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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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