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

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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-

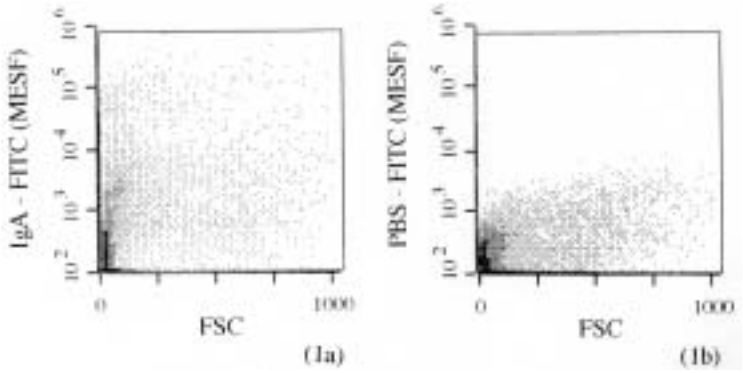

Figure 1: Forward scatter (FSC) versus fluorescence scatterplots of a sample stained for IgA (1a) or without staining (1b).

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 FlTC-$F(ab')_2$ anti-Hu-

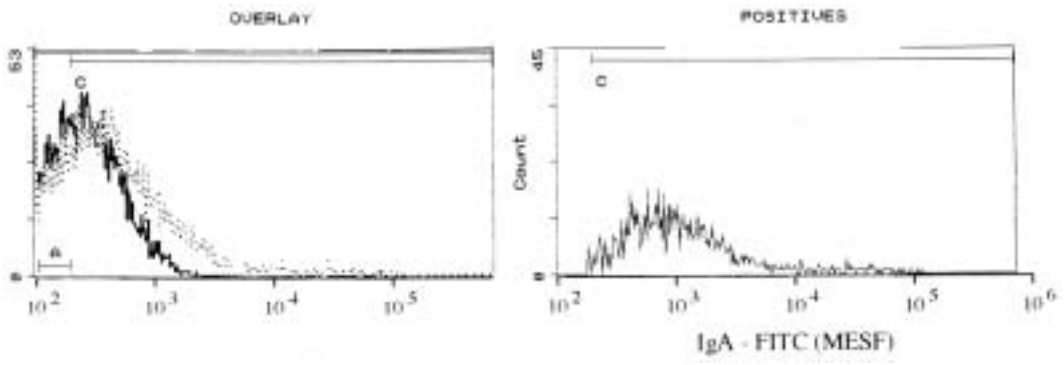

Figure 2: Typical example of fluorescence distributions of non-stained bacteria (overlay, continuous line) and bacteria labelled with FITC-anti-IgA (dotted line) as recorded by flow cytometry. The fluorescence histogram of non-stained bacteria was subtracted from that of stained bacteria by match region 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

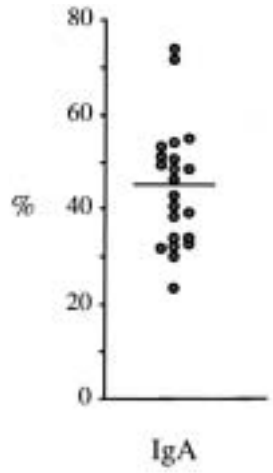

Figure 3: Percentage of faecal bacteria coated with IgA as determined by match region subtraction. The median percentage is shown.

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-

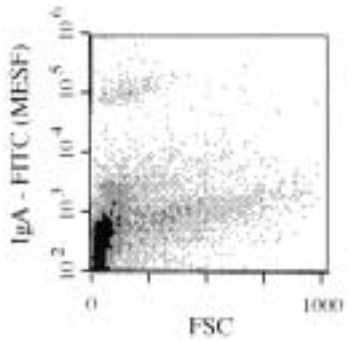

Figure 4: FSC versus FITC-fluorescence distribution of a faecal bacterial suspension stained for IgA. This sample contained a separate strongly IgA-coated population which was sorted by flow cytometry.

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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