibiotic treatment is

not longer effective for this reason. This information has urged us to speed up our attempts to collect information supporting our search for other ways than antibiotic therapy for prevention and treatment of opportunistic infections.

The present meeting was specifically focussed on the physiologic routes of contacts and interactions between the immune system and microflora as well as on techniques by which these interactions could be measured with great sensitivity and accuracy. The outcome of the discussions of the meeting could possibly be used as a basis for the design of (standardised) protocols to study new ways of prevention and possibly even therapy of opportunistic infections in hospitals.

DISCUSSIONS

General

The program of the meeting was based on the fact that the antimicrobial defence system consists basically of two mechanisms which should both be measurable in greater detail:

a. *Inter-bacterial interactions* (including competition for nutrients provided by the host organism). Inter-bacterial interactions inside the digestive tract could be regarded as a first line of defence. This part of the defence system in which the host organism is also involved is called *colonisation resistance of the digestive tract*.

b. *The antimicrobial defence system of the host organism* consisting of an *a-specific part* (phagocytosis killing and digestion of microorganisms) and a *specific part* by cells of the immune system. The specific part is involved in both opsonisation of bacteria by coating them with antibodies and the subsequent cytotoxic killing of phagocytosing cells that have been paralysed by the ingested organisms.

The discussions concerned these fundamental points. In addition techniques to measure the activity of the interactions between host and microflora have been discussed.

Colonisation resistance

The *colonisation resistance* (CR) of the digestive tract was defined as the resistance encountered by a (non-viral) microorganism when it tries to colonise a particular niche in the digestive tract upon oral intake. The resistance would be the net resultant of a concerted action of the host organism (by feeding the indigenous flora with saliva/mucus and extruded cells in the mucosal layer) and his indigenous microflora (by competition for nutrients and by producing substances with some hostile [antibiotic] activity). Presumably, secretion of antibodies into the intestines plays a role. The combination of host and flora forms a stable ecosystem.

The CR may differ between individuals of an animal species. A lower CR may imply an enhanced risk of infection, especially in compromised individuals. Therefore, it is to be expected that it will become of practical value to measure the CR in individual patients. However, it is still difficult to measure the CR directly in patients. Firstly, this is so because it requires oral contamination with living potentially pathogenic bacteria. Secondly, for each bacterial species the CR may be different although values overlap. Apperloo-Renkema reported a linear correlation between the mean ^{10}log concentration of the *E. coli* strain used for oral contamination and the CR measured by comprehensive biotyping of Enterobacteria species in four faecal samples. Indirect

measurement of the CR by comprehensive biotyping of Enterobacteria species in minimally four faecal samples is the only way for the time being. However, this is a laborious and expensive way of measuring the CR. New techniques to determine the CR clinically should therefore be developed.

Suppression of the composition of the microflora by antibiotics during treatment of an infection as well as by modulation with food additives and diet may influence the CR to foreign bacteria respectively negatively and positively. In addition, it may influence the interactions between the flora and the immune system. In previous seminars, it appeared that flora suppression with antibiotics might play an essential role in the outcome of experimental treatment of tumours in oncology. In another previous seminar, the potential role of the microflora - and thus the effect of its modulation - on the development of autoimmune phenomena such as Graft-versus-Host Disease (GvHD) has become evident.

Methods to measure the CR

Factors, which can be used to determine the intactness of the indigenous microflora, are called *Microflora Associated Characteristics* (MAC's). Several MAC's have been reported, most of which concern chemical activity of bacteria, one MAC concerned the morphology of the faecal microflora. Many of the bacteria involved in MAC's are presumably involved in the CR. However, a study in which this relation has been specifically studied is not known. The use of β-aspartylglycine as a (negative) marker for intactness of the flora as a MAC was regarded most promising. However, its concentration may fluctuate rather strongly in a series of faecal samples of individual subjects without proven concomitant fluctuations in the composition of the intestinal flora.

The entropy of the micromorphological diversity of the faecal flora as determined by *Meijer* et al. (1991) in nigrosine stained faecal smear preparations with computer software designed for this purpose, theoretically also could be considered as a measure for CR. However, no study is known to date that indicates a correlation between the micromorphological entropy and the CR for one or more potentially pathogenic bacteria. The entropy however, is easy to determine. Furthermore it has not only shown a close relation between the outcome of anaerobic culturing of faecal samples during antibiotic treatment but also showed a slight but significant decrease after some time during oral treatment of volunteers with Symbioflor 1® (a mixture of ten different strains of living *Enterococcus faecalis*). This requires further study.

Correlation between antibody titres of Ig-isotypes and the degree of CR. The relation between the CR and an anti-potentially-pathogenic antibody titre has been studied indirectly. This means that a correlation was sought between the titre of circulating antibodies to an *E. coli* strain used for oral contamination in healthy human volunteers. There was however in that study only an indication that high IgM antibody titres to *Enterobacteriaceae* species in the faecal samples did correlate with a low CR. A significant correlation was not found for IgG or IgA antibodies.

THE DEVELOPMENT OF DEFENCE SYSTEMS IN THE COURSE OF EVOLUTION

Our planet was first colonised by autotrophic bacteria; later mutants developed which could make use the feed-erlayer formed by autotrophic bacteria.

In the course of billions of years, different ecosystems have thus been formed at different places. Their defence to newcomers (bacteria developed in another niche), which may have been moved in by the wind from time to time, may have been the same as it is nowadays: i.e. competition for nutrients and the presence of toxic substances (antibiotics) for which the local population was naturally resistant. Bacteria and other primitive cell-systems had - and still have - a strong capacity to adapt to external (environmental) changes in several generations. Development of resistance to antimicrobial drugs (including the man-made and man-modified antimicrobial drugs) was therefore to be expected. The design of an antimicrobial agent for which no resistance can develop may never be possible.

From the bacterial ecosystems, eukaryotic cells may have developed as suggested by Lynn Margulis in her book "Symbiosis in cell evolution" (ISBN 0-7167-7028-8). These cells may have been comparable to our present monocellular organisms in their capacity to defend themselves to microorganisms, namely by phagocytosis, intra-cellular killing and -digestion. When later in evolution multi-cellular organisms developed a digestive tract, they may have attained a microflora in that tract similar to the ones present in primitive animals. More recent in evolution legs, wings or a tail to swim developed. New animals could actively move to different places. Arrival in new environments may only have been possible because the simultaneous development of an (primitive) immune system. The static system formed by the antibiosis by the digestive tract ecosystem may not have been adequate to protect the host organism to new bacteria encountered in other (microbial) environments. For the relatively rapid changes in the spectrum of microorganisms present in the environment, encountered by moving from one place to another, a specific flexible defence system was required. The antimicrobial defence had to become specific because it should not affect bacteria of their own gut ecosystem like antibiotics may do during treatment. The defence had to be dynamic and adaptable because of the development of resistance by bacteria. Microorganisms appear to have the capacity to vary antigens in their outer membrane so that they become less sensitive to the existing immune response.

It should be attempted to learn more from developments during the evolution, because these developments have been dictated by Nature and they may represent the most optimal format for long-term survival of the various species including man.

IMMUNE INTERACTIONS WITH MICROFLORA

General

Central in the discussion was the fact that in healthy individuals no signs and symptoms of inflammation are seen although bacteria appear to pass through the gut epithelium and therewith might provoke an inflammatory response.

Inflammation was defined as the response of white blood cells and their products to foreign antigens to such a degree that local vasodilatation occurs and more white blood cells (and their products) are attracted.

In severe cases, the inflammatory response may become systemic and involve many organs (multi-organ failure) being associated with an increase of the serum concentration of interleukins such as IL-6 and TNF.

Oral tolerance

The absence of inflammation upon penetration of bacteria (and food antigens) which have induced hyporeactivity after oral ingestion is assumed to be due to a mechanism called oral tolerance. In classical experiments, oral tolerance was induced by oral gavage of high numbers of sheep red blood cells in mice or rats. Upon subsequent parenteral challenge with sheep red blood cells, no or low titred antibody response occurred. This type of induced tolerance was transmissible with spleen cells. For this reason it is likely, that CD-8 T-suppressor cells are involved.

Interactions between intestinal bacteria and immune system

The bacteria of the resident flora which colonise the mucus of the intestinal epithelium are presumably in continuous contact with the Gut Associated Lymphoid Tissues (GALT) and/or the systemic immune system. Presumably predominantly cells of the GALT are responsible for:

1. The humoral immune hyporeactivity to bacterial and food antigens, and
2. The absence of delayed type hypersensitivity reactions to intestinal antigens (including bacteria).

In order to get in contact with the immune system, bacteria or bacterial fragments must cross the barrier formed by the epithelial lining. This transfer through the epithelial lining is called *translocation*. Translocation of bacteria may include bacterial fragments and even larger (dietary) molecules.

Translocation of pathogens such as *Salmonella typhi*, *Listeria monocytogenes*, and *Mycobacterium bovis*, may occur predominantly through the M-cells overlaying the Peyer's patches. Other bacteria however, as has become evident in EM-studies by Wells and co-workers may (also) translocate elsewhere. Translocation of intestinal bacteria may therefore also occur in the colon in which Peyer's patches are rare. *In vitro* studies with colonic epithelial cells have made likely that bacteria are actively embraced by the microvilli and once they are completely engulfed they get internalised in the cell. Intra-cellular transport to the basal membrane and the underlying lamina propria may *in vivo* complete translocation. Translocation is not confined to the digestive tract as it has also been observed in the bronchi into the Bronchus Associated Lymphoid Tissues (BALT) and by bacteria of the vaginal flora to the local draining lymph nodes.

Induction mucosal hyporeactivity

Induction of tolerance by bacteria, which are indigenous in the digestive tract, may play a role in the absence of inflammation in the lamina propria and the sub-mucosa. Disturbance of this balance between immunity and tolerance could cause local or more diffuse inflammation in the intestines upon translocation of resident (colonising) bacteria. There is indeed evidence, which makes likely that, among else, 'chronic inflammatory bowel disease' could be caused by improper function of the gut associated immune system.

Influence of circulating antibodies on the occurrence of translocation

In some publications it is reported that specific immunity would reduce translocation of corresponding bacteria. Observations reported by others, however, do question that. It was suggested that this phenomenon should be studied in SCID mice who have neither T nor B cells and in RAG-2 knock-out mice. Only in this way the precise contribution of both cell types to translocation could be studied.

Translocation in man

Translocation should also be studied more intensely in man. However, such studies are only possible in patients undergoing abdominal surgery and possibly in 'organ donors' following CVA.

In both types of subjects, however, several additional factors may influence the outcome. In surgical patients this could for example be the underlying disease and anaesthesia.

ORAL IMMUNE TOLERANCE

General

Oral tolerance (immune hyporeactivity) preventing cellular immune reactivity may result from the formation of suppressor-cells in the gut's parenchyma or lymphoid tissue. After migration of these cells to the periphery, systemic tolerance may be the result. A well-known example is the prevention of nickel allergy by oral intake of nickel chloride molecules.

Mechanisms involved

Two mechanisms were considered responsible for the absence *of delayed type hypersensitivity (DTH) reactions in the digestive tract wall*:

a. Upon intake of an antigen (could be a fragment of a bacterium) in Peyer's patches, T-helper cells instruct B cells to produce IgA. After transfer through the thoracic duct, these B cells migrate back to the gut and invade the lamina propria where they differentiate into actively IgA secreting plasma cells. Secretion of IgA into the gut lumen and its specific binding to bacteria (and other antigens), might cause enhancement (antigen-blocking) and thus prevent further immune (cytotoxic T-cell) activity to the antigen in question if translocation occurs.

b. The environment in the lymphoid tissue of the gut might be such, that only Th-2 reactivity can develop (e.g. high local production of IL-4 or IL-10). Such an environment not only prevents the development of Th-1 activation (and therefore local DTH reactivity) but might also be responsible for the formation of Th-2 cells. Antigen specific Th-2 cells will migrate to the periphery and may, after renewed antigen contact, prevent Th-1 reactions to occur by secreting IL-4 and IL-10 lymphokines.

Oral tolerance upon association with bacteria in the new-born and in adult germfree mice

In new-born mice, antibodies as well as maternal lymphocytes obtained passively by passage of the placenta and via the milk, may play an essential role in the adaptation to the development of an gastro-intestinal microflora. Conventionalisation of adult germ-free mice, however, also rarely causes disease or death due to infections. In the conventionalised adult germfree animal the idiotypic antibody network and NK cells may play an essential role in bridging the interval after conventionalisation until 'oral tolerance' has developed. Initially however, quite substantial bacterial translocation may occur. A period with enhanced bacterial translocation shortly after birth (conventionalisation) has not been described in baby mice. However, an initial phase with increased uptake of larger dietary molecules is known to exist. In both conventional new-born mice and in mice who are conventionalised at adult age, circulating antibodies to intestinal bacteria are formed. However, the titres are generally low.

This information about circulating antibodies to intestinal bacteria was reported in the fifth Old Herborn University Seminar.

VACCINES

The vaccines, defined as preparations of dead or life microorganisms, are meant to instruct the immune system. Instruction of the immune system may either result in a decrease of the response (suppression) to a subsequent challenge with the same antigen or to an enhanced response (activation). Two lines of administration have been discussed: Orally and parenterally.

Oral vaccines

Life bacteria

The results of the use of a life oral vaccine were reported on the first day of the meeting. It concerned Symbioflor 1®, a mixture of ten *Enterococcus faecalis* strains. Medical use of this vaccine would suppress inflammation in the oropharyngeal region. In nine of ten volunteers, a significant decrease of the titre of circulating IgG to *Enterococcus faecalis* was found. In addition, this vaccine appeared to influence the composition of the faecal flora of the volunteers according to a slight but significant decrease of the entropy of the micromorphology of the microflora. This change in the flora occurred concomitantly with a decrease in the IgG antibody titre to the autologous faecal flora during treatment.

The site at which *Enterococcus faecalis* may have been taken up was either in the tonsils or by the Peyer's patches in the small intestines. It was suggested to study whether the tonsils or the Peyer's patches are the predominant port of entry by application of the Symbioflor 1® mixture in enteric-coated capsules in future studies.

In this category of vaccines, the use of strains of potentially pathogenic bacteria isolated from the stools of an individual also urgently requires study. It seems plausible that these vaccines 'boost' negatively (suppressive) or positively, the pre-existing immune response to the individual strains in the vaccine. It seems plausible that these vaccines can be administered parenterally as well. However, since no information is available as yet, great caution should be taken with this mode of administration. Experience with single pure cultures of Gram-positive and Gram-negative bacteria is first required before the use of mixtures of pure cultures can be considered.

Dead bacteria

Also small peptides of intestinal bacterial (outer membrane) origin appeared to influence the immune system upon oral administration in mice. The influence of some of these molecules on the immune system was boosting, whereas the effect of other molecules was suppressive. The chemical analysis of these molecules is being done.

Parenteral vaccination

There is no information about the effect of parenteral vaccination on translocation of potentially pathogenic bacteria and subsequent stages of clearance of these bacteria. This subject requires study.

DEVELOPMENT OF TECHNIQUES TO MEASURE ANTIBODY TITRES TO COMPONENTS OF THE FAECAL FLORA

Fluoro-morphometry

With the help of a system designed and developed in Groningen, the GRID-system, it was possible to reproducibly determine the amount of circulating antibodies to objects (bacteria) in the

faecal flora. A study by Apperloo-Renkema has made likely that the amount of fluorescence measured per object correlates linearly with the classical (^{2}log) antibody titre to the same object(s). These studies have been published. Some discussion occurred on the desirability of the use of markers for bacteria. With the help of specific markers for bacteria, it may become possible to distinguish between bacteria and bacteria-like particles in faeces.

Analysis of antibody coating of faecal bacteria by flow-cytometry

Both *in vivo* coating and *in vitro* coating of bacteria has been found possible, provided the events (bacteria) are marked with propidium iodine (PI) and the correct filters are used. This technique correlated well with data obtained by fluoro-morphometry in corresponding faecal samples. Flow-cytometry analysis was reported to be faster than fluoro-morphometry.

CONCLUSIONS

Our future attempts to find alternative ways of prevention/treatment of infections could perhaps best base on the physiologic mechanisms, which have developed and survived during evolution. Only relatively little is known of the cells and substances involved in the normal control of potentially pathogenic microorganisms.

Attempts to study this new concept for prevention/treatment of infections by potentially pathogenic microorganisms should concern the design of an animal model to study the occurrence of bacterial translocation as well as studies of the mechanisms involved in the physiologic smooth (unnoticed) way in which translocated bacteria are cleared from the tissues/circulation in healthy subjects.

Meanwhile, methods have been designed and tested to measure and follow in the time, the interactions between various cells of the humoral part of the immune system and indigenous bacteria.

Because of the obvious role of intestinal bacteria in autoimmune phenomena, a study of ways to prevent/treat infections along physiologic lines, outlined by Nature during the evolution, seems important.

FORMATION OF AN INTERNATIONAL STUDY GROUP

Because there is an urgent need to search for other ways of prevention and therapy of infections, since the usefulness of antibiotics is rapidly declining, it was discussed whether an International Co-operative Study Group should be formed.

Two facts will form the basis for the study of ways for prevention/treatment of infections in the future:

a. The great majority of the infections in patients is caused by opportunistic bacteria. Changes in the physiologic defence mechanisms of the body provide these bacteria with a chance to cause clinical infections.

b. The development of resistance in opportunistic bacteria to specific immune responses has remained zero during evolution. This makes likely, that the immune system is sufficiently rapid and flexible to follow changes in the antigenic composition of opportunistic bacteria.

LITERATURE

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