Old Herborn University Seminar Monograph

2. INTERACTIONS BETWEEN THE INDIGENOUS MICROFLORA AND THE HOST IMMUNE SYSTEM

EDITORS:

PETER J. HEIDT
RODNEY D. BERG
VOLKER RUSCH
DIRK VAN DER WAAIJ

Old Herborn University Seminar Monograph 2

ISBN 3-923022-11-5
ISSN 1431-6579

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

Rodney D. Berg, Ph.D.
Department of Microbiology and Immunology
Louisiana State University Medical School
Box 33932
Shreveport, LA 71130
USA

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

Contents

Contents (continued)

Contents (continued)

Participating authors

Edward Balish, Ph.D., Department of Surgery, Center for Health Sciences, 4638 Medical Science Building, University of Wisconsin, Madison, Wisconsin 53706, USA.

Kenneth F. Bartizal, Jr., Ph.D., Infectious Disease Research, Merck, Sharp and Dome Research Laboratories, Rahway, New Jersey, USA.

Robbert Benner, M.D., Ph.D., Department of Immunology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

Rodney D. Berg, Ph.D., Department of Microbiology and Immunology, Louisiana State University Medical Center, 1501 Kings Highway, P.O. Box 33932, Shreveport, Louisiana 71130-3932, USA.

Per Brandtzaeg, M.D., Ph.D., Laboratory for Immunohistochemistry and Immunopathology, Institute for Pathology, National Hospital, University of Oslo, N-0027 Oslo-1, Norway.

Philip B. Carter, Ph.D., Department of Microbiology, Pathology and Parasitology, School of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, North Carolina 27606, USA.

Henk W.S. Goris, M.D., Ph.D., Department of Medical Microbiology, University Hospital Groningen, Oostersingel 59, 9713 EZ Groningen, The Netherlands.

Peter J. Heidt, Ph.D., Department of Microbiology and Gnotobiology, Radiobiological Institute TNO, Lange Kleiweg 151, 2280 HV Rijswijk, The Netherlands.

Hiroshi Kiyono, D.D.S., Ph.D., Department of Oral Biology, Research Center in Oral Biology, The University of Alabama at Birmingham, Birmingham, Alabama 35294, USA.

Thomas T. MacDonald, Ph.D., Department of Paediatric Gastroenterology, St. Bartholomews Hospital, St. Bartholomew' Close, West Smithfield, London EC7A 7BE, UK

John H. Schwab, Ph.D., Department of Microbiology and Immunology, University of North Carolina, Campus Box II, 7290, Chapel Hill, North Carolina 27514, USA.

Dick Veenendaal, M.D., Ph.D., Department of Medical Microbiology, University Hospital Groningen, Oostersingel 59, 9713 EZ Groningen, The Netherlands.

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

SYSTEMIC IMMUNITY

Authorised transcript of a lecture by

EDWARD BALISH

Department of Surgery, Center for Health Sciences, University of Wisconsin, Madison, Wisconsin, USA

Presented at the 2nd Old Herborn University Seminar on
"Interactions between the indigenous microflora and the host immune system"
on June 1, 1988

Ladies and Gentlemen:

Thank you for the invitation to participate in this meeting. I appreciate the opportunity to relate to you some of the experiments we have carried out at the University of Wisconsin Gnotobiotic Research Laboratory in Madison, Wisconsin.

In particular I want to talk about the interaction of intestinal microorganisms with the host and how the host, in this case a germfree rodent, responds immunologically to the colonisation of the intestinal tract with different, known, microorganisms in pure culture.

Basically, what we are dealing with is a pure culture of intestinal bacteria (as the antigen) and the germfree rodent host that will undergo immune responses after its mucosal surfaces in the alimentary tract are colonised with bacteria. Today, I will relate some of our results with both bacteria and fungi. We have also carried out studies on how the intestinal flora can alter the effects of carcinogens that are injected into the large bowel and we have carried out studies on how the intestinal flora affects naturally tumouricidal lymphocytes, i.e. lymphocytes that are capable of killing tumour cells without having had previous contact with the tumour cells. Dr. Bartizal will tell you about the latter aspect of NK cell activity and microbial flora later in this meeting.

Before we go on to talk about the effects of intestinal flora on systemic immunity, I do want to point out that many non-specific host defence mechanisms are also influenced by a hosts' contact with intestinal microbes.

Such factors as acid and proteolytic secretions, mucous layering, peristalsis, shedding of epithelial cells, adhesins and receptors on cells and many other "innate" factors are all important aspects of any host interaction with intestinal microbes. For the most part I will try to confine my brief time to systemic antibody responses that take place after a germfree rodent is colonised with a pure culture of bacteria and how such bacteria-host interactions affect the hosts' immune response. A very complex series of host-parasite interactions taken place after a germfree rodent is colonised with an intestinal bacterium and a whole array of leukocytes become involved. Epithelial cells in the alimentary tract do not turn out to be a very good barrier and bacteria can translocate into the systemic circulation. During their systemic interactions, bacteria will encounter Langerhans cells, monocytes, macrophages (activated and non-activated), neutrophils, T-cells, B-cells, T helper cells, T suppressor cells, contra-suppressor cells, etc. Many of these cells produce cytokines that can modu-

late (enhance or suppress) the immune system. Other compounds produced by the interaction of bacterial antigens with host leukocytes can have a wide variety of pharmacologic effects on the host. Again, what we can detect by various assays (i.e. antigen-specific antibody, sensitised T-cell or activated macrophages) is the end product of a long line of sophisticated, complex immunological, biochemical, physiological, and pharmacological interactions that take place in the host after interacting with antigens.

I would like to talk first about some studies we carried out some time ago (Infect. Immun. 6, 112-118, 1972). In this study we used cellulose-acetate and agar gel immuno-electrophoresis to study the serum proteins of germfree rats before and after mono-association with a pure culture of several different bacteria. All of the bacteria used could quickly colonise the germfree rats' alimentary tract and all six bacteria increased the rats' total serum protein concentration. Only *S. aureus* and *P. aeruginosa* caused a noticeable rise in serum gammaglobulins. *L. acidophilus*, *S. faecalis*, and *B. fragilis* multiplied readily in the gastrointestinal tract but caused only minimal alterations in the rats' serum proteins. *Proteus vulgaris* caused a marked increase in the alpha and beta, but not the gamma globulins.

Thus, three bacteria in the above study (*S. aureus*, *P. aeruginosa*, and *P. vulgaris*) caused substantial alterations in the serum protein of gnotobiotic rats whereas three others (*L. acidophilus*, *B. fragilis*, and *S. faecalis*) did not. These results are probably explained by the more pathogenic nature of *S. aureus*, *P. aeruginosa*, and *P. vulgaris* but do point out that not all bacteria in the intestinal tract of gnotobiotic rats are equally capable of altering the hosts' serum proteins.

We have also studied how the presence of an immunomodulating bacterium, *Propionibacterium acnes*, can affect the immune response of a gnotobiotic rodent. (Infect. Immun. 26, 473-478, 1979). We observed that *P. acnes* (oral and/or parenteral administration) had a modulating effect on antibody and cell-mediated immune responses of germfree and mono-associated (with a pure culture of *P. acnes*) rats. In conventionally reared rodents, parenteral injections of killed *P. acnes* stimulated the splenic plaque-forming cell response to sheep erythrocytes. However, in germfree rats, and in rats mono-associated with viable *P. acnes*, parenteral injection of killed *P. acnes* antigen inhibited the plaque-forming cell response to sheep erythrocytes. When compared with the response of germfree control rats, splenocytes from germfree rats parenterally injected with *P. acnes* antigen had a decreased *in vitro* blastogenic response to phytohaemagglutinin and conconavalin-A but not to homologous (*P. acnes*) antigens. Thus, the presence or absence of intestinal antigenic stimuli (in mono-associated and germfree rats) had a modulating affect on the immune response to a parenteral injection of *P. acnes* antigen. This work demonstrated that oral colonisation with a pure culture of *P. acnes* can modulate the immune response of the host.

We have also carried out some studies with *Clostridium tetani* recently (Infect. Immun. 41, 826-828, 1983). Germfree rats were challenged orally and intra-rectally with spores of *Clostridium tetani*. Although *C. tetani* spores remained viable in the intestinal tract they were unable to germinate. Germfree rats were then challenged orally with vegetative cells of *C. tetani*. Vegetative cells were able to colonise the intestinal tract, grow and produce toxin in the caecum and colon. Tetanus antitoxin but not tetanus toxin was detected in the sera of mono-associated rats. When we

repeated our studies with *C. tetani* in germfree mice we again found that the vegetative cells, but not spores, could grow and colonise the intestinal tract of germfree mice. We also observed that some gnotobiotic mice died after they were colonised with *C. tetani*. Hundreds of thousands of LD50 doses of *C. tetani* toxin could be detected in caecal contents of some mice. Mice that died, were for the most part male mice. We suspect fighting among male mice may have had something to do with these deaths. Mice that did die after colonisation with *C. tetani* died of a flaccid type of paralysis. They did not manifest the spasms so typical of classic cases of human tetanus. We also observed during these studies that the intestinal tract of neonatal mice did not become colonised with *C. tetani* until they were 14-18 days of age. Something prevented the intestinal tract of the infant mice from becoming colonised within the first 14 days after birth. I thought the latter observation might be of interest to the participants at this meeting.

I would like to shift topics now and go from studies of bacteria to some of the observations we have made on systemic immunity to *Candida albicans*. *C. albicans* is a common inhabitant of mucosal surfaces. It colonises the alimentary tract of a large segment of the human population and it is a microorganism that causes serious mucosal and systemic infections in a wide variety of patients being treated with broad-spectrum antibiotics, chemotherapeutic drugs or other immunosuppressive agents. *C. albicans* is also a very serious problem for patients who manifest congenital defects in phagocytic cell functions or T-cell mediated immunity. Our laboratory has been very interested in using gnotobiotic animal models to decipher the immune responses that are important in resistance to this pathogenic yeast. We have used gnotobiotic rodents to study mucosal and systemic forms of this disease. We also worked with athymic, germfree mice and their normal (heterozygous) littermates because we wanted to take advantage of germfree athymic mice that have a congenital deficiency in T-cell mediated immunity and their euthymic counterparts (Appl. Environ. Microbiol. 47, 647-652, 1984). Colony counts, scanning electron microscopy, and light microscopy were used to assess the capacity of *C. albicans* to colonise (naturally) and infect the alimentary tract of adult and neonatal (athymic or heterozygous littermates) germfree BALB/c mice. When inoculated with yeast phase *C. albicans*, the alimentary tract of adult germfree mice (athymic or euthymic) is quickly (within 24-48 h) colonised with yeast cells. Neither morbidity nor mortality was evident in any mice that were colonised with a pure culture of *C. albicans* for 6 months. Yeast cells of *C. albicans* predominated on mucosal surfaces in the oral cavities and vaginas of adult athymic and heterozygous mice. In both genotypes, *C. albicans* hyphae were observed in keratinised tissue on the dorsal surfaces of the tongue and in the cardia-atrium section of the stomach. Conversely, neonatal athymic or heterozygous mice, born to germfree or *C. albicans* colonised mothers, do not become heavily colonised or infected with *C. albicans* until 11 to 15 days after birth. Although yeast cells adhered to some mucosal surfaces *in vivo*, neither widespread mucocutaneous candidiasis, i.e. invasion of mucosal surfaces with *C. albicans* hyphae, nor overwhelming systemic candidiasis was evident in neonatal (athymic or euthymic) mice. Thus even in the absence of functional T-cells and a viable bacterial flora, athymic and heterozygous mice (adult or neonatal) that are colonised with a pure culture of

C. albicans manifest resistance to extensive mucocutaneous and systemic candidiasis. When we associate athymic and euthymic mice with *C. albicans*, the mice do manifest an immune response. We have shown that the mice with T-cell function form IgG and IgA antibodies to a spectrum of *C. albicans* antigens. Athymic mice without T-cell function formed very little antibody (IgG or IgA) to *C. albicans* antigens. Germfree controls have very little IgM, IgG or IgA antibodies that cross react with *C. albicans* antigens. Thus, a good number of the antibodies that form in mice colonised with *C. albicans* appear to be T-cell dependent. However, it is important to note that even in the absence of a spectrum of antibodies to *C. albicans* the athymic mice manifested resistance to systemic infections with *C. albicans*.

We have also carried out some studies on systemic infections with *C. albicans* in germfree and *Candida*-monoassociated mice (Appl. Environ. Microbiol. 47, 647-652, 1984). The heterozygous mice mono-associated with *C. albicans* were better able to clear the intravenous challenge from the kidneys sooner than their athymic mono-associated counterparts. The heterozygous littermate mice are sensitised after mono-association with *C. albicans* because their lymphocytes undergo a blastogenesis response with *Candida* antigen whereas lymphocytes from athymic mice did not.

In another study on systemic infections of germfree and flora-defined mice with *C. albicans* (J. Reticuloendothel. Soc. 31, 233-240, 1982), germfree BALB/c athymic mice and their thymus-bearing heterozygous normal littermates were intravenously or orally infected with *C. albicans*. The gastrointestinal tract of adult germfree athymic and thymus-bearing mice were readily colonised with *C. albicans* within 24 hours after oral challenge. The number of *C. albicans* cultured from the caecum of these mice remained constant ($\sim 3 \times 10^7$ *C. albicans*/g) throughout the 55 day study period. Although viable *C. albicans* was recovered from systemic organs after the first 3 days of mono-association, the number recovered was low (<l0 organisms/g tissue). Histology of the gastrointestinal tract tissues revealed that *Candida* invaded (hyphae) keratinised tissues along the cardia-atrium section of *Candida* mono-associated athymic and euthymic mice. Following intravenous challenge with *C. albicans*, germfree athymic mice and their euthymic littermates readily cleared *Candida* from their kidneys, livers, and spleens. Although similarly challenged flora-defined athymic mice and their flora-defined, thymus-bearing littermates were able to clear *C. albicans* from their livers and spleens, the number of viable *C. albicans* recovered from the kidneys of these mice increased dramatically (>100-fold) within 5 days after challenge. The observed resistance of germfree nude and germfree euthymic mice to oral and systemic candidosis combined with the observed susceptibility of their flora-defined counterparts to systemic candidosis not only indicates that innate or natural immune mechanisms play a major role in resistance to oral or disseminated candidosis, but also demonstrates that the immune mechanisms of the host can be modulated by the acquisition or presence of a complex intestinal flora.

We have also pursued the effect of a dermatophyte, *Trichophyton mentagrophytes*, on systemic immunity in germfree animals (J. Invest. Dermatol. 75, 476-480, 1980). Primary and secondary *T. mentagrophytes* dermatophytosis was studies in germfree and conventionally reared Strain 2 guinea pigs. Although the onset and early development of the primary cutaneous lesions

appeared similar in germfree and conventional guinea pigs, the *T. mentagrophytes*-mono-associated guinea pigs exhibited more severe skin ulcerations and took twice as long to heal as their conventionally-reared counterparts. Cutaneous re-infection of *T. mentagrophytes*-mono-associated guinea pigs was also protracted; however, these lesions healed in about the same amount of time as a primary infection on conventionally-reared guinea pigs. Germfree guinea pigs, sensitised by cutaneous injection with *T. mentagrophytes* manifested 3 correlates of systemic cell-mediated immunity:

1. delayed-type hypersensitivity to intracutaneous injection of trichophytin antigen,
2. *in vitro* blastogenesis of spleen and lymph node cells to polyclonal mitogens and *Trichophyton* antigens, and
3. allergic contact dermatitis 48 h following cutaneous re-infection.

These experiments confirm that the normal microbial skinflora is not required for initiation, development, or clearance of *T. mentagrophytes* dermatophytosis. A primary infection is protracted and severe in gnotobiotic guinea pigs; however, following clearance of a primary infection, a second infection is abbreviated in duration indicating that the gnotobiotic guinea pig had developed acquired resistance to a dermatophytosis during the primary infection.

Some other work that our plastic surgeons have carried out in gnotobiotic animals relates to the effect of intestinal flora on wound healing. This study was based on measuring the time period for wounds to heal in the germfree animal compared to conventional mice or mice colonised with a pure culture of a skin bacterium. The study was carried out with germfree athymic and euthymic mice. The athymic and euthymic mice healed at a comparable rate in the germfree state (~12 days to heal the induced skin lesions). In the conventional state the euthymic mice take longer to heal than the athymic mice (15-16 days vs. about 12 days for athymic mice). Thus, a conventional skin flora and an intact immune system can delay wound healing. In another study we colonised germfree mice with *Staphylococcus aureus* at various times after wounding. *S. aureus* appeared to delay wound healing and the delay seemed to be more protracted in euthymic mice than athymic mice.

The final study I would like to mention today relates to some work we carried out on the susceptibility of germfree and conventional rodents to carcinogens (J. Natl. Cancer Inst. 58, 1103-1106, 1977). Germfree and conventional rats were assessed for their susceptibility to intra-rectally injected carcinogens. In comparison to conventional rats, the colons of germfree rats were more susceptible to the direct acting carcinogens. Germfree rats had earlier morbidity and developed colon tumours sooner (50% had colon tumours within 48-50 weeks) than conventional rats. Young (30 days old at the start of the experiment) germfree rats developed colon tumours more quickly (15-20 weeks) than older (60 days) germfree rats after intra-rectal injection of carcinogens. The microflora in some way, either through immunologic stimulation or interaction with the carcinogen in the bowel, enhanced the resistance of conventional rats to a direct acting carcinogen.

To summarise, this presentation has touched briefly on some of the studies we have carried out in our laboratory. We are investigating both mucosal and systemic immunity and how both may be altered by a hosts' contact with intestinal flora. We are dealing with a very complex system that involves complicated processes of antigen proc-

essing cells, T and B-cell activation, and a host of other factors that constitute many innate and acquired immune mechanisms. We are also apparently dealing with host-intestinal flora interactions that can either enhance or at times suppress immune responses. Certainly such modulations of the immune system by intestinal flora are worth further research efforts and perhaps such research will explain in further detail some of the differences that we observe in the susceptibility of germfree, mono-associated, and conventional animals to infections and neoplastic agents.

SECRETORY IMMUNITY

Authorised transcript of a lecture by

PER BRANDTZAEG

Laboratory for Immunohistochemistry and Immunopathology, Institute for Pathology, National Hospital, University of Oslo, Oslo, Norway

Presented at the 2nd Old Herborn University Seminar on
"Interactions between the indigenous microflora and the host immune system"
on June 1, 1988

Good morning!

I am very pleased to be back here in Herborn. I was here at the extraordinary 400 years' celebration 4 years ago and it was a great pleasure to be invited.

I shall talk about secretory immunity, which probably to a large extent is separate from systemic immunity. There is a good line of evidence that these two immune systems are at least partly independent. The first glimpse we had of an independent secretory immune system was apparently published in 1922 by a British military doctor called Davies who was stationed in Jerusalem. He found time to collect stool samples from the soldiers and investigated these for antibodies against dysentery bacteria. He found that such antibodies could appear in stools from infected patients several days before they appeared in the blood. He was very pleased because Dr. Besredka, who came from Russia to the Pasteur Institute a few years earlier, had a rabbit model where he had found similar "copro-antibodies" in stools before appearing in serum. He actually published his concept of local immunity in 1919. So Dr. Davies was very pleased that he could confirm this concept in man.

Then there came a long period in which very little happened in terms of local immunity, until the structure of antibodies became known in the late 50s and in the beginning of the 60s. I will particularly mention the findings of Dr. Tomasi in Buffalo, who characterised the secretory IgA antibody molecule and showed that it was quite different from monomeric IgA which is predominant in human serum. He found that secretory IgA was a dimer and that it was associated with an epithelial glycoprotein of about 80 kD, which is now called the "secretory component". It was originally called the "secretory piece", and it is actually co-valently linked to one of the subunits in the IgA dimer. So secretory IgA is an interesting hybrid molecule consisting of a plasma cell product and an epithelial cell product. It contains an additional polypeptide called the "joining" or J chain. This was discovered in 1970 by Dr. Mestecky in Birmingham, Alabama, and independently by Dr. Mariann Koshland in the USA. Dr. Mestecky showed that same peptide was found in IgM. As you will know, both these polymeric immunoglobulins can actually be transferred selectively into exocrine secretion. So both these molecules can appear as secretory immunoglobulins. We have quite good evidence that the J chain is the part of these polymeric molecules that determines their capability to be translocated into the secretions. The secretory component is indeed the

receptor for this external transport, and the secretory component can also become linked to IgM.

This model for the active transport of dimeric IgA, and the same refers to pentameric IgM, is now well accepted; I think this is an important part of the secretory immune system which is internationally agreed upon and which is a real biological mechanism.

The dimeric IgA in man is mainly produced by local plasma cells lying close to exocrine glands. The secretory epithelial cell, not the goblet cells and other mucus cells, but the serous-type of epithelial cell, produces the secretory component as a transmembrane protein in the rough endoplasmic reticulum. It goes through the Golgi complex for terminal glycosylation. It then moves to the basolateral membrane and sticks out as a receptor protein which shows high affinity for dimeric IgA and pentameric IgM; it is in the range of an antibody-antigen reaction and is, of course, primarily a non-covalent interaction. But as I mentioned, in secretory IgA there will be a covalent stabilisation through disulphide bonds taking place during the transport. This is an endocytic type of transport in vesicles. Also excess of receptor protein will be taken through the same vesicles to the lumen. The receptor is about 20 kD larger than the actual secretory component because there is an enzymatic cleavage of the receptor just before it is taken into the secretion. The cleavage site is unknown. We don't know anything about the fate of the cytoplasmic tail or the transmembrane part of the receptor protein. It is apparently degraded. The larger part of the receptor, however, is sacrificed as secretory component to stabilise secretory IgA. This molecule is actually very stable, indeed the most stable immunoglobulin of the body. Some of it will appear in faeces as active antibody, so it can pass through the gastrointestinal tract at least in small children and provide them with antibodies from breast milk to bacteria and viruses. Infants produce first secretory IgM but it is not so stable as there is no covalent stabilisation with the secretory component. So, IgM antibodies in the secretions are much more easily degraded by proteases.

This is a picture of paired immunofluorescence staining to show you what the secretory immune system looks like in the humane large bowel. These are the colonic crypts and we have stained the plasma cells here with a red fluorescence indicating IgA. Note that this immunoglobulin class is being produced in numerous plasma cells. The secretory component has been stained green. We see here the Golgi complexes, which are purely green. Yellow means that IgA has become complexed with the secretory component and is on its way out to the lumen; yellow is thus IgA plus secretory component or, in other words, secretory IgA migrating to the apical part of the cytoplasm outside the Golgi complex on its way to the lumen.

This preponderance the IgA-producing cells in all secretory tissues throughout the body from the lacrimal glands, nasal and bronchial mucosae, salivary glands, lactating mammary glands and throughout the gastrointestinal tract, is quite striking. We find that 80% to 90% of the plasma cells producing immunoglobulins are actually of the IgA class. This is in contrast to what we see in spleen and lymph nodes. So there must be some well-tuned regulation of the mucosal immune system to drive the local responses to predominantly IgA. But we see, of course, that there are also other plasma cell classes; IgM and IgG are represented to a small and variable extent. There is one peculiar feature and that is the IgD-producing cells, which are found mainly in the

upper part of a secretory immune system like the lacrimal glands and the nasal mucosa. We very rarely see IgD production in the gastrointestinal tract. This observation has some interesting implications for immune regulation, but I don't think time will allow me to discuss it here today.

When we look at the actual numbers of plasma cells producing immunoglobulins, there are large differences among the various secretory sites. This slide shows the number per mm^2 of tissue section determined by immunohistochemistry. We see, as could be expected, that in the gastrointestinal tract the number is much higher than in the parotid gland. I think that this is a direct reflection of the influence of the indigenous microbiota on the stimulation of the local immune system. We see here, perhaps to your surprise, that the lacrimal glands are also very rich in plasma cells; I think this is a reflection of the fact the conjunctiva is rather heavily exposed to protein antigens from house dust and other stimulating factors from the environment which will give rise to this high number of plasma cells in the lacrimal glands. Conversely, the lactating mammary glands and the salivary glands are rather far away from mucous membranes and are very little exposed to bacteria.

If you consider the total number of immunoglobulin-producing plasma cells in the gut it is really unbelievable. We have calculated that there must be about 10^{10} such immunocytes per metre of small bowel in man, which should be compared with the figure for bone marrow, spleen and lymph nodes altogether, that is 2.5×10^{10}. If you multiply the figure for the gut by let us say 5 or 6 metres, you get a much higher number. Actually at least 70 to 80% of all immunoglobulin-producing cells are located in the gut. We have been a little worried about this high figure for several years, but just before Christmas there was published a paper from the Netherlands showing that more than 80% of the immunoglobulin-producing cells are located in the gut also in the mouse. So our data are very much in agreement despite the fact that they were based on different techniques. The conclusion, therefore, is that in quantity of terms, the major antibody-producing cell system is actually found in the gut. This means that the gut contains our major humoral immune system.

This conclusion fits very nicely with the work of Dr. Dominique Delacroix at the University of Louvain in Belgium. He calculated the actual amounts of antibody secreted into human gut fluid as secretory IgA from local production. That figure was 40 mg per kg of body weight per day, and we know that the total production of IgG per kg is 30 mg. This means that there is more IgA transported to the gut fluid per day as dimeric IgA than the total production of IgG in the body. So IgA is actually our major antibody protein. In the bone marrow there is mainly a monomer production adding up to about 60 mg per day of total IgA. Very little dimeric IgA normally goes from the gut and bone marrow to the circulation - only about 4.5 mg per kg every day. This adds up to about 20 mg IgA to blood and then 40 mg IgA to gut fluid; that means that the IgA system amounts to twice as much immunoglobulin as the IgG system. This is probably a great surprise to some immunologists. In man the liver is mainly involved in catabolism of IgA and apparently very little IgA goes back to the gut through the bile. This is in contrast to the rat in which the liver is actually pumping dimeric IgA back from blood through bile to the gut fluid; there is relatively much more dimeric IgA reaching the blood from the gut in the rat than in man, and the rat liver cells express the secretory component as a

receptor. Human hepatocytes do not have the secretory component as a receptor for dimeric IgA, so there is a striking species difference in this respect.

According to the present concept the secretory immune system has its basis in stimulation of B-cells in Peyer's patches. This slide is from the distal ileum of a 10-year-old girl. There are numerous patches of lymphoid tissue protruding from the mucous membrane like small domes. Collectively, there are large areas of Peyer's patches and solitary lymphoid nodules in the distal ileum, and some of these will remain up to high age.

If we look at the histology, we can see that a Peyer's patch contains several lymphoid follicles with germinal centres because the local B-cell system is activated. According to the present dogma, these structures are the origin of most B-cells that become disseminated to the secretory immune system all over the body. I am not sure that this concept is completely true, but this is what is implied by the notion "a common mucosal immune system". A specialised follicle-associated epithelium is covering the lymphoid tissue; it contains very few goblet cells in contrast to numerous such cells in the epithelium covering the villi.

The follicle-associated epithelium is supposed to take up antigens actively and bring them in contact with the immune system. This process gives rise to stimulation of B-cells which migrate rapidly from the Peyer's patches through the mesenteric lymph nodes to ductus thoracius lymph and into blood. They are then taken into secretory tissues probably by specific receptor mechanisms recognising determinants on endothelial cells. In this way we have an integrated sort of immune system; you can for example have an antigenic stimulus down in the distal ileum and end up with specific antibody production against the same antigens in the lactating mammary glands. However, there are of course other sources of stimulated B-cells. We should not forget about the tonsils, and we have bronchus-associated lymphoid tissue which probably contributes some of the B-cell blasts that circulate briefly and become activated by second singles locally in various secretory tissues. But the main source of these blasts are, according to our present concept, the Peyer's patches.

The follicle-associated epithelium is specialised as I mentioned and it contains certain cells that are called the "membrane" or M cells. This slide shows an electron microscopic demonstration of these cells made by Dr. Owen in the USA. These bell-shaped cells allow the lymphoid cells to come very close to the gut lumen and they perform inward transport of antigenic material. In this case peroxidase has been taken up and passed through the vesiculo-tubular system of the M cells directly to the underlying lymphocytes.

It is not possible to see the M cells at the light microscopic level; but there is one trick you can do and that is to look for alkaline phosphatase. The brush border with alkaline phosphatase that we have on the villi is also present on the follicle-associated epithelium except where we have the M cells. Breaks or interruptions in the surface staining for alkaline phosphatase will thus indicate the M cells according to what Dr. Owen has shown by electron microscopy. We find on an average about 5 such M cells per done of human Peyer's patches, ranging from 3 to 15.

We wanted to look at the immunological activity adjacent to such breaks in the brush border representing M cells. Firstly, we studied the number of T-cells with CD3 determinants and found clusters of such cells near the

breaks. In contrast, the intra-epithelial T-cells are found scattered along the basement membrane in the villi. T-cells, therefore, apparently accumulate adjacent the M cells in and beneath the follicle-associated epithelium.

We would like to think that the M cells might have an antigen-presenting function. In that case these cells would necessarily have to express MHC class II determinants such as HLA-DR. These self-determinants bind foreign antigens and the complex is seen by the T4 ($CD4^+$) or "helper" cells. This is what we call genetically restricted antigen presentation, which represents a very important genetic aspect to the immune response. Cells like macrophages and dendritic cells, and also certain epithelial cells that express MHC class II determinants, can actually present antigens to the T4 cells. The crucial role of the T4 cell is unquestionable. We know this from AIDS patients in whom these cells are destroyed. The whole immune-system finally breaks down. So this is a key cell in the immune system as a helper cell giving regulatory signals to T8 ($CD8^+$) suppressor/cytotoxic cells and also to the B-cells. We will hear more about these interactions by Dr. MacDonald and Dr. Kiyono later on today. Such interactions are an important initiating stage to get the B-cell system going.

The major MHC class II determinant in man is HLA-DR, which shows considerable polymorphism. We find it expressed on the gut epithelium in the small intestine including the follicle-associated epithelium. In this slide HLA-DR determinants are shown in green immunofluorescence. At the same time alkaline phosphatase is stained red and we see that the breaks indicating the M cells show no green fluoresecence. The M cells, therefore, are apparently negative for class II determinants and hence cannot be antigen-presenting cells. They probably mainly perform antigen transport. But there are, of course, numerous other cells in the Peyer's patch dome area that express class II determinants such as dendritic cells, B-cells in the follicle, and also the rest of the follicle-associated epithelium which is HLA-DR positive.

If the follicle-associated epithelium and the dendritic cells in the dome area are antigen-presenting cells in a MHC class II-restricted manner, which cells do they actually trigger in the Peyer's patches? Dr. Strober in the USA has proposed that there are particular regulatory T-cells which he has called "switch" cells; they can be directly stimulated by class II determinants as indicated in this slide and they will drive B-cells expressing surface IgM directly to an IgA-expressing stage. Dr. Kiyono will probably discuss with you that there are other subsets of T-cells such as Tα cells that can drive IgA-expressing B-cells to terminal differentiation, thus giving rise to IgA production. So there may indeed be particular immunoregulatory T-cells in the Peyer's patches. There is currently a lot of discussion about this topic. Some scientists think that there is a sort of sequential differentiation along the chromosome in the order of the Ig-heavy chain genes, and that environmental factors may drive the B-cells to terminal IgA differentiation without the help of any particular T-cells. So this is an interesting area but we have actually very little specific information. There are probably various possibilities to end up with IgA as the predominant class of immunoglobulin-producing cells in secretory tissues.

If we look at the distribution of T-cells in the gut epithelium, we see as mentioned before that their number is much higher in the Peyer's patch epithelium than in the villous epithelium. I also mentioned before that there is a concentration of T-cells adjacent to the

M-cells. There is also another interesting difference between villous and Peyer's patch epithelium, namely that the CD4-to-CD8 ratio, or "helper"-to-"suppressor" phenotype ratio, is much higher in the latter than in the former epithelium. In the villous epithelium there is this remarkable dominance of the T8 or suppressor phenotype. We don't quite know the importance of this observation, but it is quite striking and very intriguing. There is really a fantastic selection of T8 cells as you can see from this slide where the villous epithelium is indicated in red staining for keratin. Here you can see the CT4 positive cells of the helper phenotype; they are located in the connective tissue and very few are going into the epithelium. On the right we have the T8 suppressor phenotype; there are very few positive cells in the lamina propria and a tremendous selection into the epithelium. I think it means something in biological terms that we have this selective migration of T8 cells into the villous epithelium.

At the same time we also have expression of MHC class II, in this case HLA-DR, on the villous epithelium. The plasma cells producing IgA are red in this slide; they are found mainly in the crypt region and you can see IgA transport in the crypt epithelium. But the villous epithelium, where we have the T8 cells, is class II positive and IgA negative. There is thus a very interesting spatial relationship between class II expression and T8 lymphocytes in the villous epithelium. The idea which has been discussed in several papers is that the intra-epithelial T8 cells perhaps are stimulated by presentation of luminal protein antigens in relation to epithelial class II molecules, giving rise to suppression of delayed type hypersensitivity and IgG and IgE immune responses. This phenomenon is called "oral tolerance" but we don't know much about it in man. Our information comes from experimental animals. But the theory is that soluble antigens induce suppression of potentially dangerous immune responses, which may give rise to immunopathology in mucous membranes. Conversely, the IgA responses are in some way released from downregulation, perhaps by contra-suppressor cells. In this way we may have a continuing IgA response in the face of a suppression of IgG and IgE responses and delayed type of hypersensitivity. However, there is not much evidence that bacteria can induce oral tolerance, except that in man IgM responses are apparently being suppressed during long-lasting infection with endotoxin-producing bacteria. But we don't have any good evidence for immunological suppression to the endogenous microflora. So perhaps "oral tolerance" is mainly relevant to food antigens.

We can probably discuss for days theories of immune regulation in the gut. But the fact remains that it works in practice. When an infant gets breast-feeding, it will be protected to a large extent against bacteria in the environment. Immunity is induced in the Peyer's patches of the mother and stimulated B-cells will end up in the lactating mammary glands and produce specific IgA antibodies. These are to a large extent directed against the gut flora and afford protection of the infant before its own IgA system has developed. This takes at least 1 month, perhaps 2-3 months, depending on the individual and the environment. But the situation in the developing countries is that breast-feeding is decreasing and bottle feeding increasing, which is a very sad development. Because bottle milk often is prepared under the most primitive conditions with filthy water, the babies contract severe diarrhoeal diseases from bacterial and viral gastro-enteritis. So I will end up with the next slide that gives

us a glimpse of the severe reality existing in the developing countries where 500 infants die from intestinal infections every hour; this means 4-5 millions per year. And in the face of the fact that in some of these countries less than 10 dollars are used for health services per inhabitant per year, this gives us some perspective of the real world. Employment of the intestinal immune system via breast feeding on the basis of better education combined with better health services are needed to reverse this unfortunate situation.

THE EFFECT OF THE INTESTINAL MICROFLORA ON SYSTEMIC DELAYED HYPERSENSITIVITY

Authorised transcript of a lecture by

PHILIP B. CARTER, also on behalf of STEPHEN G. SIMKINS

North Carolina State University, Raleigh, North Carolina, USA

Presented at the 2nd Old Herborn University Seminar on
"Interactions between the indigenous microflora and the host immune system"
on June 1, 1988

I am pleased to speak to you today about delayed hypersensitivity (DH). Having spent ten years at the Trudeau Institute, prior to joining North Carolina State University, delayed hypersensitivity has been closely tied with cellular immunity to bacterial infections throughout my work and that of my colleagues (*Carter*, 1975; *Carter* and *Collins*, 1974a, 1974b; *Carter* et al., 1980; *Carter* and *Pollard*, 1971; *Collins* and *Carter*, 1980; *Lagrange* et al., 1975; *Lagrange* and *Mackaness*, 1975; *Lane* and *Unanue*, 1972; *Lev* and *Battisto*, 1970; *MacDonald* and *Carter*, 1978, 1979, 1980, 1981; *Mackaness*, 1969). Delayed hypersensitivity is classically determined, through the work of many people during the heyday of tuberculosis (TB), primarily, the 1930s and 1940s, as the sensitivity exhibited by TB infected animals and people to purified proteins derived from the TB bacillus (PPD). TB, as a plague in the western world, caused many investigators to study the immune response to the tuberculosis organism in great detail. Through such studies it was determined that there is a sensitivity to bacterial antigens, specifically, in this example, to TB proteins, that is not immediate and evanescent as is observed in some bacterial infections but appears later in time after challenge, and remains for a longer period. The main observation in delayed hypersensitivity (DH) is swelling or induration of tissue soon after injection of the antigen, followed by subsequent decrease in that swelling, and an increase again by 24 hours. In fact, at 48 hours the injected tissue will still be indurated, whereas, with other antigens which induce a strong antibody response, the short swelling or induration following injection of the foreign antigens will disappear and remain low. Most of the swelling is due to interstitial fluids but in DH there is a characteristic influx of mononuclear cells, T-cells and macrophages, but also some neutrophils. In other types of hypersensitivity responses, basophils can be important infiltrators and you have an induration and inflammation that is mediated primarily by inflammatory products from granulocytes with a less cellular infiltrate.

Purists would say that delayed hypersensitivity is only defined in the guinea pig and particularly the guinea pig infected with mycobacteria and then elicited with PPD. Mackaness and others at the Trudeau Institute (*Collins* and *Carter*, 1980; *Mackaness*, 1962, 1964, 1969; *North*, 1973), and at other institutions throughout the world, have shown that in fact there can be a delayed hypersensitivity response to a number of purified bacterial proteins in a number of infections, primarily those caused

by facultative intracellular pathogens. A number of workers have, therefore, gone beyond using the guinea pig TB model to using other animal species. Both the footpad and the ear are very good testing sites in the mouse. The skin of the mouse and even that of the rat is not as good, for these sorts of studies, as skin of the guinea pig, or even the human, and the footpad or the ear are the preferred sites. We have used the mouse model to study the immune response to *Listeria monocytogenes*, as well as the immune response to a number of other antigens, both infectious organisms and non-infectious agents, and inanimate agents such as sheep red blood cells (SRBC). The work which I will present today was done at the Trudeau Institute and at North Carolina State University in association with Tom MacDonald, Steve Simkins, Frank Collins and Roger Brideau.

The relationship between delayed hypersensitivity and antibody production has long been suggested to be inversely associated. That is, if you have a situation in which there is a significant serologic response, then the delayed hypersensitivity response is observed to be diminished. That is, when antibody titre rises, then the DH which is elicited is diminished. It has been observed in a number of cases, in fact, that as soon as antibody is produced, we see a diminishment of DH, but in certain circumstances, when there is a very poor antibody response, those are the situations in which DH is most easily demonstrated. In fact, this posed a problem for Philippe Lagrange, now in Paris, and George Mackaness when they were together working on the definition of DH to such inanimate antigens as SRBC (*Lagrange* et al., 1975; *Lagrange* and *Mackaness*, 1975). They showed, in support of the earlier theory of Sam Salvin at the University of Pittsburgh, that DH probably is the initial response to any antigenic stimulus and only in certain circumstances is the response extended to include antibody. You can have a very short DH response because antibody appears early and therefore DH is less easily detected. In fact, if you block the antibody response with cytoxan (*Lagrange*, *Mackaness* and *Miller*, 1974), you get an even greater demonstration of DH to mycobacterial or other microbial antigens or even the sheep red cell, which again supports the view that delayed hypersensitivity and antibody responses are inversely related.

With Tom MacDonald, we did a study of the influence of the intestinal microflora on the development of delayed hypersensitivity. There had been some concern through the 1960s that, in fact, the gnotobiotic or germfree animal was unable to exhibit delayed hypersensitivity. This was primarily due to the work of Mier Lev and Jack Battisto in New York, in which they infected germfree guinea pigs with mycobacteria and then tried to elicit, in a standard, accepted test with PPD, a delayed hypersensitivity response and were unable to do so (*Lev* and *Battisto*, 1970). Other workers in Japan, Ueda, his co-workers (*Ueda* et al., 1973; 1975), and others (*Julianelle*, 1942) using mice showed that, with renewed immunisation, it was possible to demonstrate only very low levels of DH to PPD, following exposure to mycobacterial antigens, or reactivity in other tests for cell-mediated immune responsiveness (*Seicastava* et al., 1976; *Wostrnann* et al., 1970). Since it had been well demonstrated by many groups that the antibody response of gnotobiotic animals to antigenic stimulation far exceeds that of conventional animals (*Fidler*, 1975; *Hooijkaas* et al., 1984; *Kiuchi* et al., 1972; *Nielsen* and *Friis*, 1980; *Pollard* and *Nordin*, 1971; *Wostmann* et al.,

1970; *Yasutake*, 1977), we considered that the exceptionally profound serologic response of the gnotobiote might be causing the blockage of DH responses, which Merrill Chase showed decades ago to be mediated by cells rather than serum components (especially antibody).

When Tom MacDonald came to the Trudeau Institute in 1976, we were concerned about this apparent inability of germfree animals to mount a normal T-cell response. One of the first studies we performed was with skin sensitising agents (*MacDonald* and *Carter*, 1978). Although the skin sensitising agents picryl chloride and oxasolone can be argued as not being good models for DH responses of the type that mycobacteria elicit, since the response is basophil mediated, it is mediated by cells. Nonetheless, we were able to show, using either incorporation of tritiated thymidine as an indication of DNA synthesis in the draining lymph nodes or the swelling of the ear following elicitation of sensitised germfree mice, that we could sensitise a germfree animal and, just as in sensitised conventional mice, obtain very measurable and profound delayed responses. This study was followed by the SRBC work which we published in the Journal of Immunology (*MacDonald* and *Carter*, 1979), building upon the work of Lagrange and Mackaness (*Lagrange* et al., 1975; *Lagrange* and *Mackaness*, 1975). Basically, it is observed that the germfree (GF) animal does indeed exhibit a lower response, a much lower response, in the development of delayed hypersensitivity to SRBC than the conventional (CV) mouse. Intravenous sensitisation, which in the CV mouse would give good DH, when elicited in the footpad, did not produce any reaction in the GF mouse, suggesting a relationship to the microbial flora in the intestinal tract.

Inducement via the footpad or other parenteral route of sensitisation did result in some small response following subsequent elicitation within the GF animal, but it was very small compared to what is observed in the conventional. Cell transfers were performed to determine whether this resulted from a T-cell anomaly in the GF animal or whether it was due to some accessory cell defect. In fact, it was discovered that spleen cells from CV mice given to GF mice did not result in a very profound response, suggesting that the defect in the GF mouse is with the accessory cell, the macrophages (*MacDonald* and *Carter*, 1979). This will be discussed in greater detail below in regards to the *Listeria* model. Such studies done in our laboratory and those of others, have shown that GF mice have a lower level of macrophage activation than CV animals, but still demonstrate normal DH responsiveness to contact sensitising agents, while showing no DH to SRBC after i.v. sensitisation, and only limited DH following footpad sensitisation.

Perhaps not surprisingly, we found that conventionalisation or mono-association restored the capacity of the GF mouse to produce a normal DH response to SRBC antigen (*MacDonald* and *Carter*, 1979). In mono-association, the microorganisms with which we had the best results were Gram-negative organisms, a finding which relates then to information discussed below. There was question as to whether there was a B-cell induction, rather than a T-cell induction, of anti-SRBC sensitivity in the GF spleen because of some difference in the processing of antigen in the GF animal. We considered that this might be related to the profound difference in the stimulation of the gut-associated lymphoid tissues. We observed that large germinal centres in the Peyer's patch of a CV animal stained with a monoclonal antibody (Mab) to L3T4, a Mab specific for the helper T-

cell in the mouse. Cells in the T-dependent area in the Peyer's Patches are labelled. The Peyer's patches in the GF animal, because of the lack of the antigenic stimulation by the large numbers of microorganisms ordinarily present in the lumen of the intestine, rarely show germinal centres, characteristic of B-cell and antibody responses, while the T-cell areas are well populated. Upon association with Gram-negative microorganisms, we see an anti-SRBC response similar to what is observed in CV animals and histologic sections of the Peyer's patches would show larger germinal centres. The association of Gram-negative microorganisms in the mammalian intestinal tract with the host's subsequent development of DH and their possible involvement in host immunity to infectious agents was of great interest to us and continues to be.

Related to the above observations are our studies with oral association of mice with *Listeria rnonocytogenes*, a facultative intracellular microorganism which induces a delayed hypersensitivity of some significance (*Czupryuski* and *Balish*, 1981; *Fleming* et al., 1985; *Gray* and *Killinger*, 1966; *Julianelle*, 1942; *Murray*, *Webb* and *Swann*, 1926). In the oral *Listeria* model, we see significant footpad swelling, characteristic of DH to *Listeria* antigens, at day 6; this occurs slightly later, by 2-3 days, than when it would be observed following i.v. infection and probably relates to the fact that DH does not appear until the oral infection results in a systemic infection, involving the spleen. Such a pathogenesis probably accounts for the delay in exhibition of DH and has additional significance. Interestingly, the clearance of *Listeria* from the blood and uptake by the liver and the spleen in the GF and CV animal is exactly the same. In fact, the LD_{50} varies little between the GF or CV mouse. The growth curves of *Listeria monocytogenes* following an i.v. infection in the CV and GF mouse differ slightly but follow the same pattern. The colonisation of the intestinal tract following i.v. challenge as well as oral infection was observed in the GF mouse. The population of *Listeria* achieves high levels and is secondary to the infection of the liver following IV challenge. These organisms, since there is no competition for colonisation sites in the GF animal by *Listeria*, are able to maintain very high levels in the gnotobiotic animal, in this case, mono-associated by *Listeria*, and there is no fluctuation in their populations with time. In the CV animal, *Listeria*, following oral challenge with $5x10^6$, disappears very quickly from the intestinal lumen and is not be detectable after a few days. One of the things which we found to be unusual in our study, and which relates to what was said earlier using inanimate antigens such as SRBC, is a failure of lymphocytes from immunised gnotobiotic mice to transfer resistance to that same microorganism to CV mice. In fact, although the gnotobiotic mouse infected with *Listeria* develops a normal immune response, as far as can be determined, and is resistant to challenge, we were very surprised to find that it was impossible to protect CV recipients with spleen cells from these animals. Although gnotobiotic donors were fully immune to challenge, they were unable to transfer any immunity to the CV animal. In studying this result, we formed the hypothesis that effector cells [sensitised T-cells (*Lane* and *Unanue*, 1972)] leave the spleen and localise in the gut of *Listeria monocytogenes* mono-associated mice, drawn there by the high numbers of *Listeria* in the intestinal lumen of the mono-associated mouse. We would have the reverse effect of what is seen in tuberculosis patients or TB-infected animals late in disease. Early in tuberculosis, it is easy to demonstrate

delayed hypersensitivity in the periphery, i.e., the skin. However, late in the infection, this hypersensitivity is often undetectable and it is thought that the sensitised lymphocytes, which are needed to elicit that peripheral reaction, are all localised in the lung where the severe pathology is occurring thus resulting the peripheral anergy. In the *Listeria* model, our hypothesis is that the reverse is occurring, that is to say, the *Listeria* is able to maintain such high populations levels in the gnotobiotic intestine as opposed to the CV animal, and the antigen load is so great in the gnotobiote, that the sensitised T-cells are localising in the intestine, the site of maximal antigenic stimulation. And thus, we were unable to have significant numbers of sensitised T-cells left in the spleen to transfer this immunity. Such is the basis for our hypothesis. We have considered two ways in which this can be tested: One way, of course, is to remove the T-cells from the intestinal mucosa of these animals and transfer them to recipients but, as *Hiroshi Kyono* (1980) knows, isolating these T-cells in large numbers from the intestinal tract is a significant problem. What we chose to do is the reverse, that is to remove the antigenic load from the intestine and have a reversion, or remigration, of the sensitised T-cells back to the spleen. The latter is the easier one to test and that is what we did. Following the procedures of *Srivastava* et al. (1976), we used a number of non-absorbable antibiotics to reduce the antigen load in the intestinal tracts of *Listeria*-mono-associated mice. In the normal *Listeria* infection, following oral challenge, the organisms reach high numbers in the caecum and infection of the spleen occurs but in animals given the oral antibiotics, colonisation of the gut is completely inhibited, at least based upon what is could be detected on culture media. The oral antibiotics, being non-absorbable, did not affect the pathogenesis of the systemic infection, showing an infection that was turned over at day 3, indicating a very classical response immune response. Furthermore, when spleen cells were taken from these *Listeria* infected animals, in attempting to transfer passive protection, the same insignificant transfer of protection is observed as compared to CV non-immunised mice. When antibiotics were given to reduce the antigen load in the gut, it was possible then to achieve levels of protection that were almost equivalent to those achieved with spleen cells from the CV donor. This then would support the hypothesis of a redirection of the migration of sensitised T-cells. We were still not quite happy with the level of protection exhibited and thought that perhaps there was more to this problem which related to what had been seen with the deficient anti-SRBC delayed hypersensitivity response. This was continued in a thesis study by *Simkins* (1987). Simkins used a battery of monoclonal antibodies in conjunction with flow cytometry (*Herzenberg* and *Herzenberg*, 1978; *Steinkamp*, 1984) to determine the proportion of different T-cell and macrophage populations in CV mouse spleens and Peyer's patches and the same in the GF animals. A significant change in the proportion of macrophages in the spleens of *Listeria*-infected animals as compared to the normal CV animal, and certainly as compared to the macrophage population in uninfected GF mice, was observed. The other numbers are essentially similar. Following infection, macrophages increased from 5% to 13% of total mononuclear cells. Since that cell population changed the most of any that were studied, Simkins suggested (*Simkins*, 1987) that perhaps macrophages were involved in the inability to transfer resistance to *Listeria* with spleen cells from mono-associated

mice. Following antibiotic treatment the macrophage population is observed returning to what it was in the normal GF mouse, between 5% and 6%, as opposed to the 13% seen in *Listeria*-infected gnotobiotic mice. The numbers are taken from flow cytometric profiles; the anti-macrophage Mab, M1/70, is used to label spleen cell populations in CV and gnotobiotic *Listeria*-infected animals respectively. In the gnotobiotic animal there is an even more significant increase in the $M1/70^+$ population. With these observations suggesting the existence of suppressor macrophages, we removed the plastic-adherent cells from donor animals, resulting in profound reduction of $M1/70^+$ population. Except in the case of two of the recipient animals, removal of these cells resulted in a very impressive transfer of protection, well exceeding two logs.

One of the things that strikes us, tying this all together, is that in the *Listeria*-mono-associated animal there is no evidence of germinal centres in the Peyer's patches; this probably relates to the much lower antibody response to *Listeria* infections generally, which is why it is possible to show a good DH response. It is interesting that, unlike mono-association with the Gram-negative microorganisms, we do not see germinal centre formation. What remains to be shown is the exact relationship of the Gram-negative intestinal flora to the general expansion of B-cell populations and the production of antibodies, not only in the local, mucosal lymphoid tissues, but also in the systemic lymphoid tissues. Whether the effect of these microorganisms, and the endotoxin produced by them, is related, in the broader sense, to the development of the capacity for the exhibition of DH remains moot. Apparently, such responsiveness is not induced by association with Gram-positive microorganisms, even though these microorganisms produce a significant amount of peptidoglycan, which has an immune effect similar to endotoxin, as will be discussed by Dr. John Schwab.

ACKNOWLEDGEMENT

The authors acknowledge the collaboration of T.T. MacDonald, R. Brideau and K. Kaneko in various aspects of the work reviewed in this presentation. Some of the work described in this paper, attributable to the authors, was supported in part by the UNC-NCSU Center for Gastrointestinal Biology and Disease, Grant NIH DK34987.

LITERATURE

Carter, P.B.: Pathogenicity of Yersinia enterocolitica for mice. Infect. Immun. 11, 164 (1975).

Carter, P.B. and Collins, F.M.: The route of enteric infection in normal mice. J. Exp. Med. 139, 1189 (1974).

Carter, P.B. and Collins, F.M.: Experimental *Yersinia enterocolitica* infection in mice: Kinetics of growth. Infect. Immun. 9, 851 (1974).

Carter, P.B., MacDonald, T.T., Spitalny, G.L., and Simkins, S.G.: Transfer of resistance to listeriosis in gnotobiotic and conventional mice. Abstract presented at the 18th Annual Meeting of the Association of Gnotobiotics on July 13, 1980, at the University of Texas System Cancer Center, Houston, Texas (1980).

Carter, P.B. and Pollard, M.: Host responses to normal microbial flora in germ-free mice. J.

Reticuloendothel. Soc. 9, 165 (1971).
Collins, F.M. and Carter, P.B.: Development of a delayed hypersensitivity in gnotobiotic mice. Int. Archs. Allergy Appl. Immun. 61, 165 (1980).
Czuprynski, C.J. and Balish, E.: Pathogenesis of *Listeria monocytogenes* for gnotobiotic rats. Infect. Immun. 32, 323 (1981).
Fidler, J.M.: *In vivo* immune response to TNP hapten coupled to thymus-independent carrier lipopoly-saccharide. Cell Immunol. 16, 223 (1975).
Fleming, D.W., Cochi, S.L., MacDonald, K.L., Brondum, J., Hayes, P.S., Plikaytis, B.D., Holmes, M.B., Audurier, A., Broome, C.V., and Reingold, A.L.: Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. 312, 404 (1985).
Gray, M.L. and Killinger, A.H.: *Listeria monocytogenes* and listeric infections. Immunol. Rev. 30, 309 (1966).
Herzenberg, L.A. and Herzenberg, L.A.: Analysis and separation using the fluorescence activated cell sorter (FACS). In: Handbook of experimental immunology (Ed.: Weir, D.M.). Blackwell Scientific Publications, London, 22 (1978).
Hooijkaas, H., Benner, R., Pleasants, J.R., and Wostman, B.S.: Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet. Eur. J. Immunol. 14, 1127 (1984).
Julianelle, L.A.: Biological and immunological studies of Listerella. J. Bact. 32, 367 (1942).
Kiuchi, Y., Maejima, K., Fujiwara, K., and Tajima, Y.: Serum IgG in germfree, gnotobiotic and conventional mice of different ages. Japan J. Exp. Med. 42, 53 (1972).
Kiyono, H., McGhee, J.R., and Michelek, S.M.: Lipopolysaccharide regulation of the immune response: Comparison of responses to LPS in germfree, *E. coli*-monoassociated and conventional mice. J. Immunol. 124, 36 (1980).
Lagrange, P.H., Mackaness, G.B., and Miller, T.E.: Potentiation of T cell-mediated immunity by selective suppression of antibody formation with cyclophosphamide. J. Exp. Med. 139, 1529 (1974).
Lagrange, P.H., Mackaness, G.B., Miller, T.E., and Pardon, P.: Effects of bacterial lipopolysaccharide on the induction and expression of cell-mediated immunity. I. Depression of the afferent arc. J. Immunol. 114, 442 (1975).
Lagrange, P.H. and Mackaness, G.B.: Effects of bacterial lipopolysaccharide on the induction and expression of cell-mediated immunity. II. Stimulation of the efferent arc. J. Immunol. 114, 447 (1975).
Lane, F.C. and Unanue, E.R.: Requirement of thymus (T) lymphocytes for resistance to listeriosis. J. Exp. Med. 135, 1104 (1972).
Lev, M. and Battisto, J.R.: Impaired hypersensitivity in germ-free guinea pigs. Immunology 19, 47 (1970).
MacDonald, T.T. and Carter, P.B.: Contact sensitivity in the germ-free mouse. J. Reticuloendothel. Soc. 24, 287 (1978).
MacDonald, T.T. and Carter, P.B.: Requirement for a bacterial flora before mice generate cells capable of mediating the delayed hypersensitivity reaction to sheep red blood cells. J. Immunol. 122, 2624 (1979).
MacDonald, T.T. and Carter, P.B.: Cell-mediated immunity to intestinal infection. Infect. Immun. 28, 516 (1980).
MacDonald, T.T. and Carter, P.B.: Immunity to infections across mucosal surfaces. In: Recent advances in germfree research (Eds.: Sasaki, S., Ozawa, A., and hashimoto, K.). Tokai University Press, Tokyo, 503 (1981).
MacDonald, T.T., Carter, P.B., and Phillips, P.G.: Modulation of cellular immunity by the enteric flora. Delayed type hypersensitivity in the gnotobiotic mouse. In: Clinical and Experimental Gnotobiotics (Eds.: Fliedner, T.M., Heit, H., and Niethammer, D.). Suppl. no. 7 to Zentrablatt fur Bakteriologie. Gustav Fischer Verlag, New York, 221 (1979).
Mackaness, G.B.: The influence of immunologically committed lymphoid cells on macrophage activity *in vivo*. J. Exp. Med. 129, 973 (1969).
Mackaness, M.B.: Cellular resistance to infection. J. Exp. Med. 116, 381 (1962).
Mackaness, M.B.: The immunological basis of acquired cellular resistance. J. Exp. Med. 120, 105 (1964).
Mattingly, J.A., Eardley, D.D., Kemp, J.D., and Gershon, R.K.: Induction of suppressor cells in rat spleen: Influence of microbial stimulation. J. Immunol. 122, 787 (1979).

Murray, E.G.D., Webb, R.A., and Swann, M.B.R.: A disease of rabbits characterised by large mononuclear leukocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes*. J. Pathol. Bacteriol. 29, 407 (1926).

Nielsen, E. and Friis, C.W.: Influence of an intestinal microflora on the development of the immunoglobulins EgGl, IgG2a, IgM and IgA in germ-free BALB/c mice. Acta Path. Microbiol. Scand. 88, 121 (1980).

North, R.J.: Importance of thymus-derived lymphocytes in cell-mediated immunity to infection. Cell. Immunol. 7, 166 (1973).

Pollard, M. and Nordin, A.: Immune capabilities of germ-free animals. In: Progress in Immunology. Workshop 31 (Ed.: Amos, B.). Academic Press, New York, 1295 (1971).

Simkins, S.G.: Suppression of host antimicrobial immune responses by plastic-adherent spleen cells. Master's Thesis. North Carolina State University, Raleigh, North Carolina (1987).

Smith, C.S., Pilgrim, H.I., and Steinmuller, D.: The survival of skin allografts and xenografts in germ-free mice. Transplantation 13, 38 (1972).

Srivastava, K.K., Pollard, M., and Wagner, M.: Bacterial decontamination and antileukemic therapy of AKR mice. Infect. Immun. 14, 1179 (1976).

Steinkamp, J.A.: Flow cytometry. Rev. Sci. Instrum. 55, 1375 (1984).

Szeri, I., Anderlik, P., Banos, Z., and Radnai, B.: Decreased cellular immune response of germ-free mice. Acta Microbiol. Acad. Sci. hung. 23, 231 (1976).

Ueda, K., Yamazaki, S., and Someya, S.: Studies on tubercle bacillus infection in germ-free mice. J. Reticuloendothel. Soc. 12, 545 (1973).

Ueda, K., Yamazaki, S., and Someya, S.: Hyporeactivity to tuberculin in germ-free mice. J. Reticuloendothel. Soc. 18, 107 (1975).

Widmer, M.B., Sondel, P.M., and Bach, F.H.: Responsiveness of germ-free mice in mixed leukocyte culture. J. Exp. Med. 136, 398 (1972).

Wostmann, B.S., Pleasants, J.R., Bealmear, P., and Kincade, P.W.: Serum proteins and lymphoid tissues in germ-free mice fed a chemically defined, water soluble, low molecular weight diet. Immunology 19, 443 (1970).

Yasutake, I.N., Yasui, H., and Ogura, R.: A comparative study on the humoral immune responses in germ-free and conventional mice. Immunology 32, 43 (1977).

NATURAL KILLER CELLS

KENNETH F. BARTIZAL

Infectious Disease Research, Merck, Sharp and Dome Research Laboratories, Rahway, New Jersey, USA

Lecture presented at the 2nd Old Herborn University Seminar on "Interactions between the indigenous microflora and the host immune system" on June 1, 1988

SUMMARY

Various factors were reported to influence natural cytotoxicity of natural killer (NK) cells in conventional reared animals. This report summarises the role of specific microbial elements that colonise the alimentary tract of gnotobiotes as a factor in modulation of NK cell activity. Secondly, the effects of diet, age and microbial elements on the NK cell activity of gnotobiotes were examined. Lastly, this study investigated whether microbial enhanced NK cell activity correlated with increased resistance due to systemic infections with the opportunistic fungi and if the systemic fungal infections caused modulation in NK cell activity during infection. Spleen cells from flora defined (FD) and conventionalised (CONV) athymic and euthymic mice show significantly more NK cytotoxicity than germfree (GF), mono-associated (*Candida albicans*, *Streptococcus pneumoniae*, *Streptococcus lactis*, *Morganella morganii*, *Pasteurella pneumotropica*), or di-associated (*Candida albicans* and *Bacillus* sp.) mice. Athymic FD and CONV mice exhibited significantly greater NK cell activity than their euthymic counterparts. Spleen cells from GF mice raised on a chemically defined (low antigen) diet were more cytotoxic towards tumour targets than spleen cells from GF or CV (barrier maintained) mice raised on a natural ingredient diet. No significant differences in NK cell activity were evident in GF or CV mice of different ages. An increased recycling rate of NK cells was observed due to the presence of a microflora. GF and FD mice exhibited significantly elevated NK cell activity after oral association or i.v. challenge with *Cryptococcus neoformans* but not with *C. albicans*. Enhanced NK cell activity did not correlate positively with increased resistance to *Cryptococcus neoformans* infection. Environmental and dietary changes undoubtedly alter the intestinal microflora which can have dramatic affects on the cytotoxic activity of their NK cells.

INTRODUCTION

Lymphocytes that originate from the lymphoid organs or peripheral blood that exhibit "natural" cell-mediated cytotoxicity against tumour cells and a variety of other non-tumour targets are referred to as natural killer (NK) cells.

Natural killer cells are characterised as non-T, non-B large granular lymphocytes that are cytotoxic for a wide variety of target cells including syn-, allo- and xeno-geneic tumour cells, non-malignant cells, virus infected cells and non-tissue targets. Further, NK cells apparently kill without prior exposure, stimuli or manipulation, independent of MHC recognition or immunoglobulin involvement. Because of the implied definition of "natural" killing and non-specific nature of NK cell activation we became interested in whether components of the intestinal microflora directly or indirectly influence the activity and functions of NK cell cytotoxicity.

Various factors were reported to influence natural cytotoxicity of NK cells in conventional reared animals. Factors found to augment NK activity were spontaneous or chemically induced tumours, viruses, bacteria, bacterial components, bacterial adjuvants, interferon and interferon inducers, prostaglandin inhibitors and T-cell growth factors. Factors that suppressed NK activity were certain viruses, carrageenan, cyclosporin A, hydrocortisone, cyclophosphamide, adriamycin, prostaglandins and surgical stress (*Herberman* et al., 1977,1979). Most of these findings were determined in conventional reared animals harbouring an undefined, complex microflora.

This report summarises the role of specific microbial elements that colonise the alimentary tract of gnotobiotes (otherwise devoid of variable, exogenous environmental stimuli), as a factor in modulation of NK cell activity. Secondly, the effects of diet (chemically defined or natural ingredient), age and microbial elements on the NK cell activity of gnotobiotes were examined. Lastly, experimental findings of others have indicated that NK cells may function in natural resistance against certain parasitic (*Kamiyama* et al., 1982), bacterial (*Kasahara* et al., 1981), and viral agents (*Kohl* et al., 1980), as well as NK tumouricidal functions against malignant cells (*Nunn* et al., 1976). Specifically, microbial modulated NK cell activity could play an important role *in vivo*, as an effector mechanism operational against opportunistic infections caused by eukaryotic microorganisms such as the pathogenic fungi *Cryptococcus neoformans* (*Murphy* et al., 1982) and *Candida albicans* (*Marconi* et al., 1985). Natural killer cell activity has correlated positively with the ability of murine NK effector cells to inhibit the growth of fungi *in vitro*. Additional *in vivo* studies by investigators have shown that in conventional (CV) athymic mice (with a complex microbial flora and elevated NK cell activity), the growth of *Cryptococcus neoformans* in tissues was inhibited to a greater extent than in CV euthymic mice (with lower NK cell activity than athymic mice) during the first 7 days following systemic challenge (*Murphy* et al., 1982). Conventional athymic mice were also found to be more resistant to systemic *Candida albicans* infections than euthymic counterparts (*Cutler*, 1976). Cellular components from fungi such as *Cryptococcus neoformans* capsular polysaccharide antigens (*Murphy* et al., 1982) and cell wall constituents from *Candida albicans* (*Carrow* et al., 1985) have been shown to function as immunological modulators, involving both antibody and cell mediated immunity in animals and humans. The objective of these studies were to determine if microbial enhanced NK cell activity correlated with increased resistance due to systemic infections with the opportunistic fungi *Cryptococcus neoformans* or *Candida albicans* and if the systemic fungal infections caused modulation in NK cell activity during the natural course of infections with these agents.

METHODOLOGY

Gnotobiotic animals

Inbred C3H/HeCr, BALB/c (euthymic/athymic), and C57BL/6 (beige/black) mice were used in these studies. Gnotobiotic colonies were established as described previously (*Bartizal* et al., 1983, 1984). Each gnotobiotic mouse colony was housed in a separate isolator and monitored by microbiological methodology. Conventional colonies were maintained in standard animal room quarters. Except for *Cryptococcus neoformans*, all CV and gnotobiotic animals were exposed from birth with their particular microbial flora. Conventionalised (CONV) mice were established by inoculating drinking water of 3-4 week old germfree (GF) mice with 1 ml of a caecal homogenate from a healthy CV mouse. *Cryptococcus neoformans* or *Candida albicans* mice were challenged either orally or intravenously (i.v., tail veins) with 1 LD_{50} log phase, washed yeast cells. All mice were age matched and ranged between 6-10 weeks of age when assayed.

Diets

Mice in dietary studies were divided into 3 groups. In group 1, mice were fed "Amicon thin-channelled", ultra-filtered chemically defined (low-antigen) diet, with a daily lipid supplement. In group 2, mice were fed autoclaved natural ingredient diet. The third group was fed the chemically defined diet for approximately 11-15 weeks and then switched to autoclaved natural ingredient diet for the remainder of the study.

Natural cytotoxicity assays

Effector and target cell suspensions were prepared as described previously (*Bartizal* et al., 1983). Natural killer cell activity was determined by a direct 4 h ^{51}Cr-release assay at various effector:target ratios. Lymphocytes derived from spleens of animals in different experimental groups were performed in quadruplicate. Supernatant samples were collected using the Titertek supernatant collection system (Flow Laboratories) and counted in an autogamma spectrometer. Percent cytotoxicity was calculated as follows:

$$\%\text{cytotoxity} = \frac{\text{experimental c.p.m. - spontaneous c.p.m.}}{\text{maximum c.p.m. - spontaneous c.p.m}} \times 100$$

Significance of data was analysed (Student's t-test and analysis of variance) from quadruple experimental samples, from at least 3 age-matched mice from an experimental group, assayed on the same day.

In addition, a modified single cell NK assay was used to determine NK cell parameters. Briefly, splenic NK lymphocytes and YAC-1 tumour target cells were used at 1 effector:2 target cell ratio. Effector and target cells were incubated together to form conjugates, pelleted and mixed with molten agarose then spread over agarose pre-coated slides and incubated for 4 h. Results were assessed microscopically using trypan blue dye exclusion to determine cell viability. The NK cell parameters determined were mean percent target binding cells (%TBC), mean percent target binding cells that were cytotoxic (%TBCC), and mean percent active NK cell frequency (%NK). Calculations used to derive parameters were described previously (*Liljequist* et al., 1987).

Table 1: Cytotoxicity in BALB/c mice by the ^{51}Cr-release assay

Microbial status	Phenotype	Percent Cytotoxicity[a]
Germfree	athymic	14.25[b]
	euthymic	9.76[b]
C. albicans MA	athymic	12.30[b]
	euthymic	10.80[b]
S. pneumoniae MA	euthymic	3.74[b]
S. lactis MA	euthymic	4.47[b]
M. morganii MA	euthymic	4.88[b]
P. pneumotropica MA	euthymic	4.37[b]
C. albicans + *Bacillus* sp. DA	athymic	12.54[b]
	euthymic	10.16[b]
Flora defined	athymic	31.83
	euthymic	21.21
Conventionalised	athymic	43.85
	euthymic	24.92

[a]: effector:target ratio = 100:1.
[b]: Significantly lower ($p<0.05$) than flora defined and conventionalised (mean values).

RESULTS AND DISCUSSION

Previous studies of NK cell activity in mice have not resolved whether differences exist in GF, gnotobiotic or CV mouse NK cell cytotoxicity. These studies demonstrate that spleen cells from flora defined (FD) and conventionalised (CONV) athymic and euthymic mice show significantly more NK cytotoxicity than germfree (GF), mono-associated (*Candida albicans*), or di-associated (*Candida albicans* and *Bacillus* sp.) mice (see Table 1). Spleen cells from euthymic GF mice had significantly less NK cell activity compared to athymic GF mice. Athymic FD and CONV mice exhibited significantly greater NK cell activity than their euthymic counterparts. Colonisation of the alimentary tract of GF athymic or euthymic mice with pure cultures of *Candida albicans*, *Streptococcus pneumoniae*, *Streptococcus lactis*, *Morganella morganii*, *Pasteurella pneumotropica*, alone or *Candida albicans* in combination with a *Bacillus* sp., did not significantly alter the NK cytotoxicity of their splenic lymphocytes even though these microbes were present in high numbers (10^{8-10}/g) within their alimentary tracts. Percent cytotoxicity also increased as the effector:target ratio increased. These studies indicate that the intestinal microflora can alter murine natural NK cell cytotoxicity (Table 1).

Data in Table 2 suggest that spleen cells from GF mice raised on a chemically defined (low antigen) diet were more cytotoxic towards tumour targets than spleen cells from GF or CV (barrier maintained) mice raised on a natural ingredient diet. The NK cell activity of GF mice was dramatically increased after alimentary tract colonisation with a complex CV intestinal microflora. Conventional mice raised in clean (barrier) conditions showed significantly less NK cell activity than non barrier-reared mice. Switching GF mice from a chemically defined diet to a natural ingredient diet did not enhance NK cell

Table 2: The effects of age, diet and microbial flora on NK cell activity in C3H/HeCr mice

Microbial status	Diet[a]	Age (wks)	Percent cytotoxicity[b]
Germfree	CD	6-7	16.63
Conventional	Nl	7	9.33
Germfree	Nl	9-10	8.22
Germfree	CD-NI	15-19	11.18
Germfree	CD	29-35	16.28
Germfree	Nl	30-32	11.06
Conventional[c]	NI	34-36	9.52
Conventionalised	Nl	7-8	19.88[d]
	CD-NI	8	51.26 [d]

[a]: CD= chemically defined, NI = natural ingredient, CD-NI = diet switch.
[b]: effector:target ratio = 100:1.
[c]: CV are barrier reared (clean conventional).

activity. No significant differences in NK cell activity were evident in GF or CV mice of different ages (6-10 weeks old vs. 29-36 weeks old). These results indicate that the diet and microbial flora modulate the NK cell activity of mice. Most mice purchased from commercial suppliers are designated as being "flora defined" or "specific pathogen free". After equilibration into new animal quarters these mice are usually fed different diets and acquire different species of environmental microbes. These environmental and dietary changes undoubtedly alter the intestinal microflora, which can have dramatic affects on the cytotoxic activity of their NK cells.

When NK cell parameters were evaluated in the single cell assay, no significant differences in target binding ability (%TBC) were found between GF or CV mouse spleen cells for either athymic or euthymic mice (Table 3). However, the %TBC of athymic mouse cells was significantly greater than euthymic mouse cells. Significantly lower cytotoxicity (%TBCC) was found in non-NK-enriched populations of GF and CV athymic or euthymic spleen cells. Only the spleen cells from NK-enriched GF euthymic mice showed significantly lower %TBCC compared to CV euthymic and GF or CV athymic cells from the same population. The frequency of active NK cells (%NK) appeared higher in both GF and CV athymic mice compared to GF or CV euthymic mice in either enriched or non-enriched NK spleen cell populations. Enriched populations of athymic CV effector cells showed significantly greater %NK than non-enriched athymic CV effector cells. These results emphasise that binding ability (%TBC) is influenced by mouse genotype (athymic or euthymic) and is augmented in the presence of a microflora.

The single cell cytotoxicity assay prevents NK cells from recycling in agarose medium. The observed augmentation of NK cell activity due to microbial influence may represent an increased recycling rate of CV effector NK cells which are detected in the single cell assay. The increased %NK (calculated as a factor of %TBC and %TBCC) of GF and CV athymic and euthymic mice is largely a result of the increased %TBC factor, since %TBCC is relatively equal as measured for athymic and euthymic mice. Therefore, an increased recycling rate of NK cells

Table 3: Cytotoxicity in BALB/c mice determined by the single cell assay

	Microbial Status	Phenotype	%TBC	%TBCC	%NK
Nylon wool enriched:					
	CV	athymic	20.6	17.3	3.6
	GF	athymic	18.4	17.8	3.1
	CV	euthymic	11.7	17.3	2.0
	GF	euthymic	11.0	12.4	1.4
Non enriched:					
	CV	athymic	27.9	8.9	2.4
	GF	athymic	29.5	9.6	2.7
	CV	euthymic	18.6	8.1	1.5
	GF	euthymic	18.5	8.8	1.6

may occur due to the presence of a CV microflora. Suppressor cells present in euthymic spleen cell populations may down-regulate %TBC. Since NK cells are unable to recycle in the single cell assay (in agarose) the effects of down-regulation may become more apparent in the single cell assay compared to the chromium release assay.

Both GF and FD athymic and euthymic mice exhibited significantly elevated NK cell activity after oral association or i.v. challenge with *Cryptococcus neoformans* (Table 4). Augmented NK cell activity occurred rapidly (24 h) after challenge and persisted for 14 days post challenge. Live and heat-killed *Cryptococcus neoformans* or its capsular polysaccharide enhanced NK cell activity in mice. *Cryptococcus* augmented NK cell activity was seen to a greater extent in GF athymic mice than in euthymic mice, while in FD mice the opposite effect was seen. The greatest augmentation of NK activity occurred in systemically (i.v.) challenged mice administered viable *Cryptococcus neoformans* cells, with lesser augmentation seen in orally associated mice. The least NK cell augmentation was observed in capsular polysaccharide injected mice. Augmented NK cell activity did not appear to correlate with immunity to cryptococcal infection since all mice eventually succumbed to disseminated cryptococcal infection. The route of

Table 4: Augmentation of splenic NK cell activity after *C. neoformans* challenge in BALB/c mice (^{51}Cr-release assay).

	Challenge	% Cytotoxicity at E:T Ratio of 100:1					
		Day 3		Day 7		Day 14	
		Ath	Euth	Ath	Euth	Ath	Euth
Germfree:	capsule i.v.	6.9	4.2	11.0	2.8	7.9	0.0
	live oral	12.5	8.6	16.3	5.9	34.6	5.4
	live i.v.	26.7	16.3	39.6	23.2	37.1	6.6
Flora-defined:	live i.v.	1.5	0	15.8	28.8	all died	41.6
	heat-killed i.v.	9.0	13.1	4.2	6.3	n.d.	n.d.

Table 5: NK cell activity and lethality after systemic i.v. challenge of gnotobiotes with *C. albicans* or *C. neoformans*

Microbial status	Strain/phenotype	% Cytotoxicity at E:T Ratio 100:1 Before challenge	7-21 days after challenge[a]	Survival after challenge[b]
C. neoformans challenged mice:				
Germfree	BALB/Euthymic	7.04	13.44[c]	38
Germfree	BALB/Athymic	12.35	42.52[c]	13
Flora defined	BALB/Euthymic	18.67	30.91[c]	15
Flora defined	BALB/Athymic	16.78	29.67[c]	12
Germfree	C57BL/6/black	9.95	2.49[d]	19
Germfree	C57BL/6/beige	1.09	2.90	17
Conventional	C57BL/6/black	16.73	23.05	19
Conventional	C57BL/6/beige	5.69	4.35	17
C. albicans-challenged mice:				
C. albicans MA	BALB/euthymic	10.73	6.59	no dead
C. albicans MA	BALB/athymic	16.23	16.81	no dead

[a]: Cytotoxicity was determined 7-21 days post challenge on survivors.
[b]: Mortality represents data from 12-24 mice per group from a 50 day period.
[c]: Significantly greater ($p<0.01$) than unchallenged mice of same genotype and assay time.
[d]: Significantly less ($p<0.05$) than unchallenged mice of same genotype and assay time.

challenge, host immunocompetence, nature of the antigen and host microbial status were found to be important factors in NK cell modulation by the opportunistic fungi. Additionally, enhanced NK cell activity did not correlate positively with increased resistance to *Cryptococcus neoformans* infection. In contrast, athymic mice challenged i.v. with *Candida albicans* showed enhanced NK cell activity compared to non challenged, *Candida albicans* mono-associated mice. Euthymic mice, however, showed unaltered NK cell activity following i.v. challenge (Table 5). No mortality was observed in either athymic or euthymic mice challenged i.v. with *Candida albicans* during the experimental period.

LITERATURE

Bartizal, K.F., Salkowski, C., and Balish, E.: The influence of a gastrointestinal microflora on natural killer cell activity. J. Ret. Endol. Soc. 33, 381-390 (1983).

Bartizal, K.F., Salkowski, C., Pleasants, J.R., and Balish, E.: The effect of microbial flora, diet, and age on the tumoricidal activity of natural killer cells. J. Leukocyte Biol. 36, 739-750 (1984).

Carrow, E.W. and Domer, J.E.: Immunoregulation in experimental murine candidiasis. Specific suppression induced by *Candida albicans* cell wall glycoproteins. Infect. Immun. 49, 172-181 (1985).

Cutler, J.E.: Acute systemic candidiasis in normal and congenitally thymic-deficient (nude) mice. J. Ret. Endol. Soc. 19, 121-124 (1976).

Herberman, R.B., Nunn, M.E., Holden, H.T., Staal, S., and Djeu, J.Y.: Augmentation of natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic target cells. Int. J. Cancer 19, 555-564 (1977).

Herberman, R.B., Djeu, J.Y., Kay, H.D., Rortaldo, J., Riccardi, C., Bonnard, G. D., Holden, H. T., Fagnani, R., Santoni, A., and Puccetti, P.: Natural killer cells. Characteristics and regulation of activity. Immunol. Rev. 44, 43-70 (1979).

Kamiyama, T. and Hagiwara, T.: Augmented followed by suppressed levels of natural cell mediated cytotoxicity in mice infected with *Toxoplasma gondii*. Infect. Immun. 36, 628-636 (1982).

Kasahara, T., Harada, H., Shioiri-Nakano, K., Imai, M., and Sano, T.: Potentiation of natural killer activity of human lymphocytes by *Staphylococcus aureus* bacteria and its protein A. Immunology 42, 175-183 (1981).

Kohl, S. and Loo, L.S.: Ontogeny of murine cellular cytotoxicity to herpes simplex virus infected cells. Infect. Immun. 30, 847-850 (1980).

Liljequist, B. and Bartizal, K.: Estimation of natural killer cell parameters of germfree or conventional athymic and euthymic BALB/c mice using nylon wool-enriched versus non-enriched splenic lymphocytes in the single cell assay. In: Gnotobiology and its applications (Ed.: Ducluzeau, R.). Marcel Mérieux, Lyon, 255-257 (1987).

Marconi, P., Scaringi, L., Tissi, L., Boccanera, M., Bistoni, F., Bonmassar, E., and Cassone, A.: Induction of natural killer cell activity by inactivated *Candida albicans* in mice. Infect. Immun. 50, 297-303 (1985).

Murphy J.W. and McDaniel, D.O.: *In vitro* effect of natural killer (NK) cells on *Cryptococcus neoformans*. In: NK cells and other natural effector cells (Ed.: Herberman, R.B.). Academic Press, New York, 1105 (1982).

Murphy J.W. and McDaniel, D.O.: *In vitro* reactivity of natural killer (NK) cells against *Cryptococcus neoformans*. J. Immunol. 128, 1577-1583 (1982).

Murphy J.W. and Moorhead, J.W.: Regulation of cell mediated immunity in Cryptococcosis. I. Induction of Specific afferent T suppressor cells by cryptococcal antigen. J. Immunol. 128, 276-283 (1982).

THE DEVELOPMENT AND FUNCTIONAL ACTIVITY OF T-CELLS IN HUMAN FOETAL SMALL INTESTINE

THOMAS T. MACDONALD

Department of Paediatric Gastroenterology, St. Bartholomews Hospital, London, UK

Lecture presented at the 2nd Old Herborn University Seminar on "Interactions between the indigenous microflora and the host immune system" on June 1, 1988

INTRODUCTION

The normal human and rodent small intestine is densely populated with T-cells, the function of whom is unknown. The study of the lymphocytes in human foetal small intestine gives the opportunity to investigate the development of gut associated lymphoid tissue in a sterile germfree environment. In addition, through use of an organ culture technique, we can investigate the function of foetal gut T-cells following activation. This article reviews some of the major findings.

MATERIALS AND METHODS

Tissue

Small intestine (jejunum and ileum) from therapeutically aborted foetuses was snap frozen and stored at -70°C.

Organ culture of foetal human small intestine

Small intestine from foetuses was placed in a petridish in serum-free CMRL-1066 medium (Flow) modified as described (*MacDonald* and *Spencer*, 1988). The intestine was cut into pieces 2-3 mm^2. Five pieces of tissue were cultured in 7 ml modified CMRL-1066 medium in 5 cm diameter tissue culture dishes (Sterilin). The cultures were incubated at 37°C in a 95% oxygen, 5% CO atmosphere with or without pokeweed mitogen. At the end of the culture period the 5 explants in each dish were carefully removed from the culture dish and placed on top of one another on a piece of filter paper to absorb excess moisture. The tissues were then snap-frozen in liquid nitrogen and stored at -70°C. Frozen sections profiling each of the explants were then cut and stained immunohistochemically using the peroxidase technique.

Monoclonal antibodies used were Ki67 (Dako Ltd., High Wycombe, Bucks) which identifies a nuclear antigen in all dividing cells and the anti-T-cell antibodies anti-CD3, CD4, CD8 and CD25 purchased from Becton-Dickinson, and anti-HLA-DR (a gift from Dr. K. Adams).

Quantification of IEL

Differential counts of villus or crypt epithelial cells and peroxidase-stained intra-epithelial lymphocytes in each explant were made (*Ferguson* and *Murray*, 1971). Results are expressed as the percentage IEL stained with any particular antibody per 100 epithelial

Table 1: Number of $CD3^+$ IEL, $CD4^+$ IEL and $CD8^+$ IEL per 100 epithelial cells in the tissues of different ages

Age of Tissue (weeks)	Stained cells per 100 epithelial cells CD3	CD8	CD4
11	0.0	0.0	0.0
14	1.8	0.8	0.3
16	2.9	1.5	0.3
18	3.2	2.1	0.9
22	4.2	3.9	0.1

cells. Crypt cell proliferation was measured by counting the number of crypt epithelial cell nuclei staining immunohistochemically with Ki67 (*MacDonald* and *Spencer*, 1988).

Induction of HLA-DR on HT-29 cells with organ culture supernatants to measure interferon gamma

The transformed intestinal epithelial cell line HT-29 was obtained from the European Collection of Animal Cell Cultures, Porton Down, and was grown and passaged in RPMI-1640 culture medium containing 10% foetal calf serum. 50,000 HT-29 cells were added to microculture wells on plastic slides (Labtech tissue culture chamber slides, Miles Laboratories, Napierville, Illinois) in a volume of 0.2 ml and allowed to adhere overnight. The next day the adherent cells were washed and were treated with dilutions of organ culture supernatants or recombinant gamma interferon (a gift from Dr. Allan Morris, University of Warwick). Twenty-four hours later the cells were washed and stained immunohistochemically with anti-HLA-DR. Each culture supernatant was tested in duplicate. The person reading the slides was unaware of the origin of the supernatant added to the HT-29 cells. The percentage cells expressing HLA-DR was taken as the mean of the duplicate tests of each supernatant dilution.

Sheep anti-recombinant interferon gamma (a gift from Dr. A. Meager, British Institute for Biological Standards, Potters Bar) was added to some of the wells.

RESULTS

Development of T-cells in human small intestine

Twenty-two foetal small intestines were examined, ranging in age from 11 to 22 weeks. The number of $CD3^+$ IEL, $CD4^+$ IEL, and $CD8^+$ IEL per 100 epithelial cells in the tissues of different ages are shown in Table 1.

The results clearly show an increase in intra-epithelial lymphocytes with increasing gestation. Interestingly, most of the cells are $CD8^+$, as in adult intestine. This would indicate that exogenous bacterial or dietary antigen is not responsible for the appearance of $CD8^+$ cells in the gut epithelium.

As well as an increase in IEL there was also an increase in lamina propria T-cells, but these were $CD4^+$, as is also the case in post-natal intestine.

Organised aggregates of lymphoid tissue (Peyer's patches) were not seen

Figure 1: $CD3^+$ cells in a Peyer's patch from 19 week-old foetal human small intestine.

until 19 weeks gestation. Figure 1 illustrates the appearance of a foetal Peyer's patch stained immunohistochemically with anti-CD3 to identify the T-cell zones.

T-cell activation in organ cultures of foetal intestine

Explants of human foetal intestine can be maintained in organ culture for several weeks with retention of gut structure and epithelial cell function (*MacDonald* and *Spencer*, 1988). We therefore thus attempted to directly stimulate mucosal T-cells in situ in explants of human small intestine.

Preliminary experiments indicated that the T-cell mitogens phytohaemagglutinin and wheatgerm agglutinin added directly to the cultures activated mucosal T-cells in the explants as measured by the appearance of $CD25^+$ cells, however the most striking effects were seen with pokeweed mitogen (PWM).

Associated with the appearance of $CD25^+$ cells there was also dramatic changes in mucosal morphology in the older tissues (an 18 week-old foetal gut cultured for 3 days with PWM is shown in Figure 2). At the onset of culture, villous morphology was good and there was epithelial cell division in the crypts, highlighted using the monoclonal antibody Ki67 and the peroxidase technique. After 3 days in culture the villi had become swollen as a result of transporting water across the epithelium and there were still epithelial cells in division in the crypts. However in the presence of PWM, there is increased cellularity in the lamina propria, villous atrophy, and a dramatic crypt epithelial cell hyperplasia. This effect was age-dependent in that in 14 week-old specimens PWM had no effect and in specimens aged 16-17 weeks the effect was less striking than that shown in Figure 2.

Interferon-gamma production by activated T-cells in foetal small intestine in organ culture

To determine if the T-cells activated with PWM were secreting lymphokines, the organ culture supernatants were

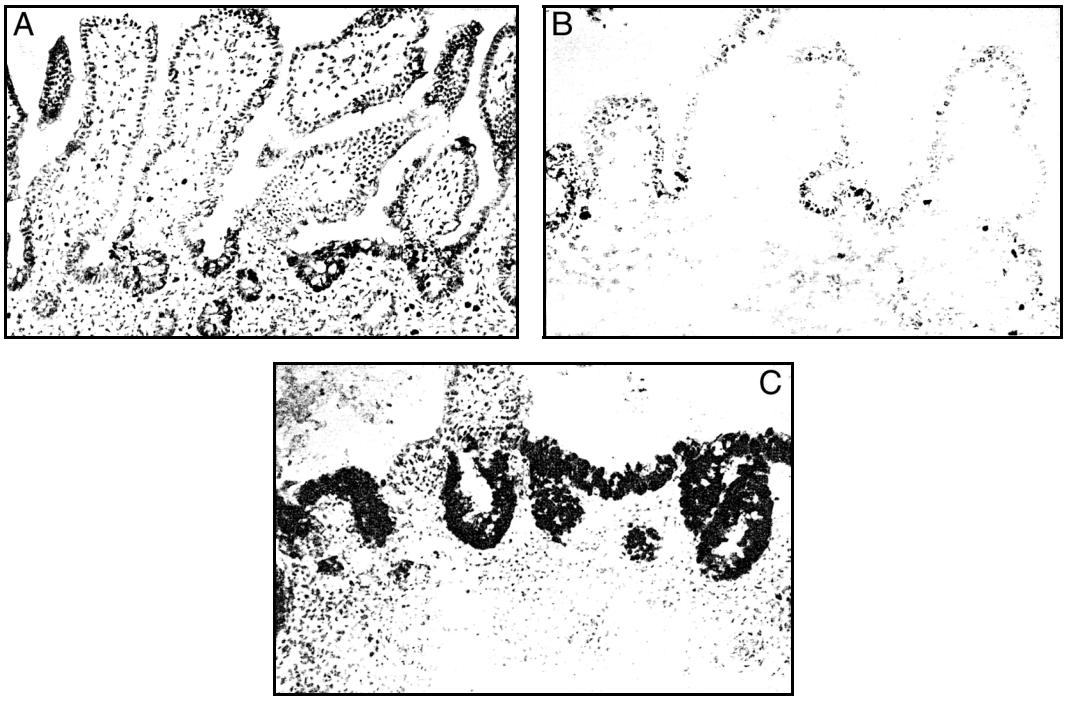

Figure 2: The immunohistochemical appearance of 18-week-old foetal gut on day 0 (A), after 3 days in culture in medium alone (B), and after 3 days in culture with PWM (15 mg/ml, C). The sections were stained with Ki67 to highlight dividing cells in the crypts.

tested for interferon-gamma. This was done by using the ability of gamma-interferon to induce *de novo* the expression of Class II MHC molecules on an epithelial cell line (*MacDonald* et al., 1988).

Less than 1% of HT-29 cells cultured in medium alone was HLA-DR$^+$. However treatment with recombinant interferon-gamma increased the number of HLA-DR$^+$ cells to 18% (Figure 3). This effect titrated out with decreasing amounts of gamma-interferon and was completely inhibited by sheep anti-gamma-interferon. Organ culture supernatants from PWM-treated explants (but not control cultures) also increased HLA-DR positivity on the HT-29 cells, and this effect was also inhibited with sheep anti- interferon-gamma.

SUMMARY AND CONCLUSIONS

These studies show that lymphocytes populate human small intestine in an antigen-independent fashion. Most noticeably, CD8$^+$ T-cells show a marked tropism for gut epithelium, even in the absence of antigen. Peyer's patches also form in the absence of antigen. In organ culture T-cells in foetal small intestinal lamina propria can be activated with PWM to secrete lymphokines.

A consequence of T-cell activation is a rapid increase in the rate of intestinal crypt cell proliferation. Thus although the function of T-cells in health remains unknown, these results indicate that T-cells may play an important role in the development of enteropathy.

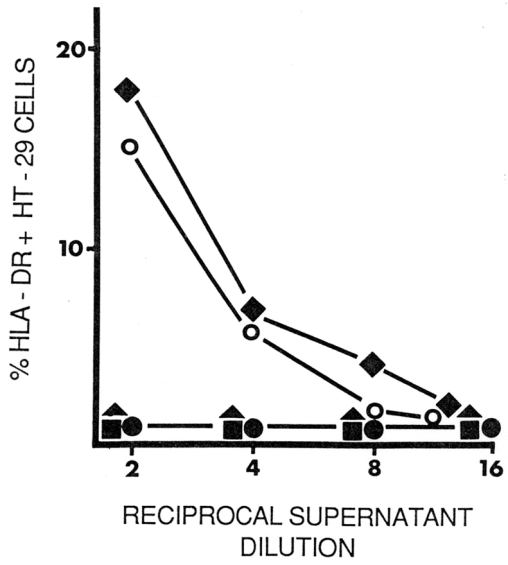

Figure 3: The induction of HLA-DR on HT-29 cells with recombinant gamma interferon and organ culture supernatants. Each point is the mean of duplicate observations per dilution of culture supernatant or recombinant interferon-gamma. In this representative experiment recombinant gamma-interferon (200 units/ml, ◆—◆) was titrated out and gave a dose dependent increase in HT-29 HLA-DR expression. Organ culture supernatants (○—○) from explant cultures of a 22 week-old foetal gut cultured for 3 days with PWM also caused an increase in HLA-DR expression. Control supernatants not stimulated with PWM (●—●) had no effect on HLA-DR expression. In the presence of sheep anti-interferon gamma (final dilution in the wells-1:200) the HLA-DR inducing effects of recombinant interferon-gamma (■—■) and the PWM-treated organ culture supernatant (▲—▲) was completely neutralised.

LITERATURE

Ferguson, A. and Murray, D.: Quantitation of IEL in human jejunum. Gut 12, 988-994 (1971).

Spencer, J.M., Dillon, S.B., Isaacson, P.G., and MacDonald, T.T.: T cell subclasses in human fetal ileum. Clin. Exp. Immunol. 65, 553-558 (1986).

Spencer, J.M., MacDonald, T.T., Finn, T.T., and lsaacson, P.G.: The development of gut associated lymphoid tissue in the terminal ileum of fetal human intestine. Clin. Exp. Immunol. 64, 536-543 (1986).

Spencer, J.M., MacDonald, T.T., and Isaacson, P.G.: Development of the gut associated lymphoid tissue in human fetal gut. In: Recent Advances in Mucosal Immunology (Eds: Mestecky, J., McGhee, J., Bienenstock, J., and Ogra, P.L.). Adv. Exp. Med. Biol. 216B, 1421-1430 (1987).

MacDonald, T.T. and Spencer, J.M.: Evidence that activated mucosal T cells play a role in the pathogenesis of enteropathy in human small intestine. J. Exp. Med. 167, 1341-1349 (1988).

MacDonald, T.T. and Spencer, J.M.: The consequences of T cell activation in human small intestine. In: Inflammatory Bowel Disease: Current status and future approaches (Ed.: McDermott, R.M.). Elsevier Science Publishers B.V., 107-112 (1988).

MacDonald, T.T., Weinel, A., and Spencer, J.M.: HLA-DR expression in human fetal intestinal epithelium. Gut 29, 1342-1348 (1988).

Monk, T.J., Spencer, J., Cerf-Bensussan, N., and MacDonald, T.T.: Activation of mucosal T-cells *in situ* with anti-CD3 antibody: Phenotype of the activated T-cells and their distribution within the mucosal microenvironment. Clin. Exp. Immunol. 74, 216-222 (1988).

CYTOKINES IN ORAL DISEASE AND MUCOSAL IMMUNE RESPONSES: INTERLEUKIN 6 REGULATION OF IgG AND IgA SYNTHESIS IN NORMAL AND INFLAMED SITUATIONS

YOSHIHARU KONO[*], KENNETH W. BEAGLEY[#], KOHTARO FUJIHASHI[*], JERRY R. McGHEE[+], MASATOMO HIRASAWA[§], MASAHARU MAKIMURA[§], SHIGEO OTAKE[§], and HIROSHI KIYONO[*]

Departments of [*]Oral Biology, [+]Microbiology and [#]Medicine, Research Center in Oral Biology, The University of Alabama at Birmingham, Birmingham, AL 35294, USA, and [§]Nihon University, School of Dentistry at Matsudo, Matsudo, Chiba 271, Japan

Lecture presented at the 2nd Old Herborn University Seminar on "Interactions between the indigenous microflora and the host immune system" on June 1, 1988

INTRODUCTION

The major antibody isotypes involved in immune responses in the oral cavity are IgG and IgA. The IgG antibodies seen in oral fluids originate from serum via the gingival crevicular fluid (GCF). Thus, the concentration of IgG antibody in GCF is very similar with those seen in serum (~12 mg/ml) (*Shillitoe* and *Lehner*, 1972). Further, some IgA antibodies, especially the monomeric form, are also derived from serum via GCF. On the other hand, predominant IgA antibody seen in oral fluids is either dimeric or larger molecular weight polymers (dIgA or pIgA, respectively) and is produced by plasma cells which reside in the salivary gland (*Mestecky* and *McGhee*, 1987; *Brandtzaeg*, 1988). This antibody isotype is extremely important in immune responses, which occur at mucosal surfaces, since most pathogens and foreign antigens enter the host through these mucous membranes. Mucosal surfaces represent over 400 square meters of surface area in humans which require immune protection (*Mestecky* and *McGhee*, 1987; *Brandtzaeg*, 1988, 1989). Both dIgA and pIgA production by plasma cells occur in mucosal associated tissues including the lamina propria regions of the gastrointestinal, upper respiratory and genitourinary tracts, and exocrine tissues. It has been estimated that greater than 10^{10} plasma cells per meter of intestine occur and approximately 80% of these are committed to the secretion of the dIgA or pIgA isotype (*Brandtzaeg*, 1989). Therefore, it is important to elucidate the exact regulatory mechanisms which modulate IgA responses at mucosal surfaces including those of the oral cavity.

Adult periodontitis (AP) is one of the most common chronic inflammatory diseases which occur in the oral tissues. AP is characterised by a local accumulation of activated B-cells and plasma cells and significant polyclonal immunoglobulin synthesis (*Brandtzaeg*, 1988). When gingival mononuclear cells (GMC) were enzymatically isolated from inflamed tissues of AP, high frequencies of plasma cells were present (*McGhee* et al., 1989a; *Ogawa* et al., 1989a). Further, when Ig producing

Table 1: Effect of cytokines on B-cells

Cytokines	Effects on B-cells
IL-2	Co-stimulation of B-cell growth and differentiation
IFN-γ	Enhances IgM and IgG2a in LPS triggered cultures Suppresses effects of IL-4 on B-cells
TGFβ	Induction of IgA isotype switching Inhibition of B-cell responses
IL-4	Induces B-cell entry into G1 Enhances IgE and IgG1 isotype switching and production in LPS and some antigen-specific B-cell systems Involvement in IgA isotype switching?
IL-5	Induces B-cell proliferation Enhances B-cell differentiation Synergises with IL-2 for B-cell proliferation Enhances IgA synthesis in $sIgA^+$ B-cells
IL-6	Induces terminal differentiation of B-cells committed to all isotypes Significantly enhances IgA synthesis in $sIgA^+$ B-cell subsets

cells were assessed by the enzyme-linked immunospot (ELISPOT) and by immunofluorescence, the GMC contained predominantly IgG followed by IgA and essentially no IgM secreting cells (*McGhee* et al., 1989a; *Ogawa* et al., 1989a). When Ig subclasses were assessed, the IgG profile was IgG1 > IgG2 > IgG3 = IgG4 and for IgA was IgA1 > IgA2 (*Ogawa* et al., 1989a). Other studies using immunofluorescence analysis of sections from gingival tissues have also shown that IgG followed by IgA are the predominant plasma cell isotypes seen in AP (*Killian* et al., 1989). Thus, it is of importance to determine the exact mechanism which regulates production of high IgG and IgA isotype responses seen in inflammatory lesions. Since recent studies with recombinant interleukins (IL) clearly showed that certain interleukins (e.g., IL-4, IL-5 and IL-6) are of particular importance in B-cell responses (*McGhee* et al., 1989b; *Kishimoto* and *Hirano*, 1988), this article summarises our recent findings on the regulation of IgA synthesis by interleukins in both the normal situation and on local production of increased IgG and IgA antibodies seen in inflamed gingiva of AP patients.

INTERLEUKIN REGULATION OF MUCOSAL IMMUNE RESPONSES

The cDNA cloning of cytokines during the past few years has led to the finding that more than one cytokine can induce B-cell responses at the stages of activation, proliferation and differentiation, and that the cytokine may also affect other immunocompetent cells including T-cells and antigen presenting cells (APCs) (*Kishimoto* and *Hirano*, 1988; *Paul* and *O'hara*, 1987). In this regard, IL-4, IL-5 and IL-6 have been shown to be key cytokines for the B-cell

activation, proliferation and differentiation (Table 1). It is generally accepted that IL-4 induces resting B-cells to enter G1, express MHC class II antigens and receptors for other cytokines (*Noelle* et al., 1984; *Rabin* et al., 1985). Activated B-cells respond to IL-5 and undergo cell division, and studies in mice have shown that this cytokine also induces B-cell differentiation and Ig synthesis (*Karasuyama* et al., 1988; *Swain* et al., 1983). However, its precise role in human Ig production remains unclear. IL-6 has been shown to induce B-cells to differentiate into plasma cells, with subsequent high rate Ig synthesis (*Hirano* et al., 1986; *Beagley* et al., 1989). In this regard, IL-6 induced high levels of IgM, IgG and IgA synthesis in mitogen-stimulated human tonsillar B-cell cultures (*Muraguchi* et al., 1988). A significant role in terminal differentiation was also suggested by the finding that anti-IL-6 antibodies, when added to pokeweed mitogen (PWM) stimulated peripheral blood mononuclear cells (PBMC), inhibited Ig synthesis of all isotypes (*Muraguchi* et al., 1988). For convenience, we will discuss major effects of IL-4, IL-5 and IL-6 on B-cell responses, especially for IgA synthesis and refer the reader to more general reviews of these cytokines for the other effects which are seen (*Kishimoto* and *Hirano*, 1988; *Paul* and *O'hara*, 1987).

Switch cytokines for IgA expression

Two important processes occur during the development of IgA producing cells which include the B-cell heavy chain switching to 3' isotypes (e.g., $\mu^+ \rightarrow \alpha^+$) and the terminal differentiation of IgA committed B-cells to high Ig secreting plasma cells. The heavy-chain gene family is present as a long sequence on a single chromosome and consists of exons (specific coding sequences) separated by introns (long stretches of non-coding DNA sequences). In the mouse, the order of heavy-chain constant-region (C_H) genes on chromosome 12 is 5'-μ-δ-γ3-γ1-γ2b-γ2a-ε-α-3'. The C_H gene order on human chromosome 14 is 5'-μ-δ-γ3-γ1-φε-α1-φγ-γ2-γ4-ε-α2-3' (*Cooper*, 1987; *Webb* et al., 1986). After the formation of V_HDJ_H gene in a developing progenitor B-cells for the antigen specificity, a V_HDJ_H-Cμ mRNA transcript is then differentially spliced for synthesis of a functional μ chain and the latter process defines the stage of a pre-B-cell (*Cooper*, 1987). Similarly, light-chain gene rearrangements (V_LJC_L) occur and, together with the V_HDJ_H exon, provide the developing B-cell with precise antigen specificity. This leads to the formation of an IgM molecule, which is inserted into the surface of cell membrane (as sIgM). Surface IgD (sIgD) of the same V_HDJ_H and V_LJC_L specificity results from differential splicing of the VDJCμ-Cδ gene complex (*Cooper*, 1987; *Webb* et al., 1986). Mature $sIgM^+$, $sIgD^+$ B-cells may be induced to switch from the production of IgM to that of any other isotype, e.g., to the expression of Cα-chain gene, a process which is broadly termed isotype switching (*Webb* et al., 1986). Generally, most agree that switching is accomplished by deletion of 5' C_H sequences to those expressed in fully differentiated plasma cells.

The best characterised interleukin which regulate 5' C_H to 3' C_H isotype switching has come from studies with IL-4 in the murine system (*Paul* and *O'hara*, 1987; *Coffman* et al., 1988). IL-4 has been shown to enhance isotype switching of $sIgG^-$ B-cells to sIgG1 bearing B-cells which lead to the enhancement of IgG1 synthesis in LPS-stimulated B-cell cultures (*Lutzker* et al., 1988). Further, IL-4 also acts directly on $sIgM^+$ B-cells for the induction

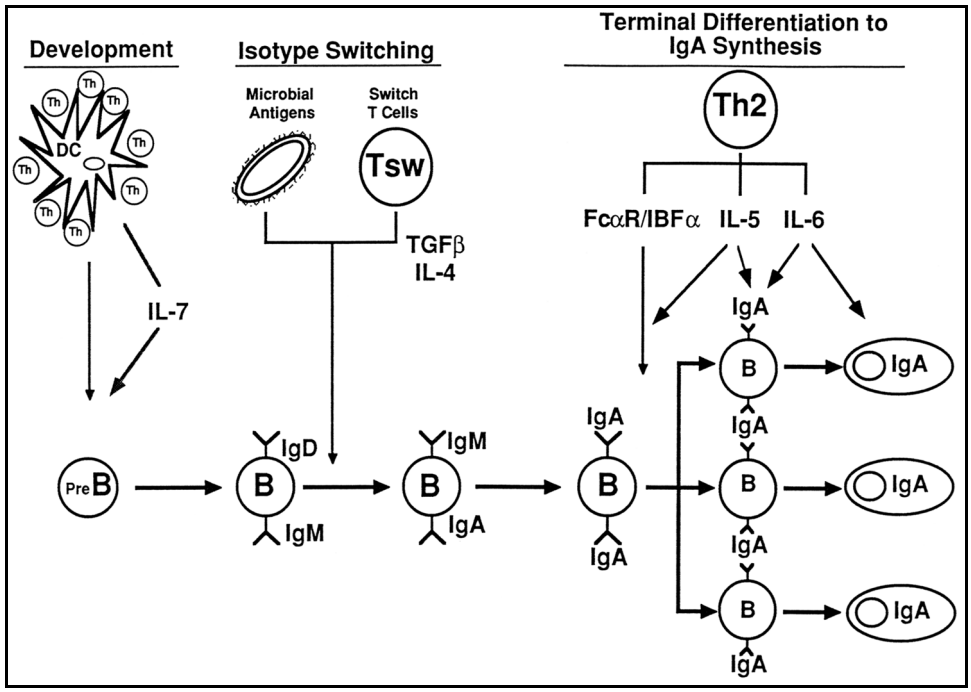

Figure 1: T-cells and cytokines involved in regulation of IgA synthesis.
DC: dendritic cells, Th: helper T-cells, Tsw: switch T-cells.

of switches to $sIgE^+$ B-cells (*Savelkoul* et al., 1988). Evidence for IL-4 involvement in IgA isotype switching has been provided by experiments using the sIgM bearing CH12LX B-cell line (*Kunimoto* et al., 1988). Incubation of CH12LX cells with IL-4 markedly enhanced numbers of $sIgA^+$ cells. On the other hand, IL-5 did not affect these cells. It was suggested that IL-4 induced the switching of $sIgM^+$ B-cells (*Kunimoto* et al., 1988). However, it is also possible that IL-4 may simply be expanding a small population of sIgA bearing cells in CH12LX cell line, since approximately 1% of these cells expresses sIgA. Therefore, it remains to be determined if IL-4 is a key interleukin for the induction of $\mu \rightarrow \alpha$ switching.

Most recent studies have provided evidence that transforming growth factor beta (TGFβ) is a switch factor for IgA (*Coffman* et al., 1989; *Sonoda* et al., 1989). Addition of TGFβ to splenic B-cells triggered with LPS gave 10-fold increases in IgA synthesis (*Coffman* et al., 1989; *Sonoda* et al., 1989). It was also shown that addition of IL-5 to TGFβ-treated cultures markedly enhanced IgA synthesis. These results have presumably suggested TGFβ induced $sIgM^+ \rightarrow sIgA^+$ commitment and IL-5 resulted in terminal differentiation. More definitive evidence for TGFβ-induced $\mu^+ \rightarrow \alpha^+$ switching was suggested by the most recent study that TGFβ induced $sIgA^+$ cells and more of the secreted form of mRNA (s-mRNA) than for the membrane form and that IL-2 enhanced these B-cells to produce more s-mRNA and total IgA synthesis (*Lebman* et al., 1990). Thus, both IL-4 and TGFβ have been shown to induce $sIgM^+$ B-cells to switch to more 3' isotypes including IgA (Figure 1).

Differentiation of IgA committed B-cells to IgA plasma cells by interleukins

It has been shown that murine IL-5 selectively enhances IgA synthesis in purified B-cell cultures (Table 1). The autoreactive T-cell lines produced both IL-4 and IL-5 after antigen stimulation and enhanced IgG1 and IgA synthesis in B-cell cultures co-stimulated with LPS (*Murray* et al., 1987). When purified IL-5 was added to LPS-driven B-cell cultures, enhanced IgA production was seen, and this effect was further augmented by IL-4 (*Murray* et al., 1987). The other studies have shown that supernatants from Th2-cell clones contained IL-5 and enhanced IgA synthesis in LPS-triggered splenic B-cell cultures (*Coffman* et al., 1987). The precise mechanisms for the IL-5 regulation of IgA synthesis was studied in more detail in Peyer's patch (PP) B-cell cultures, since PP is an important IgA inductive site which contain a high frequency of IgA committed B-cells and approximately 30-40 percent of PP B-cells are in cell cycle (*McGhee* et al., 1989b; *Beagley* et al., 1988). The addition of recombinant IL-5 (rIL-5) to PP B-cell cultures resulted in increased synthesis of only the IgA isotype (*Beagley* et al., 1988). The IL-5-induced increase in IgA synthesis was confined to the large blast B-cell subset. Further, the IL-5-induced effect was entirely restricted to the $sIgA^+$ B-cell fraction (*Beagley* et al., 1988). These studies suggest that IL-5 induces $sIgA^+$ B-cells to differentiate into cells secreting IgA. Similar findings were also noted in LPS-driven PP B-cell cultures where IL-5 induced IgA synthesis in LPS-stimulated $sIgA^+$ B-cells but not in $sIgA^-$ B-cells (*Coffman* et al., 1987; *Harriman* et al., 1988). Based on these findings, it is now clear that IL-5 is an important cytokine, which can induce IgA, committed B-cells to become IgA producing cells (Figure 1).

The major cytokine for the regulation of terminal differentiation of B-cells to plasma cells is IL-6 (Table 1), since this interleukin has been shown to induce terminal differentiation of mitogen- or antigen-activated B-cells to Ig secreting cells of all isotypes (*Kishimoto* and *Hirano*, 1988). In addition to the effect of rIL-5 on IgA, rIL-6 also induced significant increases in IgA synthesis in PP B-cell cultures (*Beagley* et al., 1989). In these studies, rIL-6 induced two- or three-fold higher levels of IgA than were seen with rIL-5 (*Beagley* et al., 1989). Further, both rIL-5 and rIL-6 induced significant increases in IgA levels in the large blast B-cell population. When PP B-cells were separated into $sIgA^+$ and $sIgA^-$ B-cell subsets by flow cytometry, removal of $sIgA^+$ B-cells abolished the effect of both rIL-5 and rIL-6 on IgA synthesis (*Beagley* et al., 1989). On the other hand, B-cells enriched for $sIgA^+$ cells and incubated with rIL5 or rIL-6 increased IgA synthesis in a dose-dependent manner; rIL-6 induced two- to fourfold higher levels of IgA synthesis than did rIL-5. Thus, both IL-5 and IL-6 induce $sIgA^+$ blast B-cell subsets to differentiate into IgA-secreting cells (*Beagley* et al., 1989). Since IL-6 induces increased numbers of B-cells which secrete IgA and in higher levels of total IgA synthesis, it is more effective for terminal differentiation than is IL-5. IL-5 and IL-6 both appear to act on B-cells already committed to IgA (Figure 1).

ROLE OF INTERLEUKINS IN ADULT PERIODONTITIS

It is well known that local infiltration of lymphoid cells, including lymphocytes, plasma cells, monocytes/macrophages and neutrophils occur in in-

Table 2: Production of IL-6 by gingival mononuclear cells isolated from patients with adult periodontitis*

Cultures contained			IL-6 activity
Samples (culture supernatants)	Con-A	Anti-IL-6	[O.D. (590-650nm) value x 1.000 ± S.D.]
GMC	-	-	411 ± 65
GMC	-	+	28 ± 13
PBMC	-	-	39 ± 20
PBMC	+	-	198 ± 29
PBMC	+	+	21 ± 14
rIL-6	-	-	395 ± 24
rIL-6	-	+	20 ± 15

*Culture supernatants from GMC or PBMC cultures were measured for IL-6 activity by biological assay using IL-6 dependent MOPC 104E kD 83 cell line. Cultures containing kD 83 cells (5 x 10^3/50 μl) and different dilution of samples (50 μl) were incubated for 48 hr. During the final 4-hr incubation, MTT was added. Isopropanol was then added for the colour development. The intensity of colour was determined by use of a Titertek Multiskan MC photometer.

flamed gingiva of AP patients (*Brandtzaeg*, 1988). These infiltrating lymphoid cells induce abnormal immunological responses in the soft tissue of gingiva, which results in the alveolar bone loss in AP. Thus, AP closely resembles the destructive reactions seen in rheumatoid arthritis (RA). In both cases, the local accumulation of activated lymphoid cells results in the increased production of both polyclonal and antigen-specific antibodies, cytokines and inflammatory mediators at the disease site which lead to the destruction of both connective tissue and bone (*Brandtzaeg*, 1988; *Snyderman* and *McCarty*, 1982). When mononuclear cells were isolated from gingiva of patients with AP, GMC contained high numbers of Ig secreting cells, especially of the IgG and IgA isotypes include their subclasses (*McGhee* et al., 1989a; *Ogawa* et al., 1989a). It was also shown that elevated local IgG and IgA subclass responses occur to the protein antigen of a major AP-associated pathogen *Porphyromonas* (*Bacteroides*) *gingivalis* (*Ogawa* et al., 1989b). Similar findings were derived from the analysis of GCF and gingival homogenates of AP patients (*Ebersole* et al., 1986; *Naito* et al., 1987; *Smith* et al., 1985). In addition, it was also shown that increased level of endogenous antigen, e.g., Type I and II collagen-specific antibody-producing cells were seen in GMC of patients with AP (*Hirsch* et al., 1988). Based upon these observations, it was important to determine the molecular mechanisms, which induce aberrant Ig responses at the local disease site. Thus, the role of cytokines, especially IL-6, in this process was reviewed in this section.

IL-6 in chronic inflammatory disease

The involvement of IL-6 in aberrant immune responses which occur in chronic inflammatory disease was originally described as a B-cell differentiation factor (BCDF) activity in the synovial fluid of RA patients (*Al-Balaghi* et al., 1984). The synovial fluid isolated from RA patients contained BCDF and induced Ig secretion in acti-

vated B-cells and in B-cell lines. Recent studies have formally proven that the synovial fluid of RA patients possesses high levels of IL-6 (*Hirano* et al., 1988). In this study, high concentrations of IL-6 were detected in synovial fluids obtained from the joints of RA patients. Further, the mononuclear cells freshly isolated from synovial fluid constitutively expressed high levels of IL-6 specific mRNA. Excess production of IL-6 in the inflamed joints of RA patients could explain the occurrence of abnormal autoantibody production by mononuclear cells in the inflamed synovia. The dysregulation of IL-6 production is also seen in other diseases. Thus, tumour cells isolated from patients with cardiac myxoma, cervical cancer or bladder carcinomas secrete abnormal amounts of IL-6 (*Kishimoto* and *Hirano*, 1988). This would contribute to the induction of high amounts of autoantibodies and autoimmune-like symptoms seen in these patients. Further, the correlation of excess production of IL-6 and induction of complement-reactive protein, an example of a non-antibody protein which appears in high quantities in serum following the onset of infection or tissue destruction has also been shown in patients with systemic lupus erythematosus (*Swaak* et al., 1989), RA (*Houssiau* et al., 1988), severe burns (*Nysten* et al., 1987) and renal transplantation (*van Oers* et al., 1988).

Involvement of IL-6 in induction of aberrant IgG and IgA synthesis at localised inflamed gingiva

Since GMC from AP patients contain high numbers of IgG and IgA antibody producing cells (*McGhee* et al., 1989a; *Ogawa* et al., 1989a,b), it was important to determine if IL-6 present in these sites account for these responses. In order to examine whether GMC from inflamed tissues produce this cytokine, GMC from AP patients were incubated for 24-72 hr and culture supernatants were then harvested for the assessment of IL-6 activity using the IL-6 dependent MOPC 104E kD 83 cell line (*Kono* et al., 1990). GMC isolated from inflamed tissues of patients with AP spontaneously produce high levels of IL-6. Thus, when freshly isolated GMC were incubated in cultures without any stimulant, high levels of biologically active IL-6 were detected within 24 hr of culture (Table 2). The addition of goat-anti-human IL-6 to GMC culture supernatants resulted in inhibition of IL-6 activity, while the same amount of normal goat serum did not affect IL-6 activity in GMC culture sups (Table 2). This formally proves that GMC spontaneously produces IL-6 (*Kono* et al., 1990). On the other hand, PBMC from AP patients did not secrete IL-6 during a 24-72 hr incubation period. In order to induce IL-6 production, it was necessary to incubate PBMC with T-cell mitogens (e.g., Con A and PHA) (Table 2). Similar findings were also seen at the mRNA level since hybridisation of total mRNA from GMC and PBMC of the same patients with a IL-6 specific cDNA probe resulted in higher levels of IL-6 specific message in GMC but not in PBMC.

Based on the finding that GMC supernatants contained high levels of IL-6, it was important to examine whether GMC produced IL-6 possesses biologically active B-cell stimulatory factor-2 (BSF-2) function. Thus, PBMC isolated from AP patients or normal subjects were incubated in the presence (or absence) of GMC supernatants for 7 days and the cultures assessed for IgM, IgG and IgA secreting cells by the ELISPOT assay. Although GMC supernatants augmented SFC of all 3 isotypes, the major increases were seen with IgG and IgA isotypes (*Kono* et al., 1990). On the other hand, stimulation of

PBMC with pokeweed mitogen (PWM) resulted in a response pattern of IgM > IgG > IgA. Addition of goat anti-IL-6 to GMC sups neutralised BSF-2 activity and completely abrogated spot forming cell (SFC) responses of all isotypes. These findings provide strong evidence that GMC isolated from inflamed gingiva of AP patients spontaneously produce high levels of IL-6 (*Kono* et al., 1990). Further, these results suggest that GMC produced other cytokines in addition to excess amounts of IL-6. One possibility is that GMC produce additional cytokines which induce switching of $sIgM^+$ B-cells to IgG or IgA bearing B-cells and trigger their responsiveness to IL-6. In this regard, it has been shown that malignant T-cells from a patient Rac with mycosis fungoides/ Sezary-like syndrome produce an uncharacterised factor(s) which induce $sIgM^+$ B-cells to switch to IgG and IgA producing cells (*Mayer* et al., 1985). Alternatively, GMC may produce cytokines which activate resting B-cells to become blasts with a $sIgD^-$ phenotype, since previous studies showed that disappearance of sIgD on the B-cell surface could lead to a stage for final maturation into Ig-producing cells (*Kuritani* and *Cooper*, 1982). Further, other studies have provided evidence that IL-6 induces Ig production in $sIgD^-$ activated B-cells (*Cooper*, 1987). Support for this latter possibility was provided by experiments where pre-incubation of normal PBMC cells or purified B-cells with GMC culture sups (even in the presence of anti-human IL-6) induced expression of IL-6R on B-cells (*Kono* et al., 1990). Further, GMC supernatant-treated PBMC were responsive to IL-6, since the addition of rhIL-6 to GMC supernatant pre-treated PBMC resulted in the enhancement of IgG and IgA SFC. These results strongly suggest that GMC produces several cytokines, which may be involved in the process of isotype switching and/or in the induction of IL6R on resting B-cells.

ACKNOWLEDGEMENTS

The authors acknowledge the excellent secretarial help of Ms. Sandra Roberts and the computer drawn figure by Mr. Masahiko Amano. The results described in this article were supported by US PHS grants DE 08228, DE 04217, DE 08182, AI 19674, AI 18958, AI 21032, and AI 28147, and by Nihon University Oota Memorial Research Fund. YK is the recipient of Nihon University Suzuki Memorial Research Fund. HK is the recipient of Research Career Development Award, DE 00237.

LITERATURE

Al-Balaghi, S., Strom, H., and Möller, E.: B cell differentiation factor in synovial fluids of patients with rheumatoid arthritis. Immunol. Rev. 78, 7-23 (1984).

Beagley, K.W., Eldridge, J.H., Kiyono, H., Everson, M.P., Koopman, W.J., Honjo, T., and McGhee, J.R.: Recombinant murine IL-5 induces high rate IgA synthesis in cycling IgA-positive Peyer's patch B cells. J. Immunol. 141, 2035-2042 (1988).

Beagley, K.W., Eldridge, J.H., Lee, F., Kiyono, H., Everson, M.P., Koopman, W.J., Hirano, T., Kishimoto, T., and McGhee, J.R.: Interleukins and IgA synthesis. Human and murine interleukin 6 induces high rate IgA secretion in IgA-committed B cells. J. Exp. Med. 169, 2133-2148 (1989).

Brandtzaeg, P.: Role of immune system - Dan-

gers of a nonholistic approach in explaining health and disease. Periodontology Today (Ed.: Guggenheim, B.). Karger, Basel, 196-208 (1988).

Brandtzaeg, P.: Overview of the mucosal immune system. Curr. Top. Microbiol. Immunol. 146,13-25 (1989).

Coffman, R.L., Shrader, B., Carty, J., Mosmann, T.R., and Bond, M.W.: A mouse T cell product that preferentially enhances IgA production. I. Biologic characterization. J. Immunol. 139, 3685-3690 (1987).

Coffman, R.L., Seymour, W.P., Lebman, P.A., Hiraki, D.D., Christainsen, J.A., Shrader, B., Cherwinski, H.M., Savelkoul, H.F.J., Finkelman, F.D., Bond, M.W., and Mosmann, T.R.: The role of helper T cell products in mouse B cell differentiation and isotype regulation. Immunol. Rev. 102, 5-28 (1988).

Coffman, R.L., Lebman, D.A., and Shrader, B.: Transforming growth factor β specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. J. Exp. Med. 170, 1039-1044 (1989).

Cooper, M.D.: Lymphocytes-B. Normal development and function. N. Engl. J. Med. 317, 1452-1456 (1987).

Ebersole, J.L., Taubman, M.A., Smith, D.J., and Frey, D.E.: Human immune responses to oral microorganisms: Patterns of systemic antibody levels of *Bacteroides* species. Infect. Immun. 51, 507-513 (1986).

Harriman, G.R., Kunimoto, D.Y., Elliott, J.F., Paetkau, V., and Strober, W.: The role of IL-5 in IgA B cell differentiation. J. Immunol. 140, 3033-3039 (1988).

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamitsu, A., Tunasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T., and Kishimoto, T.: Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature 324, 73-76 (1986).

Hirano, T., Matsuda, T., Turner, M., Miyasaka, N., Buchan, G., Tang, B., Sato, K., Shimizu, M., Maini, R., Feldmann, M., and Kishimoto, T.: Excessive production of interleukin 6 / B-cell stimulatory factor-2 in rheumatoid arthritis. Eur. J. Immunol. 18:1797-1801 (1988).

Hirsch, H.Z., Tarkowski, A., Miller, E.J., Gay, S., Koopman, W.J., and Mestecky, J.: Autoimmunity to collagen in adult periodontal disease. J. Oral Pathol. 17, 456-459 (1988).

Houssiau, F.A., Devogelaer, J.P., van Damme, J., Nagant de Deuxchaisies, C., and van Snick, J.: Interleukin 6 in synovial fluids and serum of patients with rheumatoid arthritis and other inflammatory arthritis. Arthritis Rheum. 31, 784-788 (1988).

Karasuyama, H., Rolink, A., and Melchers, F.: Recombinant interleukin 2 or 5, but not 3 or 4, induces maturation of resting mouse B lymphocytes and propagates proliferation of activated B cell blasts. J. Exp. Med. 167, 1377-1390 (1988).

Killian, M., Ellegaard, B., and Mestecky, J.: Distribution isotypes including IgA subclasses in adult, juvenile and rapidly progressive periodontitis. J. Clin. Periodontol. 16, 179-184 (1989).

Kishimoto, T. and Hirano, T.: Molecular regulation of B lymphocyte response. Annu. Rev. Immunol. 6, 485-512 (1988).

Kono, Y., Beagley, K.W., Fujihashi, K., McGhee, J.R., Taga, T., Hirano, T., Kishimoto, T., and Kiyono, H.: Cytokine regulation of localized inflammation: Induction of activated B cells and IL-6 mediated polyclonal IgG and IgA synthesis in inflamed human gingiva. J. Immunol. 146, 1812-1821 (1991).

Kunimoto, D.Y., Harriman, G.R., and Strober, W.: Regulation of IgA differentiation in CH12LX B-cells by lymphokines. IL-4 induces membrane IgM-positive CH12LX cells to express membrane IgA and IL-5 induces membrane IgA-positive CH12LX cells to secrete IgA. J. Immunol. 141, 713-720 (1988).

Kuritani, T. and Cooper, M.D.: Human B cell differentiation. II. Pokeweed mitogenresponsive B cells belong to a surface immunoglobulin D-negative subpopulation. J. Exp. Med. 155, 1561-1566 (1982).

Lebman, D.A. and Coffman, R.L.: The effects of IL-4 and IL-5 on the IgA response by murine Peyer's patch B cell subpopulations. J. Immunol. 141, 2050-2056 (1988).

Lebman, D.A., Lee, F.D., and Coffman, R.L.: Mechanism for transforming growth factor β and IL-2 enhancement of IgA expression in lipopolysaccharide-stimulated B cell cul-

tures. J. Immunol. 144, 952 959 (1990).
Lutzker, S., Rothman, P., Pollock, R., Coffman, R., and Alt, F.W.: Mitogen- and IL-4-regulated expression of germ-line Igγ2b transcripts: Evidence for directed heavy chain class switching. Cell 53, 177-184 (1988).
Mayer, L., Posnett, D.N., and Kunkel, H.G.: Human-malignant T-cells capable of inducing an immunoglobulin class switch. J. Exp. Med. 161, 134-144 (1985).
McGhee, M.L., Ogawa, T., Pitts, A. M., Moldoveanu, Z., Mestecky, J., McGhee, J.R., and Kiyono, H.: Cellular analysis of functional mononuclear cells from chronically inflamed gingival tissue. Reg. Immunol. 2, 103-110 (1989a).
McGhee, J.R., Mestecky, J., Elson, C.O., and Kiyono, H.: Regulation of IgA synthesis and immune response by T cells and interleukins. J. Clin. Immunol. 2, 175-199 (1989b).
Mestecky, J. and McGhee, J.R.: Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. Adv. Immunol. 40, 153-254 (1987).
Muraguchi, A., Hirano, T., Tang, B., Matsuda, T., Horii, Y., Nakajima, K., and Kishimoto, T.: The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. J. Exp. Med. 167, 332-344 (1988).
Murray, P.D., McKenzie, D.T., Swain, S.L., and Kagnoff, M.F.: Interleukin 5 and interleukin 4 produced by Peyer's patch T-cells selectively enhance immunoglobulin A expression. J. Immunol. 139, 2669-2674 (1987).
Naito, Y., Okuda, K., and Takazoe, I.: Detection of specific antibody in adult human periodontitis sera to surface antigens of *Bacteroides gingivalis*. Infect. Immun. 55, 832-834 (1987).
Noelle, R., Krammer, P.H., O'hara, J., Uhr, W., and Vitetta, E.S.: Increased expression of Ia antigens on resting B cells: an additional role for B-cell growth factor. Proc. Natl. Acad. Sci. USA 81, 6149-6153 (1984).
Nysten, M.W.N., de Groot, E.R., ten Duis, H.J., Klasen, H.J., Hack, C.E., and Aarden, L.A.: Serum levels of interleukin-6 and acute phase proteins. Lancet ii, 921 (1987).
Ogawa, T., Tarkowski, A., McGhee, M.L., Moldoveanu, Z., Mestecky, J., Hirsch, H.Z., Koopman, W.J., Hamada, S., McGhee, J.R., and Kiyono, H.: Analysis of human IgG and IgA subclass antibody-secreting cells from localized chronic inflammatory tissue. J. Immunol. 142, 1150-1158 (1989a).
Ogawa, T., McGhee, M.L., Moldoveanu, Z., Hamada, S., Mestecky, J., McGhee, J.R., and Kiyono, H.: *Bacteroides*-specific IgG and IgA subclass antibody-secreting cells isolated from chronically inflamed gingival tissues. Clin. Exp. Immunol. 76, 103-110 (1989b).
Paul, W. E. and O'hara, J.: B cell stimulatory factor 1/interleukin 4. Annu. Rev. Immunol. 5, 429-459 (1987).
Rabin, E.M., O'hara, J., and Paul, W.E.: B cell stimulatory factor (BSF)-1 activates resting B cells. Proc. Natl. Acad. Sci. USA 82, 2935-2939 (1985).
Savelkoul, H.F.J., Lebman, D.A., Benner, R., and Coffman, R.L.: Increase of precursor frequency and clonal size of murine IgE-secreting cells by IL-4. J. Immunol. 141, 749-755 (1988).
Shillitoe, E.J. and Lehner, T.: Immunoglobulins and complement in crevicular fluid, serum and saliva. Arch. Oral Biol. 17, 241-247 (1972).
Smith, D.J., Gadalla, L.M., Ebersole, J.L., and Taubman, M.A.: Gingival crevicular fluid antibody to oral microorganisms. III. Association of gingival homogenete and gingival crevicular fluid antibody levels. J. Periodontal Res. 20, 357-367 (1985).
Snyderman, R. and McCarty, G.A.: Analogous mechanisms of tissue destruction in rheumatoid arthritis and periodontal disease. In: Host-parasite interactions in periodontal diseases (Eds.: Genco, R.G. and Mergenhagen, S.E.). American Society for Microbiology, Washington, D.C., 354-361 (1982).
Sonoda, E., Matsumoto, R., Hitoshi, T., Ishi, T., Sugimoto, M., Arai, S., Tominaga, A., Yamaguchi, N., and Takatsu, K.: Transforming growth factor β induces IgA production and acts additively with interleukin 5 for IgA production. J. Exp. Med. 170, 1415-1420 (1989).
Swaak, A.J.G., van Rooyen, A., and Aarden, L.A.: Interleukin 6 (IL-6) and acute phase proteins in the disease course of patients

with systemic lupus erythematosus. Rheumatol. Int. 8, 263-268 (1989).

Swain, S.L., Howard, M., Kappler, J., Marrack, P., Watson, J., Booth, R., Wetzel, G.D., and Dutton, R.W.: Evidence for two distinct classes of murine B cell growth factors with activities in different functional assays. J. Exp. Med. 158, 822-835 (1983).

van Oers, M.H.J., van der Heyden, A.A.P.A.M., and Aarden, L.A.: Interleukin 6 (IL-6) in serum and urine of renal transplant patients. Clin. Exp. Immunol. 71, 314-319 (1988).

Webb, C.F., Burrow, P.D., Borzillo, G.V., and Cooper, M.D.: Normal and abnormal generation of isotype diversity. Monogr. Allergy 20, 1- 12 (1986).

BACTERIAL TRANSLOCATION AND IMMUNITY

RODNEY D. BERG and MICHAEL D. GAUTREAUX

Department of Microbiology and Immunology,
Louisiana State University Medical Center,
Shreveport, Louisiana, USA

Lecture presented at the 2nd Old Herborn University Seminar on
"Interactions between the indigenous microflora and the host immune system"
on June 1, 1988

SUMMARY

Bacterial translocation is defined as the passage of viable bacteria from the GI tract to extra-intestinal sites, such as the MLN, spleen, liver, kidney and bloodstream. In healthy adult rodents, normal flora bacteria are not normally crossing the mucosal barrier or they are translocating at a rate low enough to be eliminated by the host defences. Translocation readily occurs, however, when the mucosal barrier is physically disrupted, bacterial overgrowth occurs in the ileum or caecum, or the host immune defences are compromised. Little is known about the exact anatomical route by which bacteria translocate from the GI tract to the MLN and other organs. Most likely, except in the case of disrupted mucosal epithelium, the bacteria translocate intracellularly by endocytosis through the epithelial cells lining the intestinal tract. Also, very little is known concerning the immune mechanisms inhibiting bacterial translocation. Preliminary experiments demonstrate that bacterial translocation readily occurs in athymic (nu/nu) mice and neonatally thymectomised mice, but is inhibited in heterozygous nu/+ mice and in thymus-grafted (nu/nu) mice. Bacterial translocation is also promoted by injection with immunosuppressive agents, such as cyclophosphamide or prednisone. On the other hand, bacterial translocation is inhibited by vaccination with formalin-killed *Propionibacterium acnes*, a non-specific immunomodulator of macrophages. It is important to determine the relative roles of mucosal immunity (especially secretory-IgA), systemic immunity (serum IgG and IgM), and cell-mediated immunity (macrophages and T-cells) in preventing bacterial translocation. By elucidating the various immune mechanisms inhibiting bacterial translocation, strategies can be devised to reduce life-threatening opportunistic infections originating from the GI tract.

INTRODUCTION

The term translocation has been used by *Keller* and *Engley* (1958) and by *Hildebrand* and *Wolochow* (1962) to describe the passage of virus particles across the GI mucosal barrier. The term translocation was used subsequently by *Wolochow* et al. (1966) to describe the passage of viable bacteria from the GI tract to the lymph and mesenteric lymph nodes (MLN) of rats, and by *Fuller* and

Jayne-Williams (1970) for the passage of bacteria from the GI tract to the liver of chickens. Consequently, we employed the term bacterial translocation to describe the phenomenon of the passage of viable normal flora bacteria from the GI tract to extra-intestinal sites, such as the MLN, spleen, liver, kidney and bloodstream (*Berg* and *Garlington*, 1979).

In the healthy adult mouse, normal flora bacteria are not usually cultured from extra-intestinal sites, such as the MLN (*Berg* and *Garlington*, 1979). The bacteria either do not translocate across the mucosal barrier or they cross the mucosa but are killed in route or *in situ* in reticulo-endothelial organs. Most likely, normal flora bacteria are continuously translocating from the GI tract at very low numbers and are killed by the host's immune defences and, therefore, are not cultured from extra-intestinal sites, such as the MLN. However, very little information is available concerning spontaneous bacterial translocation in the healthy animal. For example, even the exact anatomical route is not known by which normal flora bacteria translocate across the intestinal mucosa. *Takeuchi* (1967) observed by electron microscopy *Salmonella typhimurium* translocating intra-cellularly through GI epithelial cells in pre-starved, opium-treated guinea pigs. *Staley* et al. (1968) and *Murata* et al. (1979) also found enteropathogenic *Escherichia coli* to translocate by endocytosis through the mucosal epithelial cells rather than translocating between the epithelial cells. Thus, it is likely that the relatively non-pathogenic normal flora bacteria also translocate through mucosal epithelial cells by endocytosis (an intracellular route) rather than by interrupting the tight junctions between epithelial cells (an inter-cellular route). However, if the mucosal barrier is disrupted by injury, then bacteria can easily pass through the denuded or ulcerated areas of the mucosa. It also is not known whether normal flora bacteria translocate primarily through the mucosa of the small intestine or caecum or whether they translocate with equal efficiency at all sites in the GI tract.

Bacterial translocation from the GI tract readily occurs when: (a) the mucosal barrier is physically disrupted, (b) the host immune defences are compromised, or (c) there is bacterial overgrowth in the GI tract (reviewed in *Berg*, 1980; 1981; 1983; 1985; *Berg* and *Itoh*, 1986). Deficiencies in host defence mechanisms can act synergistically to promote bacterial translocation from the GI tract, as demonstrated by animal models with multiple alterations in host defences. For example, bacterial translocation occurs to a greater degree in mice receiving the combination of an immunosuppressive agent (cyclophosphamide or prednisone) plus an oral antibiotic (penicillin or clindamycin) to cause bacterial overgrowth, than in mice receiving only the immunosuppressive agent or only the oral antibiotic (*Berg* et al., 1988). Thus, an immunosuppressive agent plus an oral antibiotic acts synergistically to promote translocation and subsequent lethal sepsis by the normal flora bacteria. Other animal models exhibiting multiple alterations in the defence against bacterial translocation from the GI tract include streptozotocin-induced diabetes (*Berg* 1985), endotoxaemia (*Deitch* and *Berg* 1987), thermal injury (*Maejima* et al., 1984), and haemorrhagic shock (*Baker* et al., 1988).

The pathogenesis of bacterial translocation from the GI tract appears to occur in several discrete stages (Figure 1). In the healthy animal, spontaneous bacterial translocation is likely occurring continuously at a very low rate but these low numbers of translocating bacteria are killed by the host immune defences.

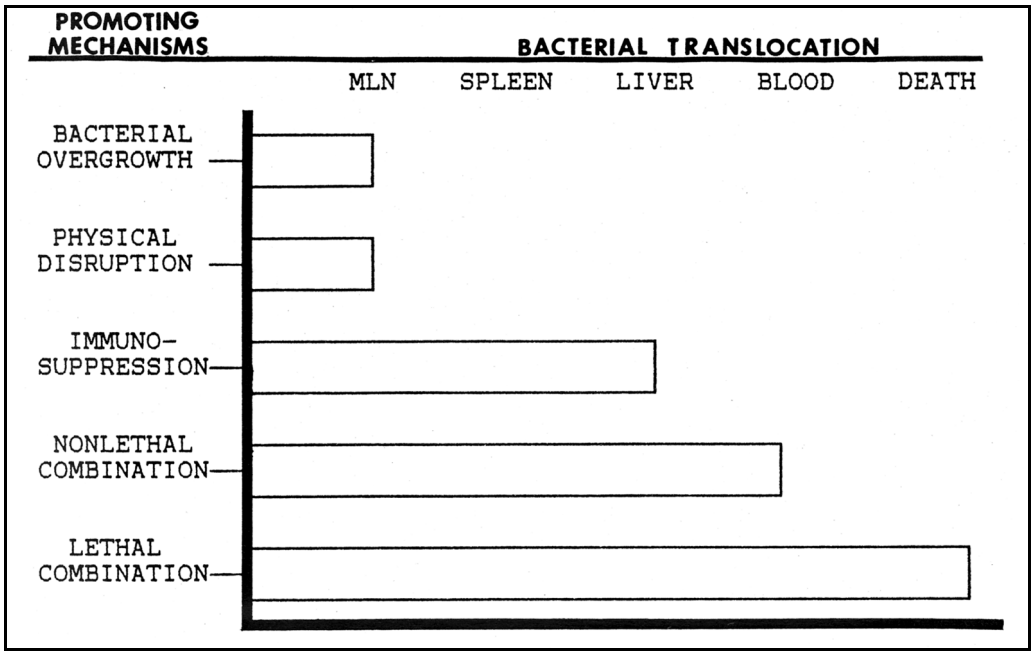

Figure 1: Mechanisms promoting bacterial translocation to extra-intestinal sites.

The administration of an oral antibiotic, however, disrupts the ecologic equilibrium in the GI tract to allow intestinal overgrowth by certain bacteria and the concomitant translocation of these bacteria from the GI tract (*Berg*, 1981). Although these bacteria readily translocate from the GI tract to the MLN in this model, they rarely spread from the MLN to other organs and sites because of the intact host immune defences. Within one day after the oral antibiotic is discontinued, translocating bacteria are eliminated from the MLN (*Berg* and *Owens*, 1979). Thus, the translocating bacteria do not multiply in the MLN but a persistent infection of the MLN is maintained by continuously translocating bacteria.

Immunosuppression promotes the next stage of bacterial translocation. For example, after the injection of an immunosuppressive agent, the host can no longer confine translocating bacteria only to the MLN. The translocating bacteria spread systemically from the MLN to the spleen, liver, kidney and even the bloodstream (*Berg*, 1983). Depending on the degree of immunosuppression and the pathogenic properties of the translocating bacteria, the host may control the infection or the infection may proceed to lethal sepsis. Thus, multiple alterations in host defences can lead to bacterial translocation and lethal sepsis by opportunistic normal flora bacteria residing in the GI tract or by recently acquired exogenous pathogenic bacteria. The host immune defences appear critical in determining whether the translocating bacteria will establish a temporary local infection of the MLN or spread systemically to cause lethal sepsis.

DISRUPTION OF PHYSICAL AND MECHANICAL BARRIER

One of the most important defences against bacterial translocation from the GI tract is the physical barrier of the GI mucosa. Increased intestinal permeability promotes the pathogenesis of various inflammatory as well as infectious diseases. *Schweinburg* et al. (1950) reported that *E. coli* can translocate from the GI tract in dogs injected intra-peritoneally with 5% mono-ethanolamine oleate to induce peritonitis and increased intestinal permeability. Irradiation also promotes bacterial translocation by damaging the architecture of the GI mucosa (*Gordon* et al., 1955). Bacterial translocation also appears to occur to a greater degree in neonates prior to intestinal closure than in mature animals (*Fuller* and *Jayne-Williams*, 1970).

We have found that ricinoleic acid, (12-hydroxy-9-octadecenoic acid) the pharmacologically active constituent of castor oil, given orally to mice severely damages the intestinal mucosa to allow bacterial translocation from the GI tract (*Morehouse* et al., 1986). A single dose of ricinoleic acid administered intragastrically to mice produces significant alterations in the mucosa of the proximal small intestine. Two hours after administration, the duodenal villi are shortened with massive exfoliation of columnar and goblet cells resulting in continuity between the intestinal lumen and the lamina propria. Because of this loss of the mucosal barrier, both strictly anaerobic and facultatively anaerobic bacteria of the normal flora translocate from the GI tract to the MLN, spleen, and liver. The peak incidence of bacterial translocation occurs 4 days after the ricinoleic acid treatment. The mucosa begins to regenerate by 4 hours after a single dose of ricinoleic acid and bacterial translocation ceases by 7 days following treatment.

We have examined other animal models in which mucosal injury appears to be particularly important in promoting bacterial translocation. For example, bacterial translocation readily occurs after the intra-peritoneal injection of endotoxin (*Deitch* et al., 1987). The ileal and caecal mucosa appear relatively intact, although there are sporadic areas where the lymphatic lacteals of the lamina propria are congested and the mucosa is exfoliated. It has been demonstrated previously that endotoxin treatment disrupts the intercellular tight junctions between intestinal epithelial cells (*Walker* and *Porvaznik*, 1978). Consequently, endotoxin may increase GI mucosal permeability due to the local action of humoral mediators acting within the gut wall.

Protein malnutrition also produces histologic atrophy of the small bowel and caecal mucosa, but the epithelial barrier remains intact and bacterial translocation does not occur (*Deitch* et al., 1987). However, the combination of protein malnutrition plus endotoxin injection produces a spectrum of histologic changes ranging from areas of moderate villus oedema to areas of ulceration and increased bacterial translocation. Similarly, physical damage to the mucosal barrier is important in the synergistic promotion of bacterial translocation after a 30% total body surface area burn and endotoxin injection (*Deitch* and *Berg*, 1987).

Haemorrhagic shock of rats for 90 minutes also produces necrosis of the ileal mucosa and subsequent bacterial translocation from the GI tract (*Baker* et al., 1988). Allopurinol, a competitive inhibitor of xanthine oxidase, administered orally prior to haemorrhagic shock reduces the mucosal damage and bacterial translocation (*Deitch* et al., 1988). Rats fed a tungsten-supplemented molybdenum-free diet to inactivate xanthine

oxidase prior to haemorrhagic shock exhibit reduced mucosal damage and bacterial translocation compared to controls fed a regular diet. Thus, bacterial translocation occurring after haemorrhagic shock appears to be due to mucosal damage mediated by oxidants generated by activation of the xanthine oxidase system. Oxygen-free radicals generated during the period of intestinal reperfusion are particularly important in the mucosal damage and associated increase in bacterial translocation. However, other factors are probably also involved, such as ischaemia-induced tissue hypoxia, when gut hypofusion persists for long periods.

These experimental animal models demonstrate conclusively that the intestinal mucosa provides a very important physical and mechanical defence against bacterial translocation, especially in conditions where there are multiple alterations in host defences. Thus, patients suffering from severe trauma with associated haemorrhagic shock or thermally injured patients may be particularly susceptible to infections originating from the GI tract due to a breakdown of the physical mucosal barrier.

SYSTEMIC IMMUNITY

Serum immunoglobulins

Serum immunoglobulins serve as opsonising antibodies to facilitate phagocytosis and clearing of bacteria from the serum and tissues. Consequently, it is likely that serum immunoglobulins also facilitate the clearing of translocating bacteria from reticulo-endothelial organs, such as the MLN.

There is abundant evidence from the literature that the passive transfer of serum antibodies protects against subcutaneous or intra-peritoneal challenge with bacteria or parasites. *Kierszenbaum* (1980) protected athymic mice from *Trypansoma crusi* infection by adoptively transferring immune sera from vaccinated mice. *Tsay* and *Collins* (1984) demonstrated passive protection with anti-*Pseudomonas aeruginosa* polysaccharide IgG in burned and normal mice challenged with *P. aeruginosa*. *Cryz* et al. (1983a), using a murine burn wound sepsis model, passively transferred protection against *P. aeruginosa* PA220 by intravenously injecting homologous anti-*Pseudomonas* lipopolysaccharide IgG. *Cryz* et al. (1983b) also protected mice rendered leukopenic by cyclosphosphamide from *P. aeruginosa* challenge by adoptively transferring serotype specific anti-LPS IgG isolated from rabbit hyper-immune sera.

Serum antibodies are also found to be effective opsonins on mucosal surfaces. *Cooper* and *Rowley* (1979) reported that pre-opsonisation of bacteria with serum antibodies enhances the clearance of these bacteria from the lungs and peritoneum by macrophages. Interestingly, they found that pre-opsonisation with secretory-IgA (s-IgA) may actually delay clearance from the lungs because the Fc portion of s-IgA is incapable of binding to Fc receptors on the macrophages.

Serum immunity has also been demonstrated to affect adversely a protective mucosal immune response. *Pierce* (1980) showed that specific serum antibody to cholera toxin can actually suppress the intestinal immune response to cholera toxin in rats. Suppression is due largely to a direct effect of hyper-immune serum antibody on the interaction of absorbed enteric antigen with lymphoid tissue in Peyer's patches and possibly in the MLN. This effect occurred after the passive transfer of hyperimmune serum from immunised do-

nor rats to normal recipients.

Mucosal immunity, by inhibiting the association or adherence of bacteria to the intestinal epithelium, may be important in the defence against bacterial translocation. However, the systemic immune system must also be important in clearing bacteria that have already translocated across the mucosa. To date, protection against bacterial translocation systemic immunity (by either serum IgM and IgG) or mucosal immunity (s-IgA or s-IgM) has not been tested. Thus, an important area of future research is the role of systemic immunity (serum IgM or IgG) in clearing translocating bacteria that have crossed the intestinal epithelial barrier and to what extent systemic immunity might interfere with the mucosal immune system.

Secretory immunoglobulins

The daily production of s-IgA exceeds that of all other immunoglobulins combined (*Mestecky* et al., 1986). Consequently, s-IgA is important in protecting the mucosal surfaces of the digestive, respiratory, and genito-urinary tracts against potential pathogens. Secretory IgA binds antigen but normally does not fix complement by the classical pathway, does not function as an opsonin, and does not directly kill microorganisms. However, s-IgA exhibits several other important direct and indirect effector functions. The direct functions are inhibition of microbial adherence and colonisation (*Abraham* and *Beachey*, 1985), toxin and enzyme neutralisation (*Brown*,1986; *Holmgren* et al., 1972; *Russell-Jones* et al., 1981; *Ogra* et al., 1984), virus neutralisation (*Spikes* et al., 1975), and inhibition of antigen absorption (*Cummingham-Rundles* et al., 1978). The indirect effector functions include interactions with innate humoral defence factors (*Kilian* et al., 1988) and the ability to potentiate the effect of certain non-specific antibacterial factors such as lactoferrin and lactoperoxidase (*Arnold* et al., 1984; *Funashok* et al., 1982; *Tenovuo* et al., 1982). It appears that s-IgA can activate the alternate pathway of complement activation; but in such a manner that C3b does not become covalently bound nor is C5a released (*Hiemstra* et al., 1987; *Cooper*, 1987).

Appreciation and understanding of the immunological importance of s-IgA has been hampered by the seemingly normal status of IgA-deficient individuals. However, complete absence of s-IgA is rare and s-IgM may compensate for the lack of s-IgA. Even with s-IgM compensating for a lack of s-IgA, increased incidences of a number of pathologic conditions, such as respiratory tract infections and atopic and autoimmune diseases, have been associated with selective IgA deficiency (*Arman* and *Hong*, 1980). Intestinal absorption of intact proteins also appears to occur more readily in patients lacking s-IgA since patients with selective IgA deficiencies have a much higher incidence than normal individuals of antibodies to certain food antigens, such as milk antigens (*Walker* et al., 1972).

Since the primary function of secretory immunoglobulins, especially s-IgA, appears to be the inhibition of the adherence and absorption of certain bacteria and particulate antigens, it seems likely that s-IgA also will reduce bacterial translocation from the GI tract. Secretory IgA inhibits the adherence of certain pathogenic bacteria, such as *Vibrio cholerae* to intestinal epithelium and *Streptococcus mutans* to tooth surfaces. Secretory IgA has not, however, been shown to prevent the close association of normal flora bacteria with the intestinal mucosa.

To date, there have not been any studies focusing on the role of s-IgA in

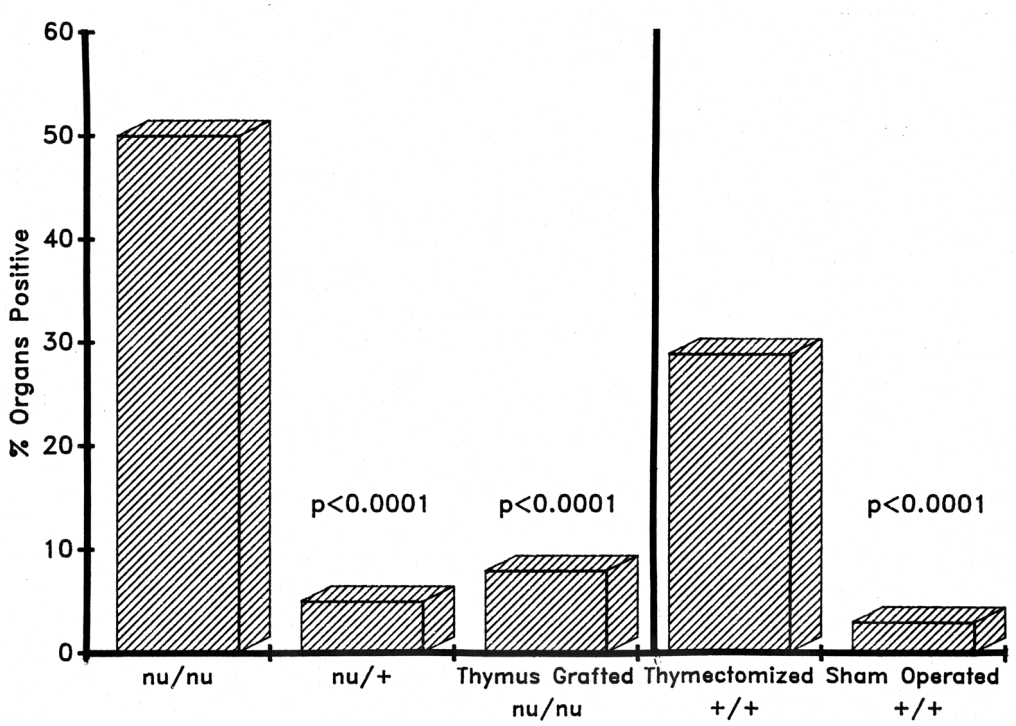

Figure 2: Inhibition of bacterial translocation by T-cell mediated immunity.
Percent positive organs represent percent of total MLNs, spleens, livers, and kidneys positive for translocating bacteria. Nu/nu percentages are compared to nu/+ ($p<.0001$) and thymus-grafted nu/nu percentages ($p<.0001$); thymectomised +/+ percentages compared to sham-operated +/+ percentages by Chi-square analysis.

the prevention of bacterial translocation from the GI tract. The bacterial translocation model seems particularly useful for studying the function of s-IgA, however, since bacteria certainly must associate closely with the intestinal epithelium prior to their translocation across the epithelial barrier.

T-CELL MEDIATED IMMUNITY

Congenitally athymic (nu/nu) mice provide a unique model for determining whether T-cell mediated immunity plays a role in preventing bacterial translocation from the GI tract. Nu/nu mice lack T-cell mediated immunity because their thymus fails to develop to produce functional T-lymphocytes (*Pantelouris*, 1971).

Athymic (nu/nu) and heterozygous (nu/+) mice were tested for translocating bacteria cultured both aerobically and anaerobically (*Owens* and *Berg*, 1980). Fifty percent (50/100) of the MLN, spleen, liver, and kidney from athymic (nu/nu) mice contain viable bacteria compared to only 5% (5/96) of the same organs from nu/+ mice (Figure 2). While the incidences of translocating bacteria found in the MLN of nu/nu versus nu/+ mice are not statistically significant, significant differences in the

translocation incidences to the spleen, liver, and kidney are present between nu/nu and nu/+ mice.

Since congenitally athymic (nu/nu) mice might possess other unrecognised abnormalities in addition to the athymic condition that could influence bacterial translocation, thymectomised mice also were tested to add strength to the role of T-cell mediated immunity in preventing bacterial translocation (*Owens* and *Berg*, 1982). After tests demonstrated the depletion of T-cell immunologic functions in the thymectomised mice, it was determined that thymectomised mice exhibit higher incidences of bacterial translocation to the MLN, spleen, liver, and kidney than sham-thymectomised controls (29% vs. 3%) (Figure 2).

Once athymic (nu/nu) and thymectomised mice were found to exhibit greater incidences of bacterial translocation than euthymic mice (nu/+ or +/+), the definitive test for determining the role of T-cell dependent immunity was to graft thymuses from donor nu/+ mice to recipient nu/nu mice. Nu/nu mice, 21 days old, were grafted with thymuses from neonatal donor mice, 1-2 days old. Four weeks after receiving the thymic grafts, the nu/nu grafted mice were tested for T-cell dependent immune responsiveness against T-cell dependent sheep erythrocyte antigens. The grafted nu/nu mice responded similarly to heterozygous nu/+ mice with increases in specific serum haemagglutinins after the sheep erythrocyte vaccine. Histologic examination of recovered thymus grafts after sacrifice also revealed normal thymus architecture with well-defined cortex and medulla regions. At 8 weeks of age, the incidence of bacterial translocation to the MLN, spleen, liver, and kidney of thymus-grafted nu/nu mice was 8% (5/64), similar to the 5% incidence (5/96) exhibited by nu/+ mice (Figure 2).

Maddus et al. (1988) in a similar study did not detect increased bacterial translocation in athymic mice compared to heterozygotes. However, the translocation assay they employed required at least ten translocating bacteria to produce a positive culture result, whereas our translocation assay requires only one viable bacterium. Also, the strain of mice they used most likely harboured a different GI microflora than the mouse strain we employed. *Cantrell* and *Jutila* (1970) found bacteria, that presumably translocated from the GI tract, in the liver, spleen and blood of thymectomised BALB/c mice that also received injections of rabbit anti-mouse thymocyte sera. *Deitch* et al. (1986) also detected increased bacterial translocation in athymic (nu/nu) compared to euthymic (nu/+) mice. *Penn* et al. (unpublished observations) also found increased *E. coli* C25 translocation to the MLN, spleen and liver of BALB/c athymic mice mono-associated with *E. coli* C25 compared with *E. coli* C25 mono-associated heterozygotes. Thus, our results have been confirmed by others and suggest that T-cell mediated immunity contributes to the host defence against bacterial translocation and, particularly, against the spread of translocating bacteria from the MLN to other sites, such as the spleen and liver.

We also have tested whether T-cell mediated immunity plays a role in preventing the bacterial translocation that occurs after thermal injury (*Deitch* et al., 1986). Athymic (nu/nu), heterozygous (nu/+), and wild type (+/+) mice, with or without 30% total body surface area burns, were tested for the translocation of normal flora bacteria on post-burn days 1, 2 and 4. No translocating bacteria were cultured from the MLN, spleen, liver, peritoneal cavity, or blood of unburned or burned euthymic mice (+/+ or nu/+). In contrast, athymic mice (nu/nu) exhibited a low level of sponta-

neous bacterial translocation, even in the absence of thermal injury, similar to our previous results described above. By the 2nd post-burn day, the numbers of viable bacteria per gram tissue were tenfold higher in the spleens and almost 150-fold higher in the livers of burned athymic mice compared with the unburned controls. These translocating bacteria were identified as *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Staphylococcus epidermidis*, and *Streptococcus faecalis*. Bacterial overgrowth was not responsible for the increased translocation from the GI tract since the population levels of these bacteria in the ilea and caeca were not increased by thermal injury.

Thus, T-cell dependent immunity appear to be an important host defence mechanism inhibiting bacterial translocation from the GI tract, and may be even more important in preventing the systemic spread of translocating bacteria. Future research will focus on identifying the T-cell subpopulations and the specific immune mechanisms whereby these T-cells inhibit bacterial translocation.

MACROPHAGES

We have examined three non-specific immunomodulators, all activators of macrophages, for their abilities to inhibit bacterial translocation from the GI tract to the MLN in specific-pathogen free (SPF) mice antibiotic-decontaminated and subsequently mono-associated with *E. coli* C25. The three immunomodulators are muramyl dipeptide (MDP), glucan, and formalin-killed *Proprionibacterium acnes*.

MDP, a small molecular weight glycopeptide, is the minimal structure responsible for the adjuvant action of *Mycobacterium*. MDP is reported to activate macrophages directly (*Nagad* et al., 1979; *Takeda* et al., 1979; *Pabst* and *Johnson*, 1980). However, we found that MDP injected intra-peritoneally does not inhibit *E. coli* C25 translocation to the MLN in the mono-associated SPF mouse model. Since MDP did not inhibit translocation in our initial experiments, and because MDP is reported to be of limited usefulness in humans due to a short half-life in the bloodstream, we have not continued these studies with MDP.

Glucan, a polyglycan isolated from the inner cell wall of the yeast *Saccharomyces cerevisae*, is associated with enhancement of both humoral and cell-mediated immunity (*Wooles* and *DiLuzio*, 1962; 1963). In addition, glucan increases the activation and proliferation of macrophages. *Kimura* et al. (1983) reported that the antibacterial activity of glucan is primarily due to enhancement of bacterial digestion by macrophages.

We found that particulate glucan injected intra-peritoneally is ineffective in reducing the translocation of *E. coli* C25 from the GI tract to the MLN of mono-associated SPF mice. The glucan vaccine induces an immune response since splenomegaly occurs in the glucan-treated mice, demonstrating a lympho-reticular response, in agreement with other reports (*Burgaleta* and *Golde*, 1977; *Patchen* and *McVittie*, 1983). *Joyce* et al. (1978) found that the major effect of intra-peritoneally injected glucan is exerted in the peritoneal cavity. This conclusion is also supported by our results in which glucan vaccine injected intra-peritoneally reduces the mortality following intra-peritoneal challenge with 10^{10} viable *E. coli* C25. Interestingly, the particulate

glucan vaccine reduces mortality due to bacterial peritonitis induced by intraperitoneal challenge with 10^{10} *E. coli* but does not inhibit the low numbers of *E. coli* translocating from the GI tract to the MLN.

The third immunomodulator tested was formalin-killed *P. acnes* (formerly called *Corynebacterium parvum*, but reclassified). Vaccination with killed *P. acnes* exerts many effects on the immune system, the most important being the non-specific activation of macrophages (*Herbert* et al., 1983). *P. acnes* vaccination increases host resistance to a variety of pathogenic bacteria, including *Salmonella enteriditis* (*Collins* and *Scott*, 1974), *Salmonella typhimurium* (*Briles* et al., 1981), *Listeria monocytogenes* (*Miyata* et al., 1980) and *Staphylococcus aureus* (*Stinnett* et al., 1979).

P. acnes vaccination reduces significantly *E. coli* C25 translocation to the MLN in antibiotic-decontaminated SPF mice mono-associated with *E. coli* C25 (*Fuller* and *Berg*, 1985). The incidence of *E. coli* C25 translocation to the MLN decreased from 75% (50/67) in control non-vaccinated mice to 41% (28/68) in *P. acnes* vaccinated mice ($p = .002$). The mean numbers of translocating *E. coli* C25 per gram MLN also decreased from 1900 to 300 ($p = .009$). The caecal population levels of *E. coli* C25 were not altered by the *P. acnes* vaccine.

These experiments were repeated with indigenous *E. coli*, *Proteus mirabilis*, and *Enterobacter cloacae* with similar results, i.e. *P. acnes* vaccination decreased translocation of these bacteria to the MLN. Furthermore, the translocation of *E. coli* or *E. cloacae* was decreased even when the *P. acnes* vaccine was given to mice in whom these bacteria were already in the process of translocating to the MLN.

The studies above demonstrate that non-specifically stimulated macrophages can inhibit bacterial translocation. In contrast, it has been suggested that macrophages actually may be helpful in the translocation of bacteria and particles from the GI tract. *Joel* et al. (1978) found that macrophages containing carbon particles after long term oral exposure (2 months) were frequently seen in the sub-epithelial region of the Peyer's patch, in the intestinal lymphatics, and the sub-capsular sinus of the lymph node, suggesting that macrophages can carry the particles from the GI tract to the lymphatics. *Harmsen* et al. (1985) using red or green fluorescent microspheres also demonstrated that particles can be carried to the tracheo-bronchial lymph nodes by lung macrophages. Since the lung macrophages contained either all red or all green microspheres, the microspheres did not travel to the lymph nodes to be phagocytised but instead were engulfed by lung macrophages and transported to the nodes. *Wells* et al. (1987) also presented preliminary evidence that macrophages may play a role in the translocation of bacteria and particles from the GI tract. They noted lower translocation rates in macrophage defective, endotoxin-resistant C3H/HeJ mice than in endotoxin-sensitive C3H/HeN mice. They also attempted experiments similar to that of *Harmsen* et al. (1985) described above except that red or green fluorescent microspheres were injected into ligated intestinal loops rather than inoculated in individual lobes of the lungs. However, since the ligated intestinal loop is subjected to internal pressure, it is not known if the transport of engulfed particles by macrophages in this model represents the normal situation in the GI tract. Furthermore, unlike the lung, the GI tract is designed for absorption and, therefore, transport of bacteria by macrophages may not play a significant role in bacterial translocation from the GI tract.

Our results suggest that the macrophage may be an important effector cell for the immunologic prevention of bacterial translocation from the GI tract since translocation is inhibited by vaccination with killed *P. acnes*, a non-specific stimulator of macrophage function. However, adoptive transfer of *P. acnes* stimulated macrophages to non-vaccinated recipient mice to prevent bacterial translocation would considerably strengthen this hypothesis.

CONCLUSION

In healthy adult rodents, normal flora bacteria are not usually cultured from extra-intestinal sites, such as the MLN, spleen, liver, or bloodstream. Either the bacteria are not crossing the mucosal barrier or they are translocating at such low numbers that they are eliminated by the host immune defences. Certain normal flora bacteria readily translocate, however, when the mucosal barrier is physically disrupted, when bacterial overgrowth occurs in the ileum and caecum, or when the host immune defences are compromised. The host might not suffer any ill effects from the translocating bacteria depending on the extent of mucosal injury, the extent of immunosuppression and on the pathogenic properties of the translocating bacteria. However, under certain circumstances the translocating bacteria spread rapidly from the MLN to infect other sites to cause fatal sepsis.

Little is known concerning the exact anatomical route by which normal flora bacteria translocate from the GI tract to the MLN and other organs. Most likely these bacteria translocate intracellularly through the epithelial cells lining the intestinal mucosa by a process of endocytosis and then exocytosis into the lamina propria. The fate of the translocating bacteria in the lamina propria is not known. For example, it is not known whether the translocating bacteria are carried free in the lymph to the MLN or whether macrophages engulf and transport the translocating bacteria to the MLN. Neither is it known if translocating bacteria are killed by the host immune defences in route through the mucosa or are cleared primarily in the MLN and other reticulo-endothelial organs. More information is required concerning the translocation route if we are to understand the initial events in the pathogenesis of bacterial translocation from the GI tract.

This paper describes experimental animal studies suggesting that T-cell mediated immunity and macrophages are important in the host defence against bacterial translocation. However, it has not yet been demonstrated that certain populations of T-cells can inhibit bacterial translocation nor have the specific inhibitory mechanism of T-cells been identified. Vaccination with the non-specific immunomodulator, killed *P. acnes*, inhibits bacterial translocation from the GI tract to the MLN presumably by activating fixed macrophages. However, the macrophage has not, as yet, been conclusively demonstrated to be the effector cell responsible for reducing translocation in this model.

Secretory immunoglobulins have not been tested as to their supposed abilities of reducing bacterial translocation by inhibiting the adherence or close association of normal flora bacteria with the intestinal mucosa. Neither have serum immunoglobulins been tested as to their effectiveness in clearing translocating bacteria from the lamina propria, MLN, spleen, liver, or even bloodstream. Consequently, it is of interest to determine the relative roles of mucosal im-

munity (especially s-IgA), systemic immunity (serum IgG and IgM), in preventing bacterial translocation and cell-mediated immunity (macrophages and/or T-cells).

The GI tract is undoubtedly a reservoir for opportunistic bacterial infections in compromised patients, such as those with AIDS, leukaemia, and haemorrhagic shock, and those suffering from severe trauma. Elucidation of the immune mechanisms inhibiting bacterial translocation would provide opportunities for devising strategies to reduce these life-threatening opportunistic GI infections.

LITERATURE

Abraham, S.N. and Beachey, E.H.: Host defenses against adhesion of bacteria to mucosal surfacee. In: Advances in Host Defense Mechanisms Vol. 4 (Eds.: Gallin, J.F. and Fauci, A.S.). Raven Press, New York, 63-88 (1985).

Amman, A.J. and Hong, R.: Disorders of the IgA system, In: Immunologic disorders in infants and children (Eds.: Stiehmn, E.R. and Tulginti, V.A.). The W.B. Saunders Co., Philadelphia, 260-273 (1980).

Arnold, R.R., Russell, J.E., Devine, S.M., Adamson, M., and Pruitt, K.M.: Antimicrobial activity of the secretory innate defense factors lactoferrin, lactoperoxidase and lysozyme. In: Cardiology (Ed.: Guggenheim, B.). S. Karger, Basel, 75-88 (1984).

Baker, J.W., Deitch, E.A., Berg, R.D., and Specian, R.D.: Hemorrhagic shock induces bacterial translocation from the gut. J. Trauma 28, 896-906 (1988).

Berg, R.D.: Mechanisms confining indigenous bacteria to the gastrointestinal tract. Amer. J. Clin. Nutr. 33, 2472-2484 (1980).

Berg, R.D.: The translocation of normal flora bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs. Microecol. Ther. 11, 27-34 (1981).

Berg, R.D.: Factors influencing the translocation of bacteria from the gastrointestinal tract. In: Recent Advances in Germfree Research (Eds.: Sasaki, S., Ogawa, A., and Hashimoto, K.). Tokai University Press, Tokyo, 411-418 (1981).

Berg, R.D. and Itoh, K.: Bacterial translocation from the gastrointestinal tract. Immunologic aspects. Microecol. Ther. 16, 131-145 (1986).

Briles, D.E., Lehmeyer, J., and Forman, C.: Phagocytosis and killing of *Salmonella typhimurium* by peritoneal exudate cells. Infect. Immun. 33, 380-388 (1981).

Brown, W.R.: Immunology of the gastrointestinal tract. In: The Gastroenterology Annual, vol 3, (Eds.: Kern, F., Jr. and Blum, A.L.). Elsevier/North Holland Publishing Co., Amsterdam, 284-316 (1986).

Burgaleta, C., and Golde, D.L.: Effect of glucan on granulopoiesis and macrophage genesis in mice. Cancer Res. 37, 1739-1742 (1977).

Collins, F.M. and Scott, M.J.: Effects of *Corynebacterium parvum* treatment on the growth of *Salmonella enteritidis* in mice. Infect. Immun. 9, 863-869 (1974).

Cooper, J.M. and Rowley, D.: Clearance of bacteria from lungs of mice after opsonizing with IgG or IgA. Aust. J. Exp. Biol. Med. 57, 279-285 (1979).

Cooper, N.R.: The complement system. In: Basic and Clinical Immunology, 6th ed. (Eds.: Stites, D.P., Solo, J.D., and Wells, J.V.). Lange Medical Publishers, Los Alto, 114-127 (1987).

Cryz, S.J., Furer, E., and Germainer, R.: Protection against *Pseudomonas aeruginosa* infection in a murine burn wound sepsis model by passive transfer of antitoxin A, antielastase, and antilipopolysaccharide. Infect. Immun. 39, 1072-1079 (1983a).

Cryz, S.J., Furer, E., and Germainer, R.: Passive protection against *Pseudomonas aeruginosa* infection in an experimental leukopenic mouse model. Infect. Immun. 40, 659-664 (1983b).

Cunningham-Rundles, C., Brandeis, W.E., Good, R.A., and Day, N.K.: Milk precipitins, circulating immune complexes and IgA deficiency. Proc. Natl. Acad. Sci. USA 75, 3387-3389 (1978).

Deitch, E.A. and Berg, R.D.: Endotoxin but not malnutrition promotes bacterial translo-

cation of the gut flora in burned mice. J. Trauma 27, 161-166 (1987).

Deitch, E.A., Berg, R., and Specian, R.: Endotoxin promotes the translocation of bacteria from the gut. Arch Surg. 122, 185-190 (1987).

Deitch, E.A., Bridges, W., Baker, J., Ma, J.W., Ma, L., Grisham, M.B., Granger, N., Specian, R.D., and Berg, R.D.: Hemorrhagic shock-induced bacterial translocation is reduced by xanthine oxidase inhibition or inactivation. Surgery 104, 191-198 (1988).

Fuller, K.G. and Berg, R.D.: Inhibition of bacterial translocation from the gastrointestinal tract by nonspecific immunostimulation. In: Germfree Research: Microflora Control and its Application to the Biomedical Sciwnces (Eds.: Wostmann, B.S., Pleasants, J.R., Pollard, M., Teah, B.A., and Wagner, M.). Alan R. Liss, Inc., New York, 195-198 (1985).

Fuller, R. and Jayne-Williams, D.J.: Resistance of the fowl (*Gallus domesticus*) to invasion by its intestinal flora. II. Clearance of translocated intestinal bacteria. Res. Vet. Sci. 11, 368-374 (1970).

Funashoki, S., Doi, T., Nakajima, T., Suyama, T., and Tokuda, M.: Antimicrobial effect of human serum IgA. Microbiol. Immunol. 26, 227-239 (1982).

Gordon, L.E., Ruml, D., Hahne, H.J., and Miller, C.P.: Studies on susceptibility to infection following ionizing radiation. IV. The pathogenesis of the endogenous bacteremias of mice. J. Exp. Med. 102, 413-424 (1955).

Gordon, L.E., Ruml, D., hahne, H.J., and Miller, C.P.: Studies on susceptibility to infection following ionizing radiation. IV. The pathogenesis of the endogenous bacteremias of mice. J. Exp. Med. 102, 413-424 (1955).

Herbert, J.C., Camelli, R.L., Foster, R.J., Jr., Chalmer, B.J., and Davis, J.H.: Improved survival after *Pneumococcus* in splenectomized and nonsplenectomized mice with *Corynebacterium parvum*. Arch. Surg. 118, 328-332 (1983).

Hiemstra, P.S., Gorter, A., Stuurman, M.E., van Es, L.A., and Daha, M.R.: Activation of the alternate pahtway of complement by human serum IgA. Eur. J. Immunol. 17, 321-326 (1987).

Holmgren, J., anderson, A., Wallenstrom, G., and Ouchterlony, 0.: Experimental studies on cholera immunization. II. Evidence for protective antitoxic immunity mediated by serum antibodies as well as local antibodies. Infect. Immun. 5, 662-667 (1972).

Joel, D.D., Laissue, J.A., and LeFevre, M.E.: Distribution and fate of ingested carbon particles. RES J. Reticuloendothelial Soc. 24, 477-487 (1978).

Joyce, L.D., Han, T., Hoffman, R., Simmons, L., and Lillehei, R.C.: Evaluation of the mechanism of zymosan induced resistance to experimental peritonitis. Surgery 83, 717-725 (1978).

Kierszenbaum, F.: Protection of congenitally athymic mice against *Trypanosoma cruzi* infection by passive antibody transfer. J. Parasitol. 66, 673-675 (1980).

Kilian, M., Mestecky, J., and Russell, M.W.: Defense mechanisms involving Fc-dependent functions of immunoglobulin A and their subversion by bacterial immunoglobulin A proteases. Microbiol. Rev. 52, 296-303 (1988).

Kimura, A., Sherwood, R.L., and Goldstein, E.: Glucan alteration of pulmonary antibacterial disease. RES J. Reticuloendothelial Soc. 34, 1-11 (1983).

Mestecky, J., Russell, M.W., Jackson, S., and Brown, T.A.: The human IgA system: A reassesment. Clin. Immunol. Immunopathol. 40, 105-114 (1986).

Miyata, H., Nomoto, and Takeya, K.: Characteristics of resistance to *Listeria monocytogens* enhanced by *Corynebacterium parvum* in mice. Immunology 40, 105-115 (1986).

Morehouse, J.L., Specian, R.D., Stewart, J.J., and Berg, R.D.: Translocation of indigenous bacteria from the gastrointesitnal tract of mice after oral ricinoleic acid treatment. Gastroenterology 91, 673-682 (1986).

Murata, H., Yaguchi, H., and Namioka, S.: Relationship between the intestinal permeability to macromolecules and invasion of septicemia-inducing *Escherichia coli* in neonatal piglets. Infect. Immun. 26, 339-347 (1979).

Nagoa, S. Tanaka, A., Yamamoto, Y., Koga, T., Onoue, K., Shiba, T., Kusumoto, K., and Kotani, S.: Inhibition of macrophage migration by muramyl dipeptides. Infect. Immun. 24, 308-312 (1979).

Ogra, P.L., Cumella, J.C., and Welliver, R.C.: Immune responses to viruses. In:

Immunology of the lung and upper respiratory tract (Ed.: Bienenstock, J.). McGraw-Hill, New York, 242-263 (1984).

Pabst, M.J., Hedegaard, H.B., and Johnston, R.B.: Altered human monocytes require exposure to bacterial products to maintain an optimal oxygen radical response. J. Immunol. 128, 123-128 (1982).

Patchen, M.L. and MacVittie, T.J.: Dose-dependent responses of murine pluripotent stem cells and myeloid and erythroid progenitor cells following administration of the immunomodulating agent glucan. Immunopharmacology 5, 303-313 (1983).

Pierce, N.F.: Suppression of the intestinal immune response to cholera toxin by specific serum antibody. Infect. Immun. 30, 62-68 (1980).

Russell-Jones, G.J., Ey, P.L., and Reynolds, B.L.: Inhibition of cutaneous anaphylaxis and arthus reaction in the mouse by antigen-specific IgA. Int. Arch. Allergy Appl. Immunol. 66, 313-325 (1981).

Schweinburg, F.B., Seligman, A.M., and Fine, F.: Transmural migration of intestinal bacteria. A study based on the use of radioactive *Escherichia coli*. New Eng. J. Med. 242, 747-751 (1950).

Staley, T.E., Jones, E.W., and Corley, L.D.: Attachment and penetration of *E. coli* into epithelium of the ileum in newborn pigs. Amer. J. Pathol. 56, 371-392 (1969).

Stinnett, J.D., Morris, M.J., and Alexander, J.W.: Macrophage activation and increased resistance to infection in immunosuppressed mice treated with *Corynebacterium parvum* vaccine or pyran copolymer. RES. J. Reticuloendothel. Soc. 25, 525-531 (1979).

Stokes, C.R., Soothill, J.F., and Turner, M.W.: Immune exclusion is a function of IgA. Nature 255, 745-746 (1975).

Takeda, H., Tsujimoto, M., Kato, K., Kotani, S., Kusumoto, S., Inage, M., Shiba, T., Yano, J., Kawata, S., and Yokogawa, K.: Macrophage activation by bacterial cell walls and related synthetic compounds. Infect. Immun. 25, 48-53 (1979).

Tenovuo, J., Moldovenanu, Z., Mestecky, J., Pruitt, K.M., and Mansson-Rahemtulla, B.: Interaction of specific and innate factors of immunity: IgA enhances the effect of the lactoperoxidase system against *Streptococcus mutans*. J. Immunol. 128, 726-731.

Tsay, G.C. and Collins, M.S.: Preparation and characterization of a nontoxic polysaccharide-protein conjugate that induces active immunity and passively protective antibody against *Pseudomonas aeruginosa* immunotype 1 in mice. Infect. Immun. 45, 217-221 (1984).

Walker, R.I. and Porvaznik, M.J.: Disruption of the permeability barrier (zonula occludens) between intestinal epithelial cells by lethal doses of endotoxin. Infect. Immun. 21, 655-658 (1978).

Wells, C.L., Maddus, M.A., and Simmons, R.L.: Role of the macrophage in the translocation of intestinal bacteria. Arch. Surg. 122, 48-53 (1987).

Wells, C.L., Maddus, M.A., Erlandsen, S.L., and Simmons, R.L.: Evidence for the phagocytic transport of intestinal particles in dogs and rats. Infect. Immun.. 56, 278-282 (1988).

Wooles, W.R. and DiLuzio, N.R.: Influence of RE hyperfunction on bone marrow transplantation. Am. J. Physiol. 203, 404-408 (1962).

THE ORIGIN OF AUTOANTIBODIES

ROBBERT BENNER and NICO A. BOS

Department of Immunology, Erasmus University, Rotterdam, The Netherlands

Lecture presented at the 2nd Old Herborn University Seminar on
"Interactions between the indigenous microflora and the host immune system"
on June 1, 1988

SUMMARY

Generally two types of autoantibodies are discriminated: "Natural autoantibodies" and "pathogenetic autoantibodies". Natural autoantibodies are normal components of the immune system. They seem not to account for autoimmune disease. These antibodies usually are of the IgM class and of low affinity. Pathogenetic autoantibodies, on the other hand, generally are of the IgG class and of high affinity. These autoantibodies are potentially harmful.

This paper presents data on the role of environmental antigens in the production of antibodies and especially autoantibodies. We made use of germfree mice fed chemically defined synthetic diet (GF-CD mice). These mice have a seemingly normal IgM production and a severely reduced production of IgG and IgA. The specificity repertoire of these IgG and IgA antibodies greatly differs from that of the same isotypes in conventional mice, and is much alike the IgM specificity repertoire. The GF-CD mice also differ from conventional mice in their higher production of natural autoantibodies. Apparently, exogenous antigenic stimulation plays an important role in the development of the actual B-cell repertoire.

Exogenous antigenic stimulation, especially infectious disease, can account not only for autoantibodies, but can also facilitate autoimmune disease. Several mechanisms have been proposed, including (a) change in endogenous antigen; (b) disturbance of the host immune response; (c) molecular mimicry; and (d) somatic diversification of antibodies to environmental antigens. Literature data supporting the latter two possibilities are discussed.

THE INFLUENCE OF ENVIRONMENTAL ANTIGENS ON IMMUNOGLOBULIN PRODUCTION

We studied the influence of environmental antigens on the immunoglobulin (Ig) production by comparing germfree mice fed with a chemically defined synthetic diet (GF-CD mice) and conventional mice fed natural ingredient diet (CV-NI mice). The GF-CD mice were bred and maintained by Drs. B.S. Wostmann and J.R. Pleasants from the Lobund Laboratory in Notre Dame, Indiana. The chemically defined diet used, consisted of sugars, amino acids, lip-

Table 1: Numbers of "background" Ig-secreting cells in spleen, bone marrow and mesenteric lymph nodes of "antigen-free" (GF-CD) and conventional BALB/c mice (CV-NI)[a]

Organ	Isotype	Ig-SC x 10^{-3}/organ GF-CD	CV-NI	Ratio CV-NI/GF-CD
Spleen	IgM	267 ± 50[b]	469 ± 75	1.8 (p>0.1)[c]
	IgG1	0.1 ± 0.1	25 ± 5	250 (p<0.05)
	IgG2a	0.2 ± 0.08	29 ± 6	145 (p<0.05)
	IgG2b	0.2 ± 0.09	20 ± 5	100 (p<0.05)
	IgG3	0.1 ± 0.07	14 ± 3	140 (p<0.05)
	IgA	0.3 ± 0.3	240 ± 41	800 (p<0.01)
Bone marrow	IgM	97 ± 25	61 ± 6	0.63 (p>0.5)
	IgG	2.0 ± 0.4	73 ± 11	37 (p<0.005)
	IgA	1.5 ± 0.7	107 ± 38	71 (p<0.05)
Lymph nodes	IgM	4.6 ± 2.5	4 ± 1.5	0.87 (p>0.1)
	IgG	0.2 ± 0.1	58 ± 18	290 (p<0.05)
	IgA	0.3 ± 0.1	31 ± 2	103 (p<0.001)

[a]Data from: *Bos* et al., 1988.
[b]Numbers represent the arithmetic means (± SEM) of three to seven individual experiments. In each experiment the organs of six animals of each group were pooled.
[c]Statistical analysis was performed with the Student *t* test.

ids, minerals and vitamins, all with a molecular weight lower than 10.000 D (*Pleasants* et al., 1986). Such GF-CD mice are tentatively called "antigen-free mice".

In the lymphoid organs of CV-NI mice many times more Ig-secreting cells (Ig-SC) occur than in GF-CD mice. Table 1 shows the numbers of IgM-, IgG- and IgA-SC in spleen, bone marrow and mesenteric lymph nodes of both groups of mice. While the numbers of IgM-SC hardly differ, the numbers of IgG- and IgA-SC are much lower in GF-CD than CV-NI mice in each of the three organs studied. The table also shows the ratio of the numbers of IgG- and IgA-SC found in CV-NI mice over those in GF-CD mice. In most cases this ratio is 100 or higher, indicating that environmental antigens affect the IgG and IgA production much more than the IgM production.

This conclusion urged for studies on selective pressures accounting for this differential production of different isotypes. Therefore, we analysed the specificity repertoire of the IgM-, IgG- and IgA-SC in both groups of mice. This was done by employing haemolytic plaque assays using sheep red blood cells (SRBC) coated with different haptens and ELISA-plaque assays. Remarkably, no substantial differences were found between GF-CD and CV-NI mice with regard to frequencies of splenic IgM-SC specific for the different antigens (Table 2). Apparently the specificity repertoire of the spontaneously occurring ("background") IgM-SC is rather stable and hardly dependent on exogenous antigenic stimulation.

For IgG and IgA, however, the situation is quite different. In CV-NI mice the frequencies of IgG- and IgA-SC specific for DNP-BSA were found to be substantially lower than the frequency of IgM-SC specific for DNP-

Table 2: Relative frequencies of background IgM-secreting cells specific for several haptens in the spleen of "antigen-free" (GF-CD) and conventional BALB/c mice (CV-NI)[a]

Antigen	GF-CD	CV-NI
NIP_4-SRBC	1 in 46 (±29)	1 in 21 (±4)[b]
$NIP_{0.4}$-SRBC	1 in 195 (±131)	1 in 188 (±85)
NNP_2-SRBC	1 in 63 (±31)	1 in 38 (±11)
$NNP_{0.2}$-SRBC	1 in 284 (±110)	1 in 1125 (±681)
TNP_{30}-SRBC	1 in 232 (±71)	1 in 122 (±36)

[a]Data from: *Bos* et al., 1986.
[b]Figures represent the mean ratio of specific IgM-antibody secreting cells to the total number of IgM-secreting cells as detected in the protein A plaque assay. The arithmetic mean ± SD has been calculated.

BSA. In GF-CD mice such a substantial difference was not found (Table 3). In such "antigen-free" mice the relatively small production of IgG and IgA seems to be independent of the selective antigenic forces that in conventional mice greatly affect the specificity repertoire of the compartments of IgG- and IgA-SC. This observation leads us to suggest that the background Ig-SC can be subdivided into an "antigen-dependent" and an "antigen-independent" compartment.

The antigen-dependent compartment consists of B-cells which, upon activation by exogenous antigens, give rise to primary and secondary humoral immune responses with antigen specific antibody production, memory induction, isotype switch and affinity maturation.

The antigen-independent compartment, in our view, consists of B-cells that are driven to develop into clones of Ig-SC by endogenous stimuli. This compartment consists mainly of IgM-SC, although the low numbers of IgG- and IgA-SC in GF-CD mice also belong to this compartment. The endogenous stimuli may be provided by idiotypes, e.g., from maternal Ig transferred via the placenta or milk (*Bernabé* et al., 1981) or other self-antigens during the development of the immune system (*Steele* and *Cunningham*, 1978). Idiotypic interactions with T-cells might also represent a driving force and/or

Table 3: Frequency of DNP_{27}-BSA-specific Ig-secreting cells in the spleen of "antigen-free" (GF-CD) and conventional BALB/c mice (CV-NI)[a]

	GF-CD		CV-NI	
Ig-secreting cells	Cells tested[b]	Frequency	Cells tested	Frequency
IgM	222	1 in 143 (±32)	52	1 in 65 (±22)
IgG	1.9	1 in 48 (±18)	989	1 in 60000 (±22060)
IgA	1.5	1 in 93 (±37)	465	1 in 2118 (±327)

[a]Data from: *Bos* et al., 1988.
[b]Total number of Ig-secreting cells (x 10^{-3}) of a particular isotype evaluated for specificity for DNP_{27}-BSA.

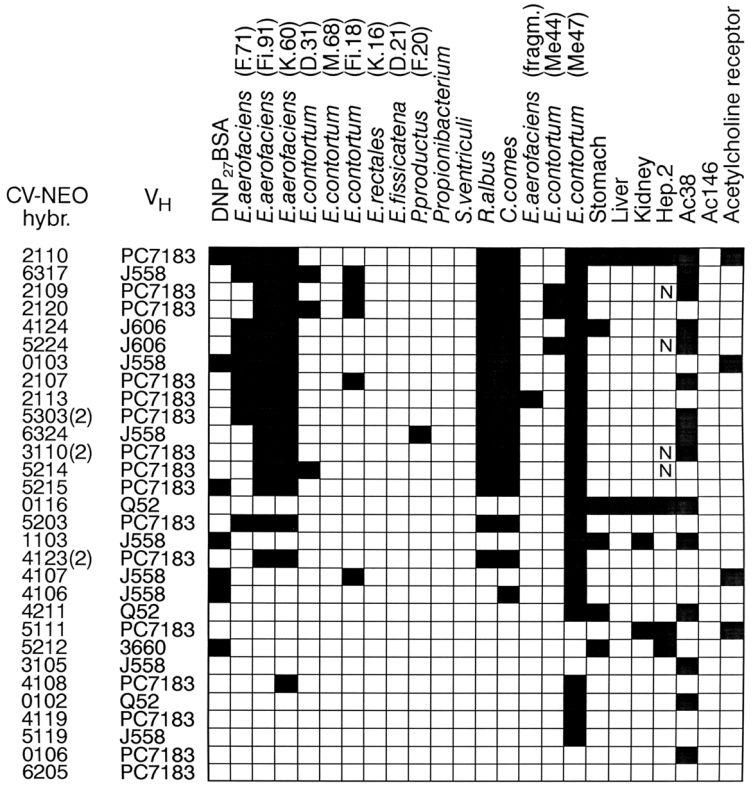

Figure 1: Reactivity pattern and V_H gene usage of a panel of hybridomas from neonatal conventional BALB/c mice. Closed squares represent positive reactions; open squares represent negative reactions; N = not done. Data from: *Bos* et al., 1989.

play a regulating role (*Martinez-A* et al., 1986). This autonomous compartment remains very stable during the lifetime and is relatively independent of exogenous stimuli (cf. Tables 2 and 3). It can be speculated that the B-cells involved belong to the naturally activated, autoreactive B-cells described by *Portnoï* et al. (1986).

NATURAL AUTOANTIBODIES

Many individuals have autoantibodies in their serum. The frequency of people with autoantibodies increases with increasing age (*Hawkins* et al., 1979). These autoantibodies include specificities for neurofilaments, tubulin, actin, transferrin, thyroglobulin etc. (*Guilbert* et al., 1982). The occurrence of autoantibodies is by far not always associated with autoimmune disease. In humans, autoantibodies can even occur for a decade or longer without signs of autoimmune disease. This discrepancy is found not only in humans, but also in mice (*Hawkins* et al., 1979; *Schattner*, 1987).

Highly informative with regard to autoantibody production are studies of hybridoma collections produced by fusing unstimulated or lipopolysaccharide (LPS) stimulated B-cells from naive mice with non-secreting murine plasmacytoma cells. Using this methodology, B-cells with the capacity of se-

creting autoantibodies have been shown to be normal components of the immune system. Such natural autoantibodies are usually of the IgM class and of low affinity (*Holmberg* et al., 1986a).

Early in ontogeny a high frequency of B-cells can be found which can bind to multiple antigens, among which autoantigens (*Dighiero* et al., 1985). Such B-cells have been called "multireactive B-cells". The antibodies produced by many of such B-cells can also recognise different antibody combining sites or determinants specific to Ig variable regions of other B-cells. They are called "highly connective" antibodies and may participate in an idiotypic network (*Holmberg* et al., 1984, 1986b).

In Figure 1 we show cross-reactivity between autoantigens and exogenous antigens for a number of hybridomas derived from unstimulated neonatal spleen cells. Probably, B-cells that produce multireactive, including autoreactive, antibodies persist throughout the mouse' lifetime, but their frequency is higher early in ontogeny (*Holmberg* et al., 1986b). This conversion, however, is much less prominent in the absence of exogenous antigenic stimulation, as shown in adult GF-CD mice (cf. Figure 2). This indicates that exogenous antigenic stimulation plays an important role in the development of the actual B-cell repertoire.

The specificity of Ig molecules is determined by the variable part of the heavy and light chains, which are largely encoded by V_H and V_L gene segments. V_H gene segments have been grouped into at least eight different families (called: PC7183, Q52, X24, 3660, J606, S107, J558, 3609) on the basis of relatedness at the nucleotide sequence level (*Brodeur* and *Riblet*, 1984; *Winter* et al., 1985). The V_H genes within a family are highly homologous, with more than 80% sequence identity, whereas the degree of homology between members of different families ranges from 50 to 70%.

Figure 3 shows the V_H gene family usage by our panels of hybridomas generated from neonatal spleen cells from conventional mice (indicated as CV-NEO) and from adult spleen cells from GF-CD mice (indicated as GF-CD). This V_H gene family usage is compared to the size and position of the V_H gene families analysed. In both hybridoma panels the PC7183 V_H gene family is preferentially used, and thus V_H gene usage is biased towards C_H proximal V_H segments. Data from the literature suggest that the V_H genes coding for the multireactive and autoreactive antibodies belong predominantly to the PC7183 family (*Painter* et al., 1986).

In hybridoma panels from adult spleen cells from conventional mice, V_H usage is primarily dependent on V_H family size instead of family position (*Dildrop* et al., 1985; *Schulze* and *Kelsoe*, 1987). Apparently, V_H family usage in conventional mice normalises during ontogeny.

If exogenous antigenic stimulation is avoided during the lifetime, clonal selection will hardly occur. Therefore, the usage of V_H genes by adult GF-CD mice will be more or less comparable to that of the neonatal B-cell pool. In adult GF-CD mice, however, besides a higher percentage of B-cells expressing the PC7183 V_H gene family, there is also a higher percentage of B-cells expressing the J558 family compared to neonatal B-cells. This suggests that normalisation has taken place to some extent in adult GF-CD mice. This could be caused by some endogenous antigenic stimulation, e.g. from idiotypes and other autoantigens, as suggested by the rise of the number of background IgG- and IgA-secreting cells in maturing GF-CD mice (*Hooijkaas* et al., 1984; *Bos* et al., 1987).

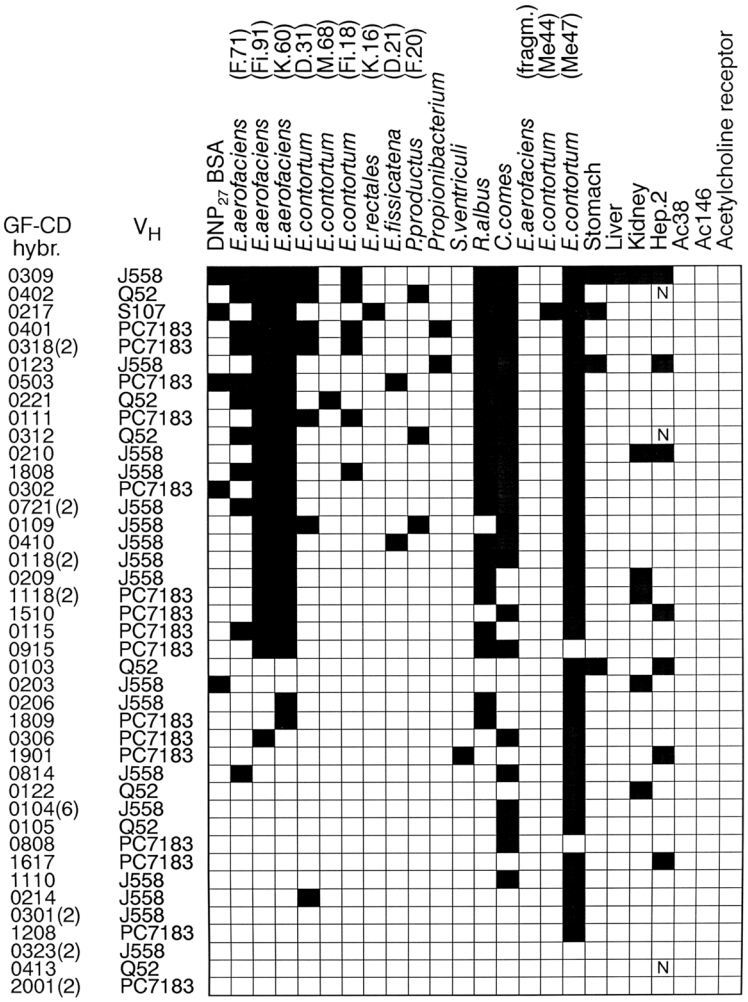

Figure 2: Reactivity pattern and V_H gene usage of a panel of hybridomas from adult "antigen-free" BALB/c mice. Closed squares represent positive reactions; open squares represent negative reactions; N = not done. Data from: *Bos* et al., 1989.

FACTORS INFLUENCING AUTOIMMUNITY

Both organism inherent factors (genetic factors etc.) and environmental factors influence the development and severity of autoimmunity.

The involvement of genetic factors is apparent from the observation that almost all autoimmune diseases show a preferential association to certain HLA alleles, especially HLA-DR2, -DR3, -DR4 and -B8 (*Nepom*, 1989). Immunodeficiencies that are genetically determined are often associated with autoimmune reactions. It is unclear, however, whether immunodeficiency directly influences autoimmunity, or that the autoimmunity is due to chronic infections, which more often occur in immunodeficiency (*Waldmann*, 1988). Also age and hormonal factors, particularly sex hormones, play a role in autoimmunity (*Wood* and *Badley*, 1986). Although many autoimmune

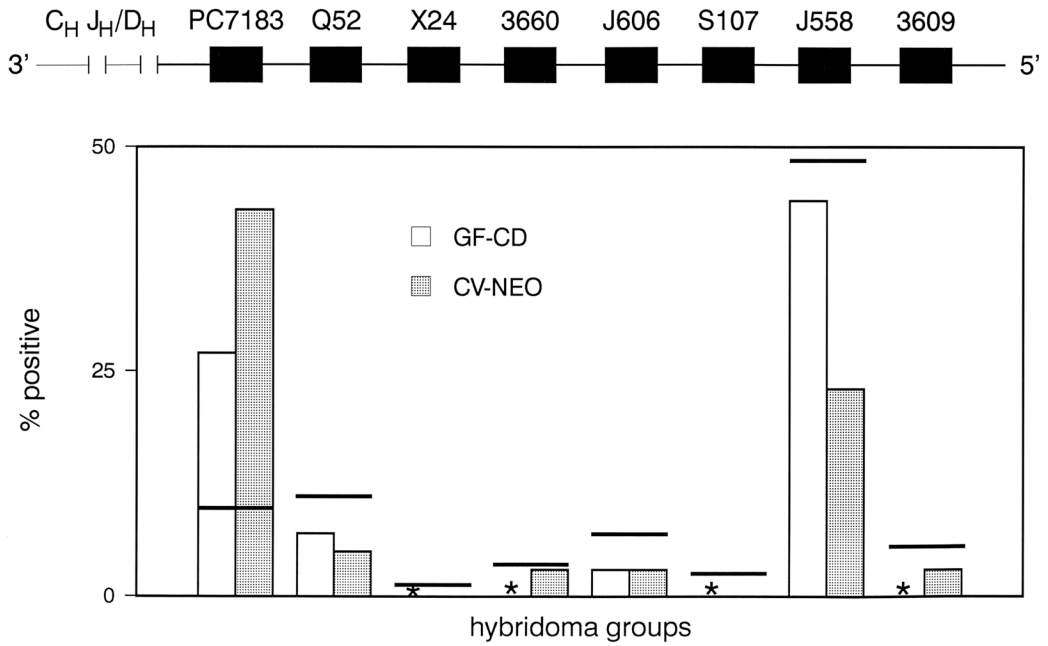

Figure 3: V_H gene family usage in GF-CD and CV-NEO hybridomas compared to the size and position of the V_H gene families. GF-CD (59) and CV-NEO (65) hybridomas were tested for V_H gene family usage by hybridisation of purified RNA to the different probes. Eleven GF-CD and 13 CV-NEO hybridomas could not be assigned to one V_H gene family. The horizontal line within each V_H gene family represents the expected percentage according to the size of that V_H gene family. The chromosomal order of the V_H gene families is shown at the top of the figures. * = not done. Data from: *Bos* and *Meeuwsen*, 1989.

diseases have an age-related peak incidence and the incidence of autoantibody production increases with increasing age (*Hawkins* et al., 1979), autoimmune diseases in general are not clearly related to older age groups (*Wood* and *Badley*, 1986).

As far as environmental factors are concerned, several drugs can induce autoimmune reactions. These are often asymptomatic and/or disappear after discontinuation of exposure. Also food, dust and other agents may cause autoimmune symptoms. Many infections by viruses, bacteria, fungi and parasites cause temporary autoimmune symptoms, particularly the production of rheumatoid factor and anti-nuclear antibodies. Usually these symptoms are reversible after eradication of the infectious agent, but tissue damage as the result of excessive immune reactions is not always reversible (*Wood* and *Badley*, 1986).

Evidence that infectious agents may be a major cause of autoimmune disease is accumulating (Table 4). In fact, many so-called autoimmune diseases may turn out to be the consequence of an infectious disease, with which the infected organism cannot cope with appropriately because of some selective immune response defect.

Table 4: Infectious agents suggested as inducers of autoimmune diseases

Rheumatoid arthritis	EB-virus related agents, Mycobacteria
Insulin-dependent diabetes mellitus	Coxsackie B virus
Multiple sclerosis	Defective measles virus
Sclerosing panencephalitis	C-type RNA virus
Sjögren's syndrome	A-type virus
Rheumatic fever	Group A streptococci
Ankylosing spondylitis	Klebsiella
Reiter's disease	Shigellae

POSSIBLE MECHANISMS OF POST-INFECTION AUTOIMMUNITY

Several mechanisms have been proposed for post-infection autoimmunity. These include: (a) change in endogenous antigen (*Bottazo* et al., 1986); (b) disturbance of the host immune response (*Marcos* et al., 1986); (c) molecular mimicry; and (d) somatic diversification of antibodies to environmental antigens (Table 5). From these, we shall discuss here the latter two.

Molecular mimicry between a microorganism and a host constituent

Research over the last few years has established that several microbial agents share determinants with human and animal proteins. This is called "molecular mimicry". An immune response mounted by the host against a specific determinant of an infecting agent may cross-react with the mimicked host-sequence, leading to an autoimmune reaction and, in some cases, tissue injury and disease. Evidence for the hypothesis that molecular mimicry can cause autoimmune disease in man comes from studies on the pathogeneses of ankylosing spondylitis and coeliac disease. *Schwimmbeck* et al. (1987) showed amino acid homology and immunological cross-reactivity between *Klebsiella pneumoniae* nitrogenase and the HLA-B27 variable domain. Similarly, *Kagnoff* et al. (1984) showed such similarities between Adenovirus 12E1B and protein A-gliadin, a dietary component of wheat gluten (Table 6). Such molecular mimicry between infectious agents and host sequences may frequently occur. However, unless the homology and subsequent immunological cross-reactivity involve a host protein that can precipitate disease, the autoimmune response is unlikely to lead to autoimmune disease (*Oldstone*, 1987).

Molecular mimicry between an antibody to a microorganism and a host constituent

The idiotypic network model of *Niels K. Jerne* (1974) implies that the variable regions of an antibody to a particular antigen can induce the production of complementary and interacting second order antibodies, the so-called anti-idiotypic antibodies. The idiotypic interconnection between first and second order antibodies and the B-cell subsets producing these antibodies is thought to regulate the immune response to the original antigen. Anti-idiotype antibodies induced as second order antibodies to a microorganism may functionally be autoantibodies (*Plotz*, 1983). That such autoantibodies may be relevant to autoimmune disease in man is suggested

Table 5: Possible mechanisms of post infection autoimmunity

Change in endogenous antigen
- tissue necrosis with release of intracellular antigen
- new antigenic determinants due to insertion of viral epitopes in cell membrane
- aberrant HLA class II expression
Disturbance of host immune response
- release of cytokines
- lymphotropic viruses
- microbial polyclonal activators (LPS, EBV, tuberculin, *B. pertussis*)
Molecular mimicry, i.e. sharing antigenic determinants between
- microorganism and a host constituent
- antibody against microorganism and a host constituent
Somatic diversification of antibodies to environmental antigens converting them into pathogenetic autoantibodies

by studies of *Dwyer* et al. (1986). They showed extensive idiotypic connectivity between antibodies against the acetylcholine receptor (AChR) and antibodies against α-1,3-dextran. Furthermore, they showed that 15% of patients with myasthenia gravis, which is caused by autoantibodies against the AChR, have serum antibodies against α-1,3-dextran. Control sera were negative for these antibodies. Certain of these anti-α-1,3-dextran antibodies were found to bind to anti-AChR antibodies via idiotypic interactions. As the α-1,3-dextran determinant is present on common opportunistic pathogens like *Enterobacter cloaca* and *Serratia liquefaciens*, these data suggest that myasthenia gravis may arise as the consequence of molecular mimicry between anti-α-1,3-dextran antibodies and the AChR. Naturally, susceptible individuals will have particular characteristics such as appropriate immune response genes (HLA-D), Ig-genes and/or T-cell abnormalities (*Dwyer*, 1988).

Somatic diversification of antibodies to environmental antigens converting them into pathogenetic autoantibodies

The antibody specificity repertoire is not only dependent on germline V-D-J gene rearrangement, deletion and other somatic diversification during B-cell differentiation, but also on somatic diversification of already expressed heavy and light chain variable regions. The

Table 6: Sequence similarities between microbial proteins and human host proteins

Disease	Protein	Residue	Sequence	Reference
Ankylosing spondylitis	*K. pneumoniae* nitrogenase	188	SRQTDREDE	*Schwimmbeck* et al., 1987
	HLA-B27	70	KAQTDREDL	
Coeliac disease	Adenovirus 12E1B	384	LRRGMFRPSQCN	*Kagnoff* et al., 1984
	Wheat gluten A-gliadin	208	LGQGSFRPSQQN	

latter form of diversification includes somatic point mutation, gene conversion and V region replacement (*Perlmutter*, 1989). Each of these events involved in the generation of antibody diversity may play a role in the production of pathogenetic autoantibodies.

Scharff and co-workers have shown that somatic point mutation in an Ig can convert an antibody reacting to an exogenous antigen into a potentially pathogenetic autoantibody (*Behar* et al., 1988). They isolated spontaneous mutants from the *in vitro* growing murine S107 myeloma cell line. The antibodies produced by this cell line bind to a number of bacterial polysaccharides found in the intestine of normal mice (*Potter*, 1972) and react specifically with phosphocholine which is present on these bacterial polysaccharides. Scharff and co-workers especially paid attention to mutants which had lost the ability to bind phosphocholine. One of these mutants was shown to have a single amino acid substitution of an alanine for glutamic acid at residue 35 in the first hypervariable region. This amino acid substitution was the result of a single base change that arose through somatic point mutation (*Giusti* et al., 1987). Most interestingly, this mutant not only had lost the ability to bind phosphocholine, but had acquired the ability to bind double-stranded DNA, phosphorylated proteins and phospholipids and therefore resembled autoantibodies found in lupus-like syndromes (*Schattner*, 1987; *Diamond* and *Scharff*, 1984). Thus, somatic mutation can convert a protective antibody into a potentially harmful autoantibody.

CONCLUSION

Normal adult humans and mice, and even new-borns, have B-lymphocytes which secrete antibodies recognising a variety of self-antigens. These "natural autoantibodies" usually are of the IgM class, of low affinity and a broad specificity (*Schattner*, 1987; *Holmberg* et al., 1986a). They seem not to account for autoimmune disease. In contrast, natural autoantibodies have been suggested to prevent autoimmune disease (*Cohen* and *Cooke*, 1986). Pathogenetic autoantibodies, on the other hand, mostly are of the IgG class, have a high affinity to the recognised antigen and have a narrow specificity (*Bottazzo* et al., 1986). In some autoimmune diseases the serum level of these autoantibodies correlate to disease activity, in other not (*Schattner*, 1987; *Holborow*, 1986), indicating that still much has to be learned about the pathophysiology of autoimmune disease.

ACKNOWLEDGEMENT

We thank Ms. Geertje de Korte for her help in the preparation of the manuscript and Mr. Tar M. van Os for preparation of the figures.

LITERATURE

Behar, S.M., Fischer, B., Diamond, B., and Scharff, M.D.: Variable region gene utilization and environmental antigens in autoimmunity. Monogr. Allergy 24, 297-306 (1988).

Bernabé, R.R., Coutinho, A., Cazenave, P.-A.,

and Forni, L.: Suppression of a "recurrent" idiotype results in profound alterations of the whole B-cell compartment. Proc. Natl. Acad. Sci. USA 78, 6416-6420 (1981).

Bos, N.A., Benner, R., Wostmann, B.S., and Pleasants, J.R.: "Background" Ig-secreting cells in pregnant germfree mice fed a chemically defined ultrafiltered diet. J. Reprod. Immunol. 9, 237-246 (1986).

Bos, N.A., Meeuwsen, C.G., Hooijkaas, H., Benner, R., Wostmann, B.S., and Pleasants, J.R.: Early development of Ig-secreting cells in young of germ-free BALB/c mice fed a chemically defined ultrafiltered diet. Cell. Immunol. 105, 235-245 (1987).

Bos, N.A., Meeuwsen, C.G., Wostmann, B.S., Pleasants, J.R., and Benner, R.: The influence of exogenous antigenic stimulation on the specificity repertoire of background immunoglobulin-secreting cells of different isotypes. Cell. Immunol. 112: 371-380, 1988.

Bos, N.A., Meeuwsen, C.G., van Wijngaarden, P., and Benner, R.: B cell repertoire in adult antigen-free and conventional neonatal BALB/c mice. II. Analysis of antigen-binding capacities in relation to V_H gene usage. Eur. J. Immunol. 19, 1817-1822 (1989).

Bos, N.A. and Meeuwsen, C.G.: B cell repertoire in adult antigen-free and conventional neonatal BALB/c mice. I. Preferential utilization of the C_H-proximal V_H gene family PC7183. Eur. J. Immunol. 19, 1811-1815 (1989).

Bottazzo, G.F., Todd, I., Mirakian, R., Belfiore, A., and Pujol-Borrell, R.: Organ-specific autoimmunity: A 1986 overview. Immunol. Rev. 94, 137-169 (1986).

Brodeur, P.H. and Riblet, R.: The immunoglobulin heavy chain variable region (Igh-V) locus in the mouse. I. One hundred Igh-V genes comprise seven families of homologous genes. Eur. J. Immunol. 14, 922-930 (1984).

Cohen, I.R. and Cooke, A.: Natural autoantibodies might prevent autoimmune disease. Immunol. Today 7, 363-364 (1986).

Diamond, B. and Scharff, M.D.: Somatic mutation of the T15 heavy chain gives rise to an antibody with autoantibody specificity. Proc. Natl. Acad. Sci. USA 81, 5841-5844 (1984).

Dighiero, G., Lymberi, P., Holmberg, D., Lundquist, I., Coutinho, A., and Avrameas, S.: High frequency of natural autoantibodies in normal newborn mice. J. Immunol. 134, 765-771 (1985).

Dildrop, R., Krawinkel, U., Winter, E., and Rajewsky, K.: V_H-gene expression in murine mlipopolysaccharide blasts distributes over the nine known V_H-gene groups and may be random. Eur. J. Immunol. 15, 1154-1156 (1985).

Dwyer, D.S., Vakil, M., and Kearney, J.F.: Idiotypic network connectivity and a possible cause of myasthenia gravis. J. Exp. Med. 164, 1310-1318 (1986).

Dwyer, D.S.: Idiotype network interactions. In: B lymphocytes in human disease (Eds.: Bird, G. and Calvert, J.E.). Oxford University Press, Oxford, 392-408 (1988).

Giusti, A.M., Chien, N.C., Zack, D.J., Shin, S.-U., and Scharff, M.D.: Somatic diversification of S107 from an antiphosphocholine to an anti-DNA autoantibody is due to a single base change in its heavy chain variable region. Proc. Natl. Acad. Sci. USA 84, 2926-2930 (1987).

Guilbert, B., Dighiero, G., and Avrameas, S.: Naturally occurring antibodies against nine common antigens in normal human sera. I. Detection, isolation, and characterization. J. Immunol. 128, 2779-2787 (1982).

Hawkins, B.R., O'Connor, K.J., Dawkins, R.L., Dawkins, B., and Rodger, B.: Autoantibodies in an Australian population. J. Clin. Lab. Immunol. 2, 211-215 (1979).

Holborow, E.J.: Autoantibodies in the rheumatic diseases. In: Copeman's textbook of the rheumatic diseases. Volume 1 (6th edition) (Ed.: Scott, J.T.). Churchill Livingstone, Edinburgh, 355-375 (1986).

Holmberg, D., Forsgren, S., Ivars, F., and Coutinho, A.: Reactions among IgM antibodies derived from normal, neonatal mice. Eur. J. Immunol. 14, 435-441 (1984).

Holmberg, D., Freitas, A.A., Portnoï, D., Jacquemart, F., Avrameas, S. and Coutinho, A.: Antibody repertoires of normal BALB/c mice: B lymphocyte populations defined by state of activation. Immunol. Rev. 93, 147-169 (1986a).

Holmberg, D., Wennerström, G., Andrade, L., and Coutinho, A.: The high idiotypic connectivity of "natural" newborn antibodies is not found in adult mitogen-reactive B cell repertoires. Eur. J. Immunol. 16, 82-87 (1986b).

Hooijkaas, H., Benner, R., Pleasants, J.R., and Wostmann, B.S.: Isotypes and specificities of immunoglobulins produced by germfree mice fed chemically defined ultrafiltered "antigen-free" diet. Eur. J. Immunol. 14, 1127-1130 (1984).

Jerne, N.K.: Towards a network theory of the immune system. Ann. Immunol. (Inst. Pasteur) 125C, 373-389 (1974).

Kagnoff, M.F., Austin, R.K., Hubert, J.J., Bernardin, J.E., and Kasarda, D.D.: Possible role for a human adenovirus in the pathogenesis of celiac disease. J. Exp. Med. 160, 1544-1557 (1984).

Marcos, M.A.R., De la Hera, A., Gaspar, M.L., Màrquez, C., Bellas, C., Mampaso, F., Toribio, M.L., and Martinez-A, C.: Modification of emerging repertoires by immunosuppression in immunodeficient mice results in autoimmunity. Immunol. Rev. 94, 51-74 (1986).

Martinez-A, C., Toribio, M.L., De la Hera, A., Cazenave, P.-A., and Coutinho, A.: Maternal transmission of idiotypic network interactions selecting available T cell repertoires. Eur. J. Immunol. 16, 1445-1447 (1986).

Nepom, G.T.: Reverse immunogenetics: investigations of HLA-associated disease based on structural and genetic identification of candidate susceptibility genes. Progr. Immunol. 7, 805-812 (1989).

Oldstone, M.B.A.: Molecular mimicry and autoimmune disease. Cell 50, 819-820 (1987).

Painter, C., Monestier, M., Bonin, B., and Bona, C.A.: Functional and molecular studies of V genes expressed in autoantibodies. Immunol. Rev. 94, 75-98 (1986).

Perlmutter, R.M.: Organization and reorganization of antigen receptor genes. Progr. Immunol. 7, 83-91 (1989).

Pleasants, J.R., Johnson, M.H., and Wostmann, B.S.: Adequacy of chemically defined, water-soluble diet for germfree BALB/c mice through successive generations and litter. J. Nutr. 116, 1949-1964 (1986).

Plotz, P.H.: Autoantibodies are anti-idiotype antibodies to antiviral antibodies. Lancet ii, 824-826 (1983).

Portnoï, D., Freitas, A., Holmberg, D., Bandeira, A., and Coutinho, A.: Immunocompentent autoreactive B lymphocytes are activated cycling cells in normal mice. J. Exp. Med. 164, 25-35 (1986).

Potter, M.: Immunoglobulin-producing tumors and myeloma proteins of mice. Physiol. Rev. 52, 631-719 (1972).

Schattner, A.: The origin of autoantibodies. Immunol. Lett. 14, 143-153 (1987).

Schulze, D.H. and Kelsoe, G.: Genotypic analysis of B cell colonies by *in situ* hybridization. Stoichiometric expression of three V_H families in adult C57BL/6 and BALB/c mice. J. Exp. Med. 166, 163-172 (1987).

Schwimmbeck, P.L., Yu, D.T.Y., and Oldstone, M.B.A.: Autoantibodies to HLA B27 in the sera of HLA B27 patients with ankylosing spondylitis and Reiter's syndrome. Molecular mimicry with *Klebsiella pneumoniae* as potential mechanism of autoimmune disease. J. Exp. Med. 166, 173-181 (1987).

Steele, E.J. and Cunningham, A.J.: High proportion of Ig-producing cells making autoantibody in normal mice. Nature (London) 274, 483-484 (1978).

Waldmann, T.A.: Immunodeficiency diseases: Primary and acquired. In: Immunological diseases. Volume 1 (4th edition) (Eds.: Samter, M., Talmage, D.W., Frank, M.M., Austen, K.F., and Claman, H.N.). Little, Brown and Company, Boston, 411-465 (1988).

Winter, E., Radbruch, A., and Krawinkel, U.: Members of novel V_H gene families are found in VDJ regions of polyclonally activated B-lymphocytes. EMBO J. 4, 2861-2867 (1985).

Wood, P.H.N. and Badley, E.M.: Epidemiology of individual rheumatic disorders. In: Copeman's textbook of the rheumatic diseases. Volume 1 (6th edition) (Ed.: Scott, J.T.). Churchill Livingstone, Edinburgh, 59-142 (1986).

THE ROLE OF BACTERIAL COMPONENTS IN AUTOIMMUNITY AND CHRONIC INFLAMMATORY DISEASES

Authorised transcript of a lecture by

JOHN H. SCHWAB

Department of Microbiology and Immunology,
University of North Carolina, Chapel Hill, North Carolina, USA

Presented at the 2nd Old Herborn University Seminar on
"Interactions between the indigenous microflora and the host immune system"
on June 1, 1988

CONTRIBUTIONS OF THE MICROBIAL FLORA TO AUTOIMMUNITY

I will first briefly review some of the work of other laboratories relating microbial antigens to autoimmunity.

Immunogens cross-reactive with mammalian tissue (Table 1)

The first example is the induction of antibodies against several antigens of mammalian heart, apparently through cross-reactivity with antigens of group A streptococci (*Streptococcus pyogenes*) These, and other autoimmune responses stimulated by bacterial immunogens, have been studied by several laboratories (*Dale* and *Beachey*, 1982; *van de Rijn* et al., 1977; *Cunningham* et al., 1984). It should be noted that in none of these examples it has been proven that the autoantibodies have a direct role in the pathogenesis of the associated diseases.

More recently, *Karounos* and colleagues (1988) have examined the immunogenesis of autoantibodies against DNA in patients with systemic lupus erythematosus (SLE). They report that

Table 1: Cross-reactive bacterial immunogens

Tissue	Immune response	Bacteria	Reference
Heart myosin actin	Antibody	*Streptococcus pyogenes* (M protein) *Streptococcus pyogenes* (membrane peptide)	Dale and Beachey, 1982 van de Rijn et al., 1977; Cunningham et al., 1984
DNA	Antibody	*Micrococcus lysodeikticus* *Staphylococcus epidermidis* *Escherichia coli* *Pseudomonas aeruginosa*	Karounos et al., 1988
Cartilage proteoglycan	T-cell	*Mycobacterium bovis* and over 50 other bacteria which share a common epitope in a heat shock protein	Holoshitz et al., 1983 Holoshitz et al., 1986 van Eden et al., 1988

anti-DNA in normal individuals and the anti-DNA, which is elevated in SLE patients, seems to be primarily a result of antigenic stimulation by DNA from a variety of bacteria which are part of the normal microflora.

The third example I have listed in Table I involve extensive studies by Cohen and colleagues in which they describe T-cells isolated from rats with adjuvant arthritis. Two T-cell clones specific for a protein antigen (MT) from the *Mycobacterium tuberculosis* organisms in complete Freund's adjuvant have been examined. One clone can induce joint inflammation when injected into irradiated Lewis rats. A second clone of the same specificity did not induce arthritis, but did protect rats against adjuvant-induced arthritis (*Holoshitz* et al., 1983). These T-cell clones also recognise a human cartilage proteoglycan antigen. T-cells from rheumatoid arthritis patients also respond to the MT antigen (*Holoshitz* et al., 1986). The 65 kD, MT antigen has been cloned in *E. coli* and the critical sequence is amino acids l80 to 188, with 4 of the 9 amino acids identical to the link protein of rat proteoglycan. The 65 kD MT antigen cannot induce arthritis, but does induce resistance to adjuvant arthritis. Finally, the MT antigen has some sequence homology with a heat shock protein shared with many other bacteria (*van Eden* et al., 1988). It is important to note that classical adjuvant arthritis requires a petidoglycan component and the MT protein is unrelated to the structure of bacterial cell wall petidoglycan. Therefore, it would appear that the T-cells specific for MT antigen do not have an essential role in adjuvant arthritis. Rather, when such T-cells cross-reactive with cartilage are induced in classical adjuvant arthritis their function may be to maintain and/or increase the severity of inflammation which has been initiated by cell wall peptidoglycan.

Non-antigen-driven autoimmunity

Another mechanism by which autoantibodies can be stimulated by bacteria may involve B-cell mitogens derived from bacteria, rather than antigenic mimicry. More evidence for this concept is presented by *Swartzwelder* et al. (1988). It has been known for some time that *Streptococccus mutans* vaccines can stimulate production of autoantibodies specific for heart antigens. However, autoantibody in rabbit anti-*S. mutans* serum, affinity purified from heart myosin antigen, does not react with *S. mutans* antigens. One interpretation is that the bacterial polyclonal B-cell mitogens can theoretically stimulate the expression of a large part of the B-cell repertoire, including antibodies against self antigens (*Swartzwelder* et al., 1988).

CHRONIC GRANULOMATOUS INFLAMMATION INDUCED BY PEPTIDOGLYCAN-POLYSACCHARIDE POLYMERS FROM BACTERIAL CELL WALLS

This section will summarise some of the recent work our laboratory has been doing on experimental inflammatory diseases induced by bacterial cell walls. The participation of autoimmunity in the pathogenesis of these diseases remains unproven. The petidoglycan-polysaccharide (PG-PS) structures which initiate and maintain the inflammation can be derived from a variety of infectious bacteria as well as bacteria which are part of the normal human microflora

(*Stimpson* et al., 1986a). The PG-PS polymers, which we use most frequently, are isolated from group A streptococci. The petidoglycan moiety has a number of relevant pro-inflammatory activities (*Stimpson* et al., 1986b). The polysaccharide also has biological activity (*Dalldorf* et al., 1988) but its primary function is to protect the petidoglycan from *in vivo* degradation which allows it to persist in tissue for a prolonged period. The relative degree of N- and O-acetylation also contributes to resistance of PG-PS to biodegradation.

Chronic, erosive, recurrent arthritis

Intraperitoneal injection of an aqueous suspension of PG-PS into rats induces an acute inflammation of joints, which reaches a peak in about 3 to 5 days and then recedes. About 2 to 3 weeks after injection, depending upon polymer size and fine structure, the inflammation of joints recurs and repeated cycles of waxing and waning occur over a period of several months. This prolonged, recurrent process can result in a severe erosive arthritis and loss of function (*Cromartie* et al., 1977).

Granulomatous enterocolitis

Sartor and colleagues (1985) have been investigating the intestinal and extra-intestinal pathology in rats injected locally or systemically with PG-PS. A chronic, recurrent, granulomatous enterocolitis, histologically resembling Crohn's disease, develops after submucosal injection of the small intestine or caecum. By about l2 weeks there appears to occur a reactivation of the inflammation at the injection sites, as evidenced by oedema and renewed infiltration of neutrophils, as well as accumulations of lymphocytes and macrophages. At 6 months there is still an active chronic inflammation with the presence of neutrophils. This prolonged disease can be induced by PG-PS from group D streptococci, part of the normal intestinal flora, but not by injection of a protein antigen such as human serum albumin.

Gastrointestinal lymphoid tissue haemorrhage (GALT)

Within 3 minutes after the i.v. injection of PG-PS into rats macroscopic haemorrhage appears in the lamina propria, organised lymphoid aggregates of the caecum and small intestine, and in the mesenteric lymph nodes (*Sartor* et al., 1986). Haemorrhage is not seen in the lung, kidney, liver, spleen, adrenal, or submandibular or popliteal lymph nodes. The response is maximal at 5 minutes and resolves completely by 3 days. Only erythrocytes appear in the tissue; neutrophils, oedema, vasculitis and necrosis are not seen. Since vascular changes are the initial event common to all inflammatory responses, we believe this haemorrhagic reaction provides a model for investigation of the earliest mediators of inflammation induced by PG-PS polymers.

Granulomatous hepatitis

Lichtman has been investigating the pathological consequences of small bowel bacterial overgrowth in the rat (*Lichtman* et al., 1990). This develops following surgical creation of a self-filling blind loop in which there is a 4-log increase in the number of anaerobic bacteria. Within 6 to l2 weeks, depending upon the strain of rat, an extensive hepatitis develops and granulomatous lesions occur in the mesenteric lymph nodes and spleen, PAS-positive macrophages are present in these lesions, which is presumptive evidence for PG-PS. The hypothesis is that the large amount of bacterial cell wall debris accumulating from the bacterial overgrowth is transported across the gut wall and systemically distributed. This

could represent one way by which PG-PS derived from the intestinal microflora can be delivered to joint and become part of the pathogenesis of inflammatory arthritis.

ACKNOWLEDGEMENT

The studies on the pathogenetic properties of PG-PS summarised here were conducted in the laboratories of Dr. R.B. Sartor, Dr. S, Lichtman, R.R. Brown and S.K. Anderle.

LITERATURE

Cromartie, W.J., Craddock, J.G., Schwab, J.H., Anderle,S.K., and Yang, C.H.: Arthritis in rats after systemic injection of streptococcal cells or cell walls. J. Exp. Med. 146, 1585-1602 (1977).

Cunningham, M.W., Krisher, K., and Graves, D.C.; Murine monoclonal antibodies reactive with human heart and group A streptococcal membrane antigens. Infect. Immun. 46, 34-41 (1984).

Dale, J.B., and Beachey, E.H.: Protective antigenic determinant of streptococcal M protein shared with sarcolemmal membrane protein of human heart. J. Exp. Med. 156, 1165-1176 (1982).

Dalldorf, F.G., Anderle, S.K., Brown, R.R., and Schwab, J.H.: Mast cell activation by group A streptococcal polysaccharide in the rat and its role in experimental arthritis. Am. J. Pathol. 132, 258-264 (1988).

Holoshitz, J., Naparstek, Y., Ben-Nun, A., and Cohen, I.R.: Lines of T lymphocytes induce or vaccinate against autoimmune arthritis. Science 2l9, 56-58 (1983).

Holoshitz, J., Drucker, I., Yaretzky, A., van Eden, W., Klajman, A., Lapidot, Z., Frenkel, A., and Cohen, I.R.: T lymhocytes of rheumatiod arthritis patients show augmented reactivity to a fraction of *Mycobacteria* cross-reactive with cartilage. Lancet ii, 305-309 (1986).

Karounos, D.G., Grudier, J.P., and Pisetsky, D.S.: Spontaneous expression of antibodies to DNA of various species origin in sera of normal subjects and patients with systemic lupus erythematosus. J. Immunol. l40, 451-455 (1988).

Lichtman, S.N., Sartor, R.B., Keku, J., and Schwab, J.H.: Hepatic inflammation in rats with experimental small intestinal bacterial overgrowth. Gastroenterology 98, 414-423 (1990).

Sartor, R.B., Cromartie, W.J., Powell, D.W., and Schwab, J.H.: Granulomatous enterocolitis induced in rats by purified bacterial cell wall fragments. Gastroenterology 89, 587-595 (1985).

Sartor, R.B., Anderle, S.K., Cromartie, W.J., and Schwab, J.H.: Localized gut-associated lymphoid tissue hemorrhage induced by intravenous peptidoglycan-polysaccharide polymers. Infect. Immun. 51, 521-528 (1986).

Stimpson, S.A., Brown, R.R., Anderle, S.K., Klapper, D.G., Clark, R.L., Cromartie, W.J., and Schwab, J.H.: Arthropathic properties of cell wall polymers from normal flora bacteria. Infect. Immun. 51, 240-249 (l986a).

Stimpson, S.A., Schwab, J.H., Janusz, M.J., Anderle, S.K., Brown, R.R., and Cromartie, W.J.: Acute and chronic inflammation induced by peptidoglycan structures and polysaccharide complexes. In: Biological properties of peptidoglycan (Eds.: Seidl, P.H. and Schleifer, K.H.). Walter de Gruyter and Co., Berlin, 273-290 (1986b).

Swartzwelder, F.J., Barua, P.K., Albini, B., and Stinson, M.W.: Heart-reactive antibodies in rabbit anti-*Streptococcus mutans* sera fail to cross-react with *Streptococcus mutans*. J. Immunol. 140, 954-961 (1988).

van de Rijn, I., Zabriskie, J.B., and McCarty, M.: Group A streptococcal antigens cross-reactive with myocardium. Purification of heart-reactive antibody and isolation and characterization of the streptococcal antigen. J. Exp. Med. 146, 579-899 (1977).

van Eden, W., Thole, J.E.R., van der Zee, R., Noordzij, A., van Embden, J.D.A., Hensen, E.J., and Cohen, I.R.: Cloning of the mycobacterial epitope recognized by T lymhocytes in adjuvant arthritis. Nature 331, 171-173 (1988).

INTESTINAL FLORA AND HAEMOPOIESIS

HENK W.S. GORIS

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

Lecture presented at the 2nd Old Herborn University Seminar on
"Interactions between the indigenous microflora and the host immune system"
on June 1, 1988

INTRODUCTION

The relationship between the intestinal flora and haemopoiesis can be studied in several ways. One of the possibilities is to associate germfree animals with several well-defined bacterial strains and to study the effects on haemopoiesis. In a series of recent experiments we have, however, chosen for another approach. We administered non-absorbable antibiotics to conventional mice in order to either totally decontaminate (*van der Waaij* and *Sturm*, 1968) or selectively decontaminate (SD) conventional animals (*van der Waaij* and *Berghuis-de Vries*, 1974). SD was applied to selectively suppress facultatively anaerobic Gram-negative bacteria. We focussed on the Gram-negative bacteria, because it was known from the literature that these bacteria might release a cell-wall-component which is commonly known as endotoxin. It is also known from the literature that haemopoiesis is extremely susceptible to this bacterial component (*Joshi* et al., 1969; *Staber* et al., 1978). Therefore, it appears likely that intestinal Gram-negative bacteria are involved in the regulation of haemopoiesis by releasing endotoxin. Free endotoxin may pass through the intestinal epithelium, particularly if the intracellular junctions are affected by stress or radiation treatment (*Walker* et al., 1978; *Gans* and *Matsumoto*, 1974).

In Table 1 the different steps in which we studied the relationship between the intestinal flora and haemopoiesis is depicted. The first step is intestinal flora modulation by administration of non-absorbable broad-spectrum antibiotics. The second step is the relationship between intestinal Gram-negative bacteria and intestinal endotoxin and the third step is intestinal flora associated endotoxin and haemopoiesis. The last part of this communication will deal with the effect of intestinal flora modulation on haemopoietic recovery.

In Table 2 information is provided about the experimental protocol. Non-absorbable antibiotics were orally ad-

Table 1: Study-design (relatyionship between intestinal flora and haemopoiesis)

- Intestinal flora modulation
- Intestinal Gram-negative bacteria and intestinal endotoxin
- Intestinal flora associated endotoxin and haemopoiesis
- Haemopoietic recovery after cytotoxic treatment and intestinal flora associated endotoxin

Table 2: Experimental protocol

- Oral administration of non-absorbable antibiotics to C3H/Law mice
- Intestinal contents assayed for Gram-negative bacteria and endotoxin
- Haemopoiesis:
  - Femoral nucleated cell content
  - Femoral CFU-S content
  - Femoral CFU-GM content
  - Proliverative state of CFU-S and CFU-GM, hydroxyurea-kill

ministered to C3H/Law mice. The intestinal contents were then daily assayed for the presence of gram-negative bacteria and the free endotoxin level in intestinal contents, i.e. the endotoxin which is released by Gram-negative bacteria during growth and death (*Rothfield* and *Pearlman-Kothencz*, 1969). The haemopoietic parameters followed, were the femoral nucleated cell content, the CFU-s content, CFU-GM content as well as the proliferative state of the CFU-s and the CFU-GM by hydroxyurea-kill. Hydroxyurea is an S-phase specific drug. Therefore, the susceptibility of certain cell types to this drug may be an indication for the proliferative state of these cells. To clarify the abbreviations used, they will briefly be discussed. All blood cells originate from the CFU-s, i.e. the pluripotent haemopoietic stem cell. This cell is called a CFU-s on basis of the spleen colony assay for this cell (*Till* and *McCulloch*, 1961). This cell forms colonies, i.e. spleen colonies, in lethally irradiated recipients. In case of the granulocytes, these CFU-s differentiate into the CFU-C or the CFU-GM. This is the progenitor cell of the granulocytes and macrophages. This cell can be cultured *in vitro* and therefore is called colony-forming-unit (CFU-C) culture or colony-forming-unit-granulocyte-macrophage (CFU-GM).

INTESTINAL TOTAL DECONTAMINATION AND SELECTIVE GRAM-NEGATIVE ELIMINATION: INTESTINAL FLORA MODULATION

In Table 3, four different antimicrobial regimens are shown as well as the effect of these antimicrobial regimens on the colonisation pattern of faecal aerobic Gram-negative bacteria. As shown in this table, all Gram-negative strains were eliminated after 4 days of treatment by these different antimicrobial regimens, i.e. the faecal cultures *Escherichia coli*, *Enterobacter* spp. and *Klebsiella* spp. The difference between the four antimicrobial regimens is in the fraction of the intestinal flora that is suppressed. The first three antimicrobial regimens differ in this respect from the last one. Polymyxin, aztreonam and temocillin were used for selective elimination (SE) of Gram-negative strains, while the combination of cephalotin/neomycin was used for total decontamination (TD) of the intestinal tract.

In Figure 1 the effects of antimicrobial treatment on the faecal endotoxin concentration is shown. The faecal endotoxin concentration is given as a percentage of the initial control during treatment with the four antimicrobial

Table 3: Effect the antimicrobial regimens on the colonisation pattern of faecal aerobic Gram-negative bacteria

Antimicrobial drug(s)	Days of treatment 0	1	2	4	8
Polymixin	●■▲	-	-	-	-
Aztreonam	●■▲	●▲	●▲	-	-
Temocillin	●■▲	▲	-	-	-
Cephalotin/neomycin	●■▲	-	-	-	-

●: *Escherichia coli*
■: *Enterobacter cloacae* >10^2 bacteria/g faeces
▲: *Klebsiella pneumoniae*

regimens. By SE of Gram-negative bacteria with polymyxin, aztreonam or temocillin the faecal endotoxin concentration decreased to 10% of the control level. Following TD with cephalotin and neomycin however, the faecal endotoxin concentration was decreased in two steps: In the first two days, concomitant with the disappearance of aerobic Gram-negative bacilli the faecal endotoxin concentration was reduced in a similar way as found during SE. However, after 2 days of TD an additional reduction of the faecal endotoxin concentration followed to 1% of the control.

One of the control experiments required for this type of study concerns the question whether these antibiotics, which were administered orally to mice, interfere with the Limulus assay for endotoxin (*Jorgensen* and *Smith*, 1974; *Gardi* and *Arpagaus*, 1980). Therefore, these antibiotics were administered to

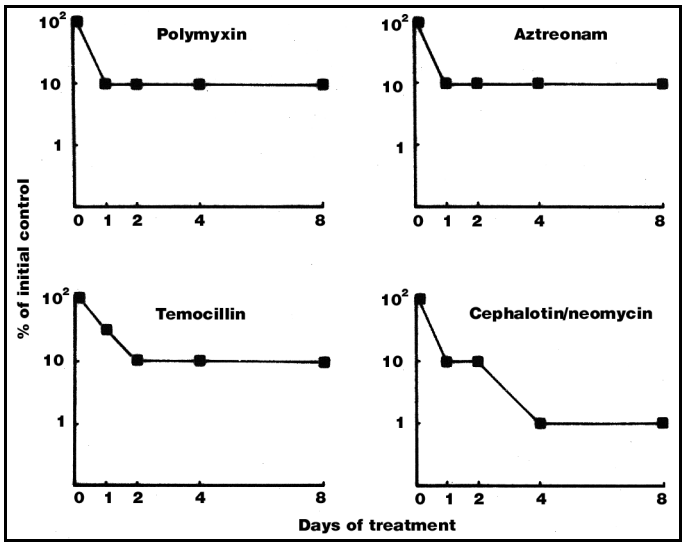

Figure 1: Effect of antimicrobial treatment on the faecal endotoxin concentration.

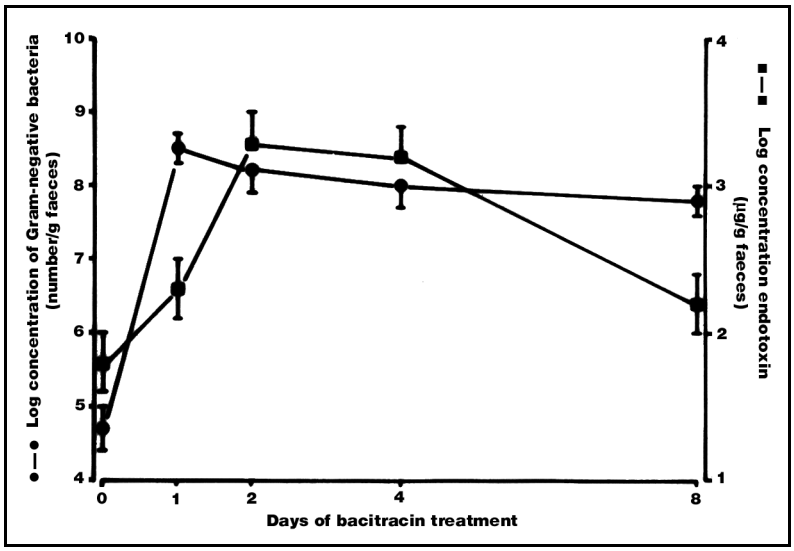

Figure 2: Faecal concentration of Gram-negative bacteria and endotoxin during bacitracin treatment.

faecal supernatants of control mice in concentrations similar to the concentrations which were measured after oral administration. The antibiotics used in the antimicrobial regimens did not influence the outcome as we found 100% recovery of endotoxin. On the basis of these results we could conclude that there is no interference of the antibiotics with the Limulus assay.

The SE experiments, therefore, indicate that 90% of the faecal free endotoxin is due to continuous release of endotoxin by aerobic Gram-negative bacteria. Secondly, on the basis of the total decontamination experiment it can also be concluded that 1% of intestinal endotoxin in mice could be ascribed to oral intake with food and water, while 9% of the faecal endotoxin is most probably due to release by anaerobic Gram-negative bacteria.

SELECTIVE GROWTH STIMULATION

We did not restrict our studies to the elimination of particular strains. We also selectively stimulated growth of Gram-negatives in the intestines of our mice. The results of these experiments are given in Figure 2. To accomplish Gram-negative growth enhancement we administered bacitracin orally via the drinking water. This antibiotic has small spectrum anti Gram-positive activity and was administered to the drinking water to reduce the colonisation resistance (*van der Waaij* et al., 1971). Gram-negative strains are essentially resistant to this antimicrobial regimen. Soon after the start of bacitracin treatment an immediate increase occurs of the level of Gram-negative bacteria in the faeces. Already after 1 day of oral bacitracin treatment a 4-log increase in the level of Gram-negatives was observed. The Gram-negatives remained at a high concentration level during the entire treatment period of eight days of bacitracin treatment. In these mice the faecal endotoxin concentration was also determined. During the first two days of bacitracin treatment, we observed an

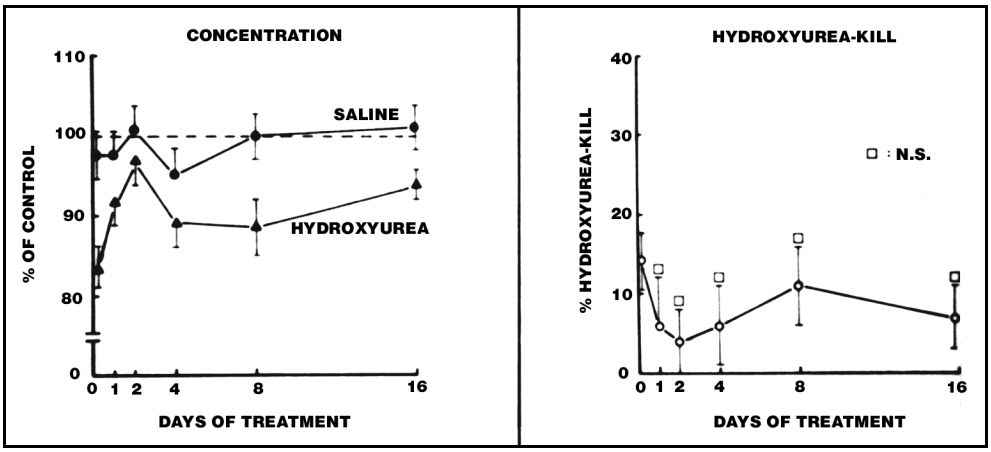

Figure 3: Femoral CFU-S and hydroxyurea-kill during polymyxin treatment.

increase of the faecal endotoxin concentration from 60 μg per g of faeces to 2000 μg per g of faeces. This is a very strong and significant increase in the faecal endotoxin concentration. Remarkably, after eight days of bacitracin treatment, however, we observed a gradual decline of the faecal endotoxin concentration. Several days after this relatively short observation period, the faecal endotoxin level was no longer significantly different from the control level; i.e. before bacitracin treatment. On basis of these results we may conclude that *in vivo* there is no strict relationship between the concentration of aerobic Gram-negative bacteria and the level of faecal endotoxin. It supports the conclusion that endotoxin is predominantly a product of highly proliferative Gram-negative bacteria.

THE EFFECT OF INTESTINAL FLORA MODULATION ON THE PLURIPOTENT AND THE COMMITTED HAEMOPOIETIC STEM CELL

Polymyxin was administered to SE mice and the femoral CFU-s were followed to determine the pool size of the CFU-s and the hydroxyurea-kill. As can be seen in Figure 3 we did not observe a change in the femoral CFU-s pool size during polymyxin treatment. Upon administration of hydroxyurea, a reduction of the hydroxyurea-kill was observed in these mice in comparison to controls. However, this reduction did not reach significance, possibly because of the relatively high initial value and because of the variance of the assay.

In Figure 4 the effects of polymyxin treatment on the femoral CFU-GM are shown as well as the CFU-GM pool size and the CFU-GM hydroxyurea-kill. In contrast to what was found in the CFU-s determination, there was a significant reduction in the femoral CFU-GM pool size to approximately 60% of the control after 4 days of polymyxin treatment. Also following hydroxyurea-kill, a significant reduction was found. The hydroxyurea-kill was reduced from 30% to approximately 10% already after one day of polymyxin

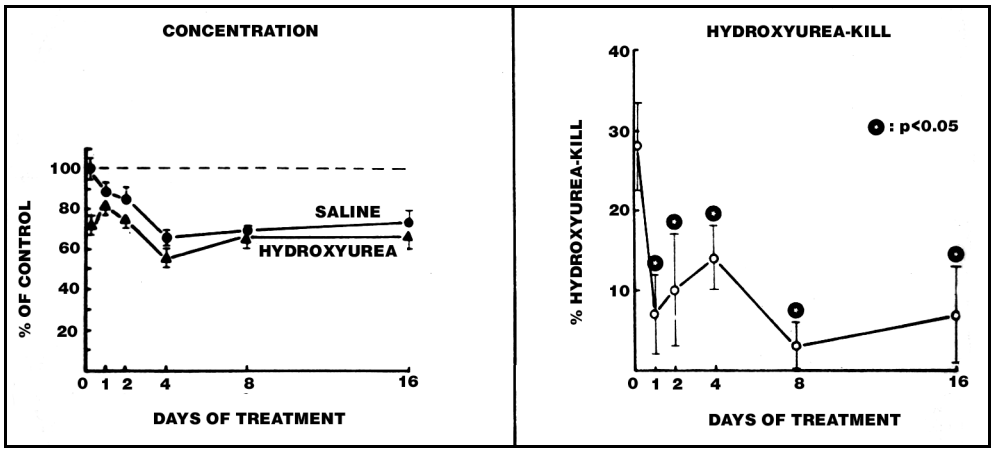

Figure 4: Femoral CFU-GM and hydroxyurea-kill during polymyxin treatment.

treatment. We have shown that polymyxin treatment reduces the proliferative state of haemopoietic stem cells. However, it is not certain after the experiment that the polymyxin effect represents a flora-mediated effect. This was studied in a subsequent experiment, during which we administered polymyxin also to germfree mice. In the germfree mice, this treatment did not cause a reduction of the CFU-GM during oral polymyxin treatment. Furthermore, there was no significant change in the hydroxyurea-kill in comparison to what was found in the polymyxin treated conventional mice.

Based on these findings in the germfree mice it can be concluded that the aerobic Gram-negative intestinal bacteria play an obvious role in the regulation of haemopoiesis.

HAEMOPOIETIC RECOVERY

Mice were treated with ARA-C, i.e. 3 sequential injections in conventional mice treated with polymyxin or bacitracin treatment. Because the former experiment had indicated that polymyxin decreased haemopoietic stem cell activity, and that bacitracin would enhance growth of Gram-negatives in the first week and therewith would increase the proliferative state of stem cells because of the high endotoxin level associated with Gram-negative proliferation, we decided to study the effect of these different flora modulating regimens. Just before ARA-C injection as a result of polymyxin, the endotoxin level was present in our mice as a low faecal endotoxin level. In contrast, the bacitracin-treated mice were found to have a very high endotoxin faecal level, as was to be expected. Figure 5 shows the effect of these pre-treatment regimens on the recovery of the femoral nucleated cell content and the CFU-GM content. In case of bacitracin treatment as well as in case of polymyxin SE, a bi-phasic recovery of the femoral CFU-GM was found after ARA-C injection. Following a first rebound of the femoral CFU-GM content on day 2, the second rebound started on day 6 or day 8. It is clear from these data that, in case of bacitracin treatment (i.e. in case of a high intestinal endotoxin level), the first re-

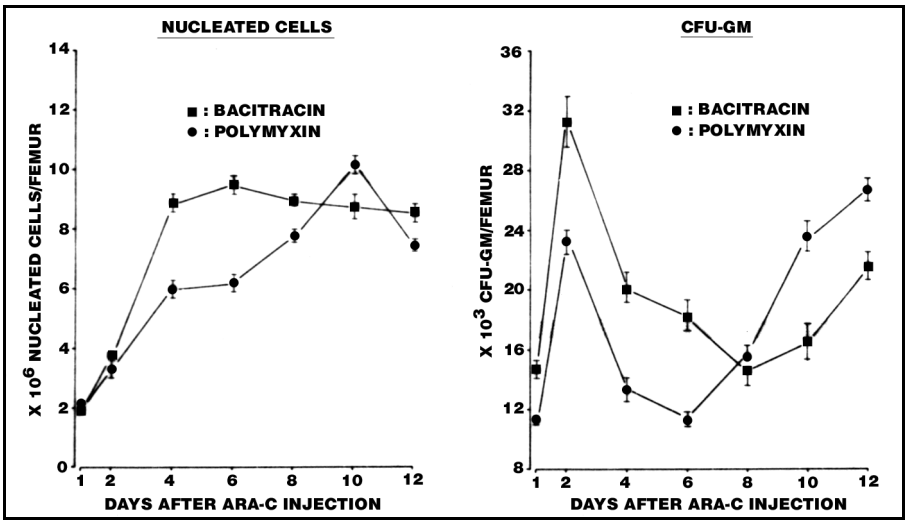

Figure 5: Effect of bacitracin and polymyxin treatment on femoral nucleated cells and CFU-GM after ARA-C injection.

bound increase of the femoral CFU-GM was significantly higher than in case of polymyxin treatment. These effects of endotoxin were also reflected in the recovery of the femoral nucleated cell content. In case of polymyxin pre-treatment it took 10 days after ARA-C injection before the femoral nucleated cell content was restored to the control value. In case of bacitracin pre-treatment, however, the nucleated cells were already recovered after 4 days. This means most probably, that at a high intestinal endotoxin level the recovery of the femoral nucleated cell content is accelerated with as much as 6 days.

CONCLUSION

Elimination of Gram-negative bacteria by antimicrobial treatment, which reduces the intestinal endotoxin level, may cause a delayed haemopoietic recovery after cytotoxic insult.

LITERATURE

Gans, H. and Matsumoto, K.: The escape of endotoxin from the intestine. Surg. Gynaecol. Obstet. 139, 395-402 (1974).

Gardi, A. and Arpagaus, G.R.: Improved microtechnique for endotoxin assay by the limulus amebocyte lysate test. Anal. Biochem. 109, 382-385 (1980).

Jorgensen, J.H. and Smith, R.F.: Measurement of bound and free endotoxin by the limulus assay. Proc. Soc. Exp. Biol. Med. 162, 44-47 (1974).

Joshi, J.H., Entringer, M.A., and Robinson, W.A.: Bacterial stimulation of serum colony-stimulating activity and neutrophil production in germfree mice. Proc. Soc. Exp. Biol. Med. 162, 44-47 (1969).

Rothfield, L. and Pearlman-Kothencz, M.: Synthesis and assembly of bacterial membrane components. A lipopolysaccharide-phospholipid-protein complex excreted by living bacteria. J. Mol. Biol. 44, 477-492 (1969).

Staber, F.G., Taressay, L., and Dukor, P: Modulation of myelopoiesis *in vivo* by chemically pure preparations of cell wall components from Gram-negative bacteria: Effects at different stages. Infect. Immun. 20, 40-49 (1978).

Till, J.E. and McCulloch, E.A.: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Research 14: 212-222 (1961).

van der Waaiy, D. and Sturm, C.A.: Antibiotic decontamination of the digestive tract of mice. Technical procedures. Lab. Animal Care 18, 1-10 (1968).

van der Waaij, D., Berghuis, J.M., and Lekkerkerk, J.E.C.: The colonization of the digestive tract in conventional and antibiotic treated mice. J. Hyg. 69:405-411 (1971).

van der Waaij, D. and Berghuis-de Vries, J.M.: Selective elimination of *Enterobacteriaceae* species from the digestive tract in mice and monkeys. J. Hyg. 72, 205-211 (1974).

Walker, R.I., MacVittie, I.J., Sinha, B.L., Ewald, P.E., Egan, J.E., and MacLung, G.L.: Antibiotic decontamination of the dog and its consequences. Lab. Animal Science 28, 55-61 (1978).

CHANGES IN MURINE GRAFT-VERSUS-HOST DISEASE AFTER PARENTERAL INJECTION OF THE DONOR WITH INTESTINAL MICROBIAL FLORA

DICK VEENENDAAL, FRAUKJE DE BOER, and DIRK VAN DER WAAIJ

Department of Medical Microbiology, University Hospital Groningen, Groningen, The Netherlands

Lecture presented at the 2nd Old Herborn University Seminar on "Interactions between the indigenous microflora and the host immune system" on June 1, 1988

SUMMARY

Graft-versus-Host Disease (GvHD) is a well-known complication of allogeneic bone marrow transplantation (allo-BMT). Mice engrafted with allogeneic bone marrow (BM) cells may suffer late onset GvHD (LO-GvHD), previously called secondary disease. LO-GvHD is known to be determined by the presence and the composition of intestinal microbial flora (I-MF) in the recipient as well as the donor. Anti-MF antibodies have been postulated to cross-react with tissue antigens and subsequently induce LO-GvHD. In this study allo-BMT experiments were carried out with specific pathogen free (SPF) C57Bl/6J (B6) (H-2b) donors and lethally irradiated SPF C3H/He (C3) (H-2k) recipients. In order to obtain different starting points at the donor site with regard to anti-MF antibody responses, B6 donors received single (day -10) or repeated (day -38 and -10) intra-peritoneal (i.p.) injections either with MF of their own (SELF-MF) or C3 recipients (RECIP-MF). Lethal LO-GvHD was found significantly ($p<0.01$) the highest (15/35=43%), in C3 recipients engrafted with BM from single SELF-MF injected B6 donors. On the other hand mortality was found the lowest in recipients engrafted with BM from donors twice injected with SELF-MF (1/25= 4%). Low mortality rates were also found in C3 recipients engrafted with BM from other B6 donors, i.e. saline injected controls (4/26=15%), and B6 donors injected once (6/32=19%) or twice (5/26=19%) with RECIP-MF. Isotype nor MF-specific serum antibodies in the different B6 donor groups showed any correlation with the occurrence of LO-GvHD in C3 recipients. It is discussed how LO-GvHD might have occurred in C3 recipients due to an interaction between RECIP-MF and 'activated' BM-cells from SELF-MF challenged donors.

INTRODUCTION

Graft versus Host Disease (GvHD) may occur as a serious complication after allogeneic bone marrow transplantation (allo-BMT) in lethally irradiated (9

Gy) mice. Transfer of allogeneic bone marrow (BM) cells together with spleen cells will cause death of all recipients within three weeks after transplantation (*Heidt* et al., 1981; *van Bekkum* et al., 1974a). However, only part of the recipients may die due to lethal GvHD after transfer of 10^7 allo-BM-cells without spleen cells (*Heidt* et al., 1981; *Veenendaal* et al., 1988) and clinical symptoms become manifest late, three weeks after transplantation. This type of GvHD, which has previously been named secondary disease, is therefore referred to as minor, delayed type, or late onset GvHD (LO-GvHD) (*Heidt* et al., 1981; *Pollard* et al., 1976; *Rappaport* et al., 1979; *van Bekkum* et al., 1974a).

LO-GvHD appears to be associated with the presence of intestinal microbial flora (I-MF) in the recipient, since it is strongly mitigated or even absent in germfree (GF), antibiotic decontaminated, and "clean" specific pathogen free (SPF)* mice (*Heidt*, 1989; *Jones* et al., 1971; *van Bekkum* et al., 1974a,b). LO-GvHD does not occur when GF or totally decontaminated recipients are (re)conventionalised 40 days after marrow transplantation (*van Bekkum* et al., 1974a). Thus there is a window of approximately 40 days during which the LO-GvHD 'inducing' capacity of the recipient's intestinal microbial flora decreases.

There is evidence that not only the recipient's I-MF but also the I-MF of the donor plays a role in MF-associated LO-GvHD (*Heidt* et al., 1989). Previous experiments in mice have shown that modulation of donor-MF by oral treatment with non-absorbable small spectrum antibiotics increased the occurrence of LO-GvHD (*Veenendaal* et al., 1988). Moreover, there is evidence that modulation of the I-MF by oral antibiotic treatment decreases myelopoiesis in the bone marrow. This effect may add to the changes of the cellular composition of the BM-graft (*Goris* et al., 1985; 1986a).

It is still unknown by which mechanism LO-GvHD occurs. Bacteria in the digestive tract have been postulated to share antigens common to the recipient host and thus induce cross-reacting antibodies (*van Bekkum* and *Knaan*, 1977). Since the induction of systemic humoral and cellular tolerance to intestinal antigens, generally referred to as oral tolerance, is found to be inhibited during GvHD (*Strobel* et al., 1985; *Strobel* and *Ferguson*, 1986), unopposed B-cell proliferation may contribute to high levels of anti-MF antibodies (*Hamilton* and *Parkman*, 1983; *van der Waaij* and *Heidt*, 1987). If so, immunostimulation by intestinal (bacterial) antigens is likely to occur in the first week(s) after marrow engraftment.

Whether or not engrafted BM-cells are able to react with MF-antigens present in the recipient host and thus induce and/or aggravate LO-GvHD may depend on the composition and the activated state of the engrafted cells. The BM as a major site of long living immunoglobulin (Ig) producing cells during secondary responses has been documented (*Benner* et al., 1981). An increase of IgA producing B-cells in the BM has been found during the secondary response against orally given antigens (*Alley* et al., 1986). So far there is a lack of information on the possible relationship between anti-RECIP-MF antibodies in the donor and LO-GvHD in allogeneic recipients.

---

* Clean SPF means strict protection against (microbial) contamination from the outside world, which makes the flora exceptionally stable in composition. For generations the entire I-MF may be (immunologically) tolerated.

In the present study we investigated whether modulation of the immune response by allogeneic C57Bl/6J (B6) (H-2b) donors against their own I-MF (SELF-MF) or that of C3H/He (C3) (H-2k) recipients (RECIP-MF) affected LO-GvHD. A difference in the immunoreactivity against RECIP-MF and SELF-MF antigens could not be excluded on forehand with regard to primary or secondary responses upon parenteral challenge. For this reason B6 donors were injected intra peritoneally (i.p.) once or twice with either SELF-MF or RECIP-MF prior to allo-BMT. The I-MFs of both mouse strains used in this study were found to be similar regarding species of genera of facultative anaerobic bacteria such as *Enterobacteriaceae*. However, the composition of (highly concentrated) obligate anaerobic bacteria was found morphologically different at microscopic examination of Gram-stained faecal pellets.

MATERIALS AND METHODS

Mice

Five to seven weeks old male C57Bl/6J (B6) (H-2b) and C3H/He (C3) (H-2k) mice were purchased from the SPF unit of the Zentral Insitut für Versuchstierzucht (Hannover, FRG) and used at 10-12 weeks of age. Weights varied between 25 and 30 grams per mouse.

Housing

Animals were housed in a conventional animal room at the Central Animal Facility (University of Groningen, The Netherlands), fed with rodent pelleted food (RMH-B, Hope Farms B.V., Woerden, The Netherlands), and tap water *ad libitum*. No antibiotics were used.

Sampling of faeces

Fresh faeces from B6 donor mice and C3 recipient mice were sampled 2 weeks after arrival, pooled and stored at -70°C in small aliquots for immunisation and for detection of microbial flora (MF) specific antibodies.

Table 1: Outline i.p. injection of B6 donors with their own microflora (SELF-MF) or C3 recipient MF (RECIP-MF) before BMT

		Days before BMT		
Group	(donors)	38♥	10♥	
A	(2xsaline)	Saline♠	Saline	
B	(1xSELF-MF)	Saline	SELF-MF	
C	(2xSELF-MF)	SELF-MF	SELF-MF	→C3H/He♣
D	(1xRECIP-MF)	Saline	RECIP-MF	(10^7 BM-cells)
E	(2xRECIP-MF)	RECIP-MF	RECIP-MF	

♥) Injection dose: 0.5 ml of 1:50 suspension of (washed) faeces holding approximately $5x10^9$ m.o./ml.

♠) Saline = Pyrogen free saline.

♣) C3H/He recipients received 9 Gy total body irradiation (speed: 0.45 Gy/min.) 24 hrs before BMT.

Bacteriological culturing and typing

Faeces were cultured quantitatively for *Enterobacteriaceae*, *Staphylococci*, *Enterococci* and yeasts as described previously (*Veenendaal* et al., 1988). *Enterobacteriaceae* were biotyped according the API 20E classification system (Analytab Products Ind., France). The composition of highly concentrated obligate anaerobic species in the MF of B6 and C3 mice was examined microscopically after Gram-staining of washed faeces.

MF-immunisation

B6 donors were divided into 5 groups of 10-15 mice. Each mouse was injected intra peritoneally (i.p.) according the outline given in Table 1. Briefly, mice were injected i.p. either with 0.5 ml of washed faeces or with pyrogen free saline. Washing of faeces was carried out as follows: frozen aliquots of faeces were thawed, suspended in pyrogen free saline (1:9 w/v) and centrifuged at 12g for 5'. Supernatants were centrifuged 10,000 g for 20'. The pellets were re-suspended in pyrogen free saline (5x the volume of the supernatant). This 1:50 suspension of washed faeces contained approximately $5x10^9$ bacteria per ml. This processing procedure will have killed practically all anaerobic bacteria in the suspension.

Irradiation

C3 recipients received lethal total body irradiation 24 hr. before BMT as described previously (*Veenendaal* et al., 1988). The total dose given was 9 Gy; radiation speed 0.45 Gy/min.

Bone marrow transplantation

B6 donors were exsanguinated before harvesting BM-cells. Sera were stored at -20°C. Isolation, preparation, and pooling of BM-cells was carried out as described previously (*Veenendaal* et al., 1988). For each group (Table 1) of approximately 30 C3 recipients, 10-15 B6 donors were needed. Recipients were injected intravenously in the orbital plexus with 10^7 nucleated B6 BM-cells in 0.25 ml (conc. $4x10^7$ nucleated cells/ml). During injection, the recipients received general anaesthesia by N_2O, O_2 (2:1) and fluothane 3.0%. Radiation Controls received total body irradiation only. Chimaerism was assessed on day 35 and 100 by Hb-electrophoresis as described previously (*Veenendaal* et al., 1988).

Monitoring GvHD

Between day 0 and 100 after irradiation, recipients were daily screened for clinical symptoms, mortality and histopathological changes.

Clinical symptoms

Body weights were determined individually at weekly intervals. Mice showing progressive weight loss, hunched back, ruffled fur, diarrhoea, skin lesions, and persistent dullness after stimulation were defined as moribund and killed by cervical dislocation after exsanguination via the plexus orbitalis.

Mortality

Mortality was divided into three periods according to *Rappaport* et al. (1979); 1st phase in which mice die of graft failure (day 0-10), 2nd phase in which mice die when suffering from acute GvHD (day 11-18), and a 3rd phase in which mortality occurs delayed (day 19-100). Animals that died during the first phase were excluded from this study.

Histopathology

At autopsy mice were examined macroscopically for aplasia of the thymus. Histology was carried out on spleen,

thymus (if present), skin, liver, colon, small intestine, and lungs from moribund and surviving mice with and without symptoms. Organs were fixed in Zenker's solution, stained with H&E or Brachet (methylgreen-peponin) and histologically examined for GvHD features as described by *Hamilton* and *Parkman* (1983) and *Rappaport* et al. (1979).

Anti-MF immune response

In order to obtain information about possible differences in the immune response of B6 donors against SELF-MF or RECIP-MF the spleen weight index as well as immunoglobulins in serum were determined at the time of BM harvesting.

Spleen weight index (SWI)

The SWI of each donor mouse (formula C) was calculated by dividing the individual relative spleen weight (formula A) by the mean relative spleen weight of saline injected controls (formula B):

$$\text{(A):}\quad \text{Relative spleenweight} = \frac{\text{Spleenweight}}{\text{Bodyweight}}$$

$$\text{(B):}\quad \frac{\sum_{1}^{n} (\text{Relative spleenweight})}{n}$$

$$\text{(C):}\quad \text{SWI}\,(x) = \frac{\text{A}\,(x)}{\text{B (controls)}}$$

Serum immunoglobulins

Isotype serum immunoglobulin (Ig) M, IgG and IgA as well as microflora specific antibodies were detected by using an enzyme linked immuno sorbent assay (ELISA). Briefly, 96 wells flat bottom trays (Greiner, Nürtingen, FRG) were incubated with goat anti mouse (GAM) IgM, IgG or IgA isotype specific antisera (Sigma, St. Louis, USA) for 1.5 hr. at 37°C. The antisera were diluted (1:1000) in 0.1 M carbonate buffer (pH 9.6). Trays were coated with a suspension of washed faeces 1:80 in 50 mM citrate buffer (pH 4.6); incubation: 1 hr. 30' at 37°C, for detection of anti B6 (=SELF)-MF and anti C3 (=RECIP)-MF isotype specific antibodies. After coating and each subsequent step, the trays were emptied and washed 3x5' with washing buffer (0.01 M Tris, 0.15 M NaCl, 0.05% Tween-20). Incubation with test sera was carried out for 45' at 37°C. The test sera were two-fold serially diluted in incubation buffer (0.01 M Tris, 0.15 M NaCl, 0.05% Tween-20, 1% Bovine Serum Albumin) starting at: 1:1000 for total serum IgM and IgG levels, 1:100 for total serum IgA, and 1:10 for microflora specific antibodies. Trays were incubated for 30' at 37°C with Horseradish Peroxidase conjugated GAM-IgM, -IgG and -IgA isotype specific antibodies (Sigma, St. Louis, USA). Conjugates were diluted 1:1000 in incubation buffer. Substrate (0-phenylene diamine dihydro chloride 0.04% and ureum peroxide) was added to each well for the final step and incubated for 30'

Table 2: Analysis of pooled faeces from B6 and C3 mice

Microorganism	B6 (n=5)♥	C3 (n=5) ♥
E. coli (API: 5144552)	1.6×10^3	2.0×10^3
Prot. mirabilis (API: 0536000)	2.0×10^4	2.5×10^4
Enterococcus spp.	5.0×10^4	3.0×10^4
Staph. Aureus	1.0×10^4	1.5×10^4
Bacillus spp.♣	ND♦	ND
Obligate anaerobic bacteria♠	10^{11}	10^{11}

♥) Data represent concentrations in m.o./g. faeces.
♣) Qualitative aerobic culturing only.
♠) Microscopic analysis by eye revealed a morphological difference between highly concentrated obligate anaerobic fractions in B6 and C3 faeces.
♦) ND: not determined.

at room temperature. The substrate conversion was stopped by adding 4N H_2SO_4 and the extinction measured photometrically at OD492 on an ELISA reader (Titertek Multiskan). In each tray a pooled reference serum was measured to verify the ELISA procedure.

Statistical analysis.

Statistical evaluation of the significance of differences in survival rates between group was carried out by Kaplan-Meier analysis. Statistical evaluation of the occurrences of clinical manifestations of GvHD was carried out by the chi-square or Fischer-exact test with Bonferroni correction. Differences in the bacterial concentrations were evaluated by the Mann-Whitney rank sum test. Differences in total and anti-MF specific serum antibody levels, and relative spleen weight index were evaluated by Newman-Keuls analysis of variance with Bonferroni correction for multiple comparison. Significance levels were taken at $p<0.05$.

RESULTS

Faecal analysis

Culturing

Results of quantitative and qualitative culturing of B6 and C3 faeces are shown in Table 2. No differences were found in the concentrations or biotypes of *Enterobacteriaceae*; e.g. *Escherichia coli* (*E. coli*) API20E: 5144552 and *Proteus mirabilis* API20E: 0536000, and *Staphylococci* and *Enterococci*. Yeasts were not detected. *Bacillus* spp. were detected in either faeces, but not quantified nor identified.

Micromorphology

Micromorphological examination of Gram-stained samples of washed faeces showed a clear difference in the composition of highly concentrated, predominantly obligate anaerobic, bacteria in the faeces of B6 and C3 mice. Gram-positive and Gram-negative fusiform and long shaped bacteria were predominant in B6-MF, whereas Gram-negative small rods and coccoid bacteria predominated in C3-MF.

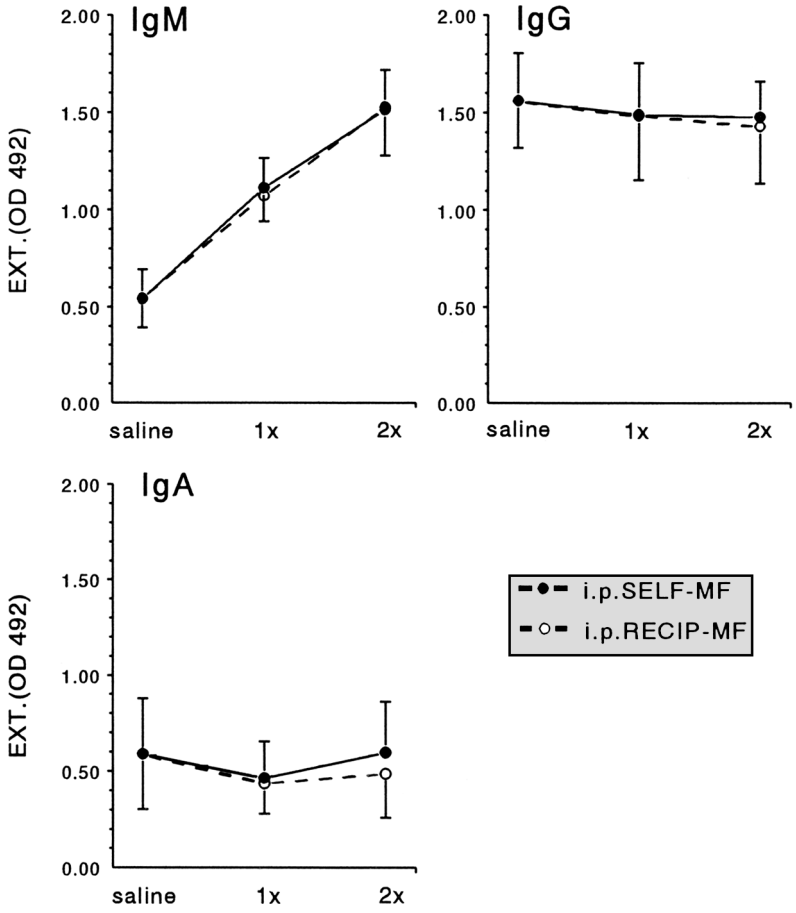

Figure 1: Isotype serum immunoglobulins in C57Bl/6J (B6) donors i.p. injected with saline (n=10), washed faecal flora of their own (SELF-MF) or washed faecal flora of C3H/He recipients (RECIP-MF). I.p. injection was performed 10 days before serum sampling for single injection; 1xSELF-MF (n=12), 1xRECIP-MF (n=16). Repeated injection was performed on day -38 and -10; 2xSELF-MF (n=12), 2xRECIP-MF (n=12). IgM antibodies increased significantly ($p<0.05$) after single injection and raised further ($p<0.05$) after repeated injection.

Clinical response to i.p. MF injection

Mice did not show clinical signs of disease upon i.p. injection of I-MF. This could be due to the fact that the majority of bacteria was dead due to contact with O_2 during the processing of the MF-suspension for injection.

Immune reactions

Isotype serum antibodies

Isotype IgG, IgM, and IgA levels in serum of donors were determined in 6 individual sera per group. Each serum was tested *in duplo*. The antibody levels are presented as mean extinction levels at OD492 for single serum dilutions (Figure 1). The following single serum dilutions were used: 1:2000 for IgM, 1:16,000 for IgG, and 1:400 for IgA. At these dilutions the highest differences were found between the various groups (data not shown). IgM antibodies in donors increased significantly ($p<0.05$) after single (group B,D) and repeated (group C,E) injection with

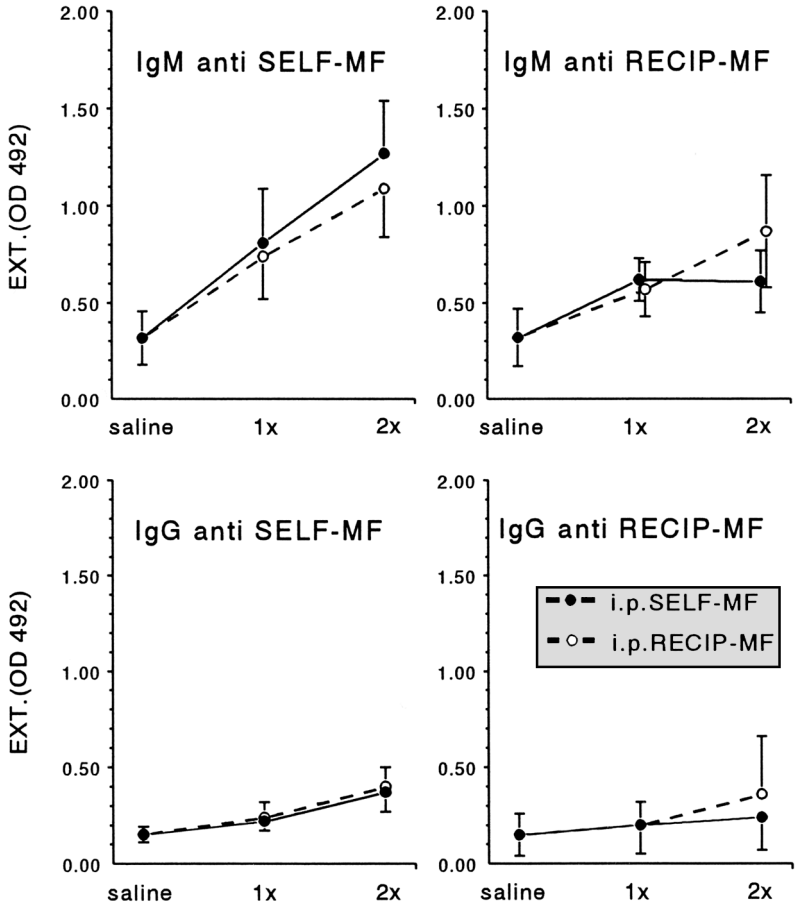

Figure 2: Anti-microflora antibodies in serum of C57Bl/6J (B6) donors i.p. injected with saline (n=10), washed faecal flora of their own (SELF-MF) or washed faecal flora of (C3H/He) recipients (RECIP-MF). I.p. injection was performed 10 days before serum sampling for single injection; 1xSELF-MF (n=12), 1xRECIP-MF (n=16) and 38 and 10 days before sampling in case of repeated injection; 2xSELF-MF (n=12), 2xRECIP-MF (n=12).

SELF-MF as well as with RECIP-MF compared to saline injected (group A) donors. No significant differences were observed for IgG or for IgA in each donor group.

Anti-MF serum antibodies

IgM, IgG, and IgA anti SELF-MF and anti RECIP-MF specific antibody levels are presented as mean extinction levels at OD492 for single serum dilutions (Figure 2). The serum dilutions were 1:20 for IgM and 1:10 for IgG. IgA anti-MF serum levels were not detectable at the 1:10 serum dilution i.e. stayed below the extinction rate of 0.2 at OD492. IgM anti-SELF-MF and IgM anti-RECIP-MF antibodies both increased equally significant ($p<0.05$) in all 1xSELF-MF (group B) and 1xRECIP-MF (group D) injected donors compared to saline injected controls (group A) (Figure 2). IgM anti-SELF-MF further increased significantly in donors twice injected with SELF-MF (group C) as well as with

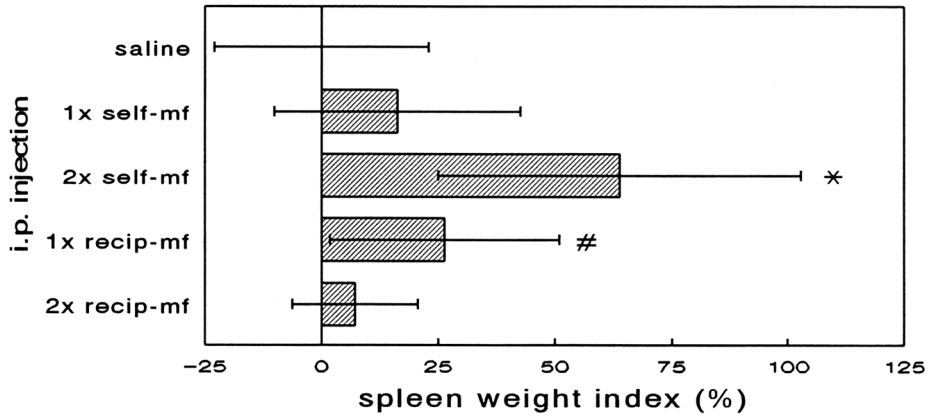

Figure 3: Spleen weight index in C57Bl/6J donors i.p. injected either with saline (n=7), 1xSELF-MF (n=13), 1xRECIP-MF (n=5), 2xSELF-MF (n=12), and 2xRECIP-MF (n=10). I.p. injection was performed on day -10 before BMT for single injection and day -38 and -10 for repeated injection. (*) = $p<0.01$ compared to all other groups; (#) = $p<0.05$ compared to saline injected animals.

RECIP-MF (group E). IgM anti-RECIP-MF antibodies increased further in 2xRECIP-MF injected donors (group E) but not in 2xSELF-MF donors (group C) (Figure 2). No significant differences were found in the IgG anti-SELF-MF levels in either donor group. However, IgG anti RECIP-MF antibodies significantly increased ($p<0.05$) in 2xRECIP-MF injected B6-donors compared to saline injected controls (Figure 2).

Spleen weight

Mean spleen weight index of each of the B6-donor groups is shown in Figure 3. The spleen weight index was found to be increased ($p<0.01$) in 2xSELF-MF injected donors compared to all other groups. The spleen weight index in 1xRECIP-MF injected donors was found significantly higher ($p<0.05$) compared to saline injected controls.

Chimaerism

Mice tested for chimaerism carried 100% the Hb pattern of B6 donors on day 35 after transplantation.

Clinical symptoms

Early mortality and symptoms

Irradiated control C3 mice, not engrafted with BM, all died by day 10; mean survival time 7.5±1.5; range 5-10 days. 'Engrafted' mice that died during this period were recorded as "graft failures" and were excluded from this study. By day 11 the graft will have taken and starts functioning. "Early mortality" was defined as mortality between day 11 and 18 after engraftment according to *Rappaport* et al. (1979); results shown in Table 3. Mortality during this period ranged from 8% (2/27) in group C (C3 recipients engrafted with BM from 2xSELF-MF injected donors) to 0% in group A (n=26) and B (n=35). There was no significant difference between the groups.

Weight loss was found in all mice that died before day 18. In surviving C3 recipients, however, early weight loss was found to be the strongest in 17/26 (65%) of the animals engrafted with BM from 2xRECIP-MF injected donors (group E). This was significantly the highest ($p=0.01$) compared to animals in group A, B and C (Table 3). Except

Table 3: 'Early' mortality and weight loss (>10%) in C3 recipients engrafted with bone marrow from MF injected B6 donors; 11-18 days after BMT

Group (donor)♥	'Early' mortality (%) 11-18 days after BMT	Weight loss in survivors 11-18 days after BMT
A (saline)	-/26 (-)	6/26 (23)
B (1xSELF-MF)	-/35 (-)	10/35 (29)
C (2xSELF-MF)	2/27 (8)	9/25 (36)
D (1xRECIP-MF)	1/33 (3)	14/32 (44)
E (2xRECIP-MF)	1/27 (4)	17/26 (65) ♣

♥) C57Bl/6J donors i.p. injected once (day -10) or twice (day -38 and -10) with washed faeces from their own (SELF-MF) or C3 recipients (RECIP-MF). Control donors were injected with saline.

♣) p=0.01 compared to group A, B, and C (Chi-square test with Bonferroni correction).

for one animal, which had been engrafted with BM from 2xSELF-MF injected B6-donors and died on day 16, no diarrhoea was seen before day 18 after transplantation in any of the C3 recipients.

'Late' mortality and symptoms at 18-100 days after BMT

Survival rates, weight loss and the occurrence of diarrhoea between day 18 and 100 after BMT are shown in Figure 4 and Tables 4 and 5 respectively. Mortality after day 18 was found the highest; 15/35 (43%) ($p<0.01$) in C3 recipients engrafted with BM from 1xSELF-MF injected donors (group B) (Figure 4). Non-surviving animals in this group also died earlier (mean 45.9 days, range 23-91) compared to mice suffering from lethal GvHD in other groups (overall mean: 65.0 days; range: 43- 100 days).

Table 4: Weight loss (>10%) 19-100 days after BMT in surviving and non-surviving C3 recipients engrafted with bone marrow from microflora injected B6 mice

		Weight loss (>10%) 19-100 days after BMT		
Group (donor)♥	Number of recipients (at day 19)	Non-survivors	Survivors	Total (%)
A (saline)	26	3	6	9 (35)
B (1xSELF-MF)	35	13♣	6	19 (54)♠
C (2xSELF-MF)	25	1	2	3 (12)
D (1xRECIP-MF)	32	3	4	7 (22)
E (2xRECIP-MF)	26	4♣	10	14 (52)♠

♥) C57Bl/6J donors i.p. injected once (day -10) or twice (day -38 and -10) with washed faeces from their own (SELF-MF) or C3 recipients (RECIP-MF). Control donors were injected with saline.

♠) p≤0.02 compared to group A, C, and D (Chi-square test with Bonferroni correction).

♣) p=0.03 between non-survivors and survivors in group B and E (Fisher-exact test).

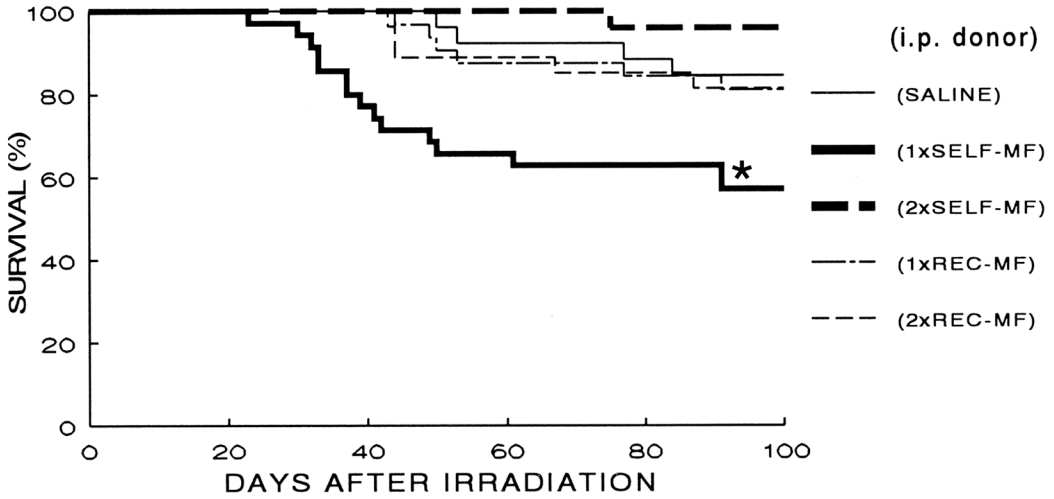

Figure 4: Survival rates of lethally irradiated (9 Gy) C3H/He (H-2k) recipients engrafted with 10^7 nucleated BM cells from C57Bl/6J (H-2b) donors 19-100 days after BMT. Donors had been pre-treated by i.p. injected with saline, washed faecal flora of their B6-strain (SELF-MF) or C3 recipients (RECIP-MF). I.p. injection was performed on day -10 for single injection and day -38 and -10 for repeated injection. The number of recipients at day 19 were: group A (2xsaline) n=26, group B (1xSELF-MF) n=35, group C (2xSELF-MF) n=25, group D (1xRECIP-MF) n=32, and group E (2xRECIP-MF) n=26. (*) = $p<0.01$ compared to all other groups (Kaplan-Meier analysis).

The overall occurrence of weight loss after day 18 was found to be the highest (p=0.003) in group B (1xSELF-MF donors) and in group E (p=0.02) (2xRECIP-MF donors) compared to recipients in the other groups (Table 4). With regard to survivors and non-survivors, however, weight loss appeared to be significantly the highest (p=0.03) in non-survivors in group B (1xSELF-MF injected donors) and significantly the lowest in non-survivors in group E (2xRECIP-MF injected donors).

Table 5: Diarrhoea 19-100 days after BMT in surviving and non-surviving C3 recipients engrafted with bone marrow from microflora injected B6 donors

Group (donor)♥	Number of recipients (at day 18)	Diarrhoea 19-100 days after BMT		
		Non-survivors	Survivors	Total (%)
A (saline)	26	3	2	5 (20)
B (1xSELF-MF)	35	5	4	9 (26)
C (2xSELF-MF)	25	1	-	1 (4)
D (1xRECIP-MF)	32	4	1	5 (16)
E (2xRECIP-MF)	26	4	2	6 (22)

♥) C57Bl/6J donors i.p. injected once (day -10) or twice (day -38 and -10) with washed faeces from their own (SELF-MF) or C3 recipients (RECIP-MF). Control donors were injected with saline.

In contrast to the early period after BMT (<day 18), diarrhoea was observed regularly in mice that died after day 18. The incidence of diarrhoea in the non-survivors varied between 33% and 100%. Like mortality, the overall occurrence of diarrhoea was found at the highest but not significant level in mice of group B (1xSELF-MF donors) (Table 5).

Arthritis

Around day 80 five out of all surviving mice that also showed skin changes as dyskeratosis of the ears and tail, started to display redness and swelling of the paws. These changes were self-limiting and healed spontaneously by day 100. There was no preference for arthritis to occur in any of the groups.

Histological examination

Lungs

Histological examination of lung tissue showed moderate to severe carnification in all mice. Only in moribund mice infiltrates with granulocytes were seen, whereas none of the tissues examined showed infiltrates with lymphocytes.

Thymus

All moribund mice that died after day 18 showed total aplasia of the thymus. Histologically, regeneration of the thymus was only found in mice that displayed clinical symptoms but survived until day 100. Animals surviving 100 days without any clinical symptom had a normal thymus.

Spleen

Histological examination of spleen sections of C3 recipients that died after day 18 revealed absence of follicle centres as well as the absence or a strongly decreased number of plasma cells. Evidence of haematopoiesis, however, was still found in these mice as granulocytes and megakaryocytes predominated in the splenic red pulpa.

Intestines

Changes in the intestines were only found in moribund mice that suffered from diarrhoea. A decrease was found in the number of goblet cells as well as a decrease of the length of the villi. No submucosal lymphocytic infiltrates were seen.

Kidneys

No histological changes were found in the kidneys of the animals examined.

DISCUSSION

This study provides additional evidence to our previous hypothesis (*Veenendaal* et al., 1988) that the incidence of intestinal microbial flora (I-MF) associated late onset GvHD (LO-GvHD) in mice (previously called secondary disease) is affected by both the I-MF of the recipient and the donor. LO-GvHD in C3 recipients was found to be affected when B6 donors had been i.p. injected with the autochthonous B6-strain I-MF (=SELF-MF) either once (10 days) or twice (38 and 10 days) before BM harvesting. On the one hand LO-GvHD mortality increased to 43% (control 15%) when using BM from once SELF-MF injected donors, whereas on the other hand LO-GvHD mortality decreased to 4% when twice SELF-MF injected donors were used. However, unlike our previous supposition, no correlation was found between LO-GvHD and the serological response by the donor against allochthonous

RECIP-MF or SELF-MF. Thus we conclude that anti-MF antibody levels alone are of no predictive value for I-MF associated LO-GvHD in mice. However, the presence or absence of anti-MF antibodies may still be a factor which indicates immune reactivity or immune suppression respectively.

An explanation for the donor I-MF to influence LO-GvHD is given by the assumption that the composition of the BM (graft) is determined by the composition of I-MF in the donor. Experiments in mice have shown that antibiotic modulation of I-MF not only affects haemopoiesis but also LO-GvHD when donor mice are treated as such (*Goris* et al., 1985, 1986a; *Heidt* et al., 1989; *Veenendaal* et al., 1988). A difference in the immunoregulation of autochthonous SELF-MF versus non-indigenous or allochthonous RECIP-MF may best be illustrated in B6 donors twice injected with either flora. IgM anti-RECIP-MF antibodies were found at a higher level in 2xRECIP-MF compared to 2x SELF-MF injected mice (Figure 2). On the other hand the mean spleen weight index was found significantly the highest in 2x SELF-MF injected donors, whereas it returned to a normal level in 2xRECIP-MF injected B6 mice (Figure 3). These data show that B6 mice respond immunologically different to allochthonous C3 recipient-MF (RECIP-MF) compared to their own autochthonous B6-MF (SELF-MF).

The bacterial subset in either SELF-MF or RECIP-MF to which the B6 immune system reacts differently, apparently should not be searched for within the low concentrated facultative anaerobes, like the *Enterobacteriaceae*, as this fraction was found to be similar in faeces of both mouse strains. This confirms previous reports on the minor role of *Enterobacteriaceae* in the pathogenesis of LO-GvHD (*Heidt* et al., 1989; *Veenendaal* et al., 1988). Instead, the highly concentrated fraction of the intestinal MF, predominantly containing obligate anaerobic bacteria, may be most important in this respect as this fraction was found to be morphologically different between the B6 and C3 mice used in this study. Within the fraction of highly concentrated obligate anaerobic bacteria, "non-immunogenic" and/or tolerogenic indigenous species may be of utmost importance in LO-GvHD, since any correlation was lacking between the levels of antibodies in serum mounted by B6 donors against immunogenic bacteria in either SELF-MF or RECIP-MF on the one hand and the induction of LO-GvHD in C3 recipients on the other.

In euthymic mice, there is evidence that the majority of the highly concentrated obligate anaerobic bacteria in the GI-tract are non-immunogenic. By using an indirect immuno-fluorescence technique we previously found that B6 as well as C3 mice, as were used in this study, both mount a detectable serum antibody response to only 10-15% of highly concentrated bacteria in their I-MF (unpublished results). B-cell tolerance has been described for autochthonous (host related) bacterial species as well (*Berg* and *Savage*, 1975; *Foo* and *Lee*, 1972), suggesting that these bacteria have become part of self and therefore are favoured to colonise the GI-tract of that particular host. The kind of defence against these indigenous species may be limited to granulocytes, macrophages and dendritic cells, i.e. the innate defence system.

The significant increase of the spleen weight index in 2xSELF-MF i.p. injected donors, may point at maximal stimulation of reticular endothelial cells, i.e. dendritic cells and macrophages, instead of lymphoid cells. This postulation is supported by the fact that only an increasing IgM response was found whereas an isotype switch to IgG or

IgA remained absent indicating the absence of T-cell mediated germinal centre reactions. However, unfortunately no histology was carried out on donor spleens in order to establish this postulation.

Stimulation of innate defence cells, i.e. granulocytes, macrophages, and dendritic cells together with the absence of a secondary response at the donor site still does not explain the differences in LO-GvHD in recipients engrafted with BM from single versus twice SELF-MF injected donors. Single i.p. injection with SELF-MF 10 days before BM harvesting in a way increased the LO-GvHD inducing capacity of the BM. However, this effect was fully reversed by repeated injection with SELF-MF. This indicates that the BM changes, which occurred after single i.p. injection with SELF-MF and aggravated GvHD, were only temporary. The first injection possibly created a new level of balance between I-MF and the immune "suppressor" system. Apparently, this level remained unchanged after i.p. challenge 28 days later.

LO-GvHD in this study was characterised by total aplasia of the thymus and depletion of germinal centres in the spleen. These features are similar to previous reports (*Hamilton* and *Parkman*, 1983; *Rappaport* et al., 1979; *Veenendaal* et al., 1988). Total lymphoid aplasia is responsible for T- and B-cell unresponsiveness during GvHD (*Hamilton* and *Parkman*, 1983; *Wall* et al., 1988). These findings correlated well with mortality, despite the persistent presence of granulocytes and macrophages in (the spleens of) mice suffering from lethal LO-GvHD. How LO-GvHD features are related to the temporary BM changes induced by SELF-MF remains subject of further study.

In conclusion, we postulate that the key for MF-associated LO-GvHD should be searched for within the non-immunogenic part of highly concentrated obligate anaerobic bacteria in the intestinal tract of the recipient. The degree at which engrafted BM cells in some way interact with this fraction and either cause or prevent LO-GvHD greatly depends on the level of 'activation' of BM cells mediated by the donor's own I-MF. The thymus becomes functionally damaged in the allo-transplanted recipient by allo-cells which have stimulated shortly before BMT. The "suppressor" system, which normally controls the response to SELF-MF, plays an important role in this process. If this is true, LO-GvHD in some part may be caused by I-MF antigens in the recipient which are shared by the donor, i.e. the degree of homology between donor and recipient-MF. Further study will be needed to elucidate this postulation.

ACKNOWLEDGEMENTS

This study was financially supported by the Dutch Cancer Foundation (GUKC 85-08). Thanks are due to drs. E. Zwart for working out the Elisa, J. Feringa of the Department of Histology and Cell Biology of the University of Groningen, and drs. W. Hofstra and the employees of the mouse breeding and housing unit of the Central Animal Facility of the University of Groningen for their skilful contribution.

LITERATURE

Alley, C.D., Kiyono, H., and McGhee, J.R.: Murine Bone marrow IgA responses to orally administered sheep erythrocytes. J. Immunol. 136, 4414-4418 (1986).

Benner, R., Hijmans, W., and Haaijman, J.J.: The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. Clin. Exp. Immunol. 46, 1-8 (1981).

Berg, R. and Savage, C.S.: Immune responses of specific pathogen-free and gnotobiotic mice to antigens of indigenous and non-indigenous microorganisms. Infect. Immun. 11, 320-329 (1975).

Crispe, N.: Mechanisms of self tolerance. Immunol. Today 9, 329-331 (1988).

Foo, M.C. and Lee, A.: Immunological response of mice to members of the autochthonous intestinal microflora. Infect. Immun. 6, 525-532 (1972).

Goris, H., de Boer, .F, and van der Waaij, D.: Myelopoiesis in experimentally contaminated Specific-Pathogen-Free mice during oral administration of polymyxin. Infect. Immun. 50, 437-441 (1985).

Goris, H., de Boer, F., and van der Waaij, D.: Oral administration of antibiotics and intestinal flora associated endotoxin in mice. Scand. J. Infect. Dis. 18, 55-63 (1986a).

Goris, H., Daenen, S., Halie, M.R., and van der Waaij, D.: Effect of intestinal flora modulation by oral polymyxin treatment on haemopoietic stem cell kinetics in mice. Acta Haematol. 76, 44-49 (1986b).

Hamilton, B.L. and Parkman, R.: Acute and chronic Graft-versus-Host Disease induced by minor histocompatibility antigens in mice. Transplantation 36, 150-155 (1983).

Heidt, P.J., Wagemaker, G., Knaan-Shanzer, S., and van Bekkum, D.W.: Two distinct types of late onset graft-versus-host-disease after bone marrow transplantation in lethally irradiated mice. Transplantation 32, 263-264 (1981).

Heidt, P.J., Solleveld, H.A., Mulder, A.H., and Gerritsen, W.R.: Identity of the microflora in donor and recipient mitigates graft versus host disease after allogeneic bone marrow transplantation in mice. In: Heidt, P.J.: Gnotobiotics and bone marrow transplantation (Thesis, University of Leiden), 21-37 (1989).

Heidt, P.J.: Mitigation of graft versus host disease after allogeneic bone marrow transplantation in gnotobiotic mice: limitation by the number of T-lymphocytes in the graft In: Heidt, P.J.: Gnotobiotics and bone marrow transplantation (Thesis, University of Leiden), 38-45 (1989).

Heit, H., Wilson, R., Fliedner, T.M., and Kohne, E: Biological effect of gnotobiotic environment. In: Germfree research, biological effect of gnotobiotic environments (Ed.: Heneghan, J.B.). Academic Press, New York, 477-483 (1973).

Jones, J.M., Wilson, R., and Bealmear, P.M.: Mortality and gross pathology of secondary disease in germfree radiation chimeras. Rad. Res. 45, 577-580 (1971).

Kagnoff, M.F.: Effects of antigen-feeding on intestinal and systemic immune responses II. Suppression of delayed-type hypersensitivity reactions. J. Immunol. 120, 1509-1513 (1978).

Keast, D.: Role of bacterial endotoxin in the graft-versus-host syndrome. J. Infect. Dis. 128 (suppl), 104-109 (1973).

Marrack, P. and Kappler, J.: The T-cell repertoire for antigen and MHC. Immunol. Today 9, 308-315 (1988).

Moore, R.H., Lampert, I.A., Chia, Y., Aber, V.R., and Cohen, J.: Influence of endotoxin on graft versus host disease after bone marrow transplantation across major histocompatibility barriers in mice. Transplantation 43, 731-736 (1987).

Pollard, M., Chang, C.F., and Srivastava, K.K.: The role of microflora in development of graft-versus-host disease. Transplant. Proc. 8, 533-536 (1976).

Rappaport, H., Khalil, A., Halle-Pannenko, O., Pritchard, L., Dantchev, D., and Mathé, G.: Histopathologic sequence of events in adult mice undergoing lethal graft-versus-host reaction developed across H-2 and/or non-H-2 histocompatibility barriers. Am. J. Pathol. 96, 121-142 (1979).

Rogers, M.J., Moore, R.H., and Cohen, J.: The relationship between faecal endotoxin and faecal microflora of the C57Bl mouse. J. Hyg. (Camb.) 95, 397-402 (1985).

Strobel, S., Mowat, A., and Ferguson, A.: Prevention of oral tolerance induction and enhanced antigen presentation during a graft

versus host reaction in mice. Immunology 56, 57-64 (1985).

Strobel, S. and Ferguson, A.: Modulation of intestinal and systemic immune responses to a fed protein antigen, in mice. Gut 27, 829-837 (1986).

Swarbrick, E.T., Stokes, C.R., and Soothill, J.F.: Absorption of antigens after oral immunisation and the simultaneous induction of specific systemic tolerance. Gut 20, 121-125 (1979).

van Bekkum, D.W., Roodenburg, J., Heidt, P.J., and van der Waaij, D.: Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. J. Natl. Cancer Inst. 52, 401-404 (1974a).

van Bekkum, D.W., de Vries, M.J., and van der Waaij, D.: Lesions characteristic of secondary disease in germfree heterologous radiation chimaeras. J. Natl. Cancer Inst. 38, 223-231 (1974b).

van Bekkum, D.W. and Knaan, S.: Role of bacterial microflora in the development of intestinal lesions from graft-versus-host-reaction. J. Natl. Cancer Inst. 58, 787-789 (1977).

van der Waaij, D. and Heidt, P.J.: Development of immunological tolerance to the mucosa-associated flora: A possible prerequisite for mitigation of graft-vs.-host disease in bone marrow transplant recipients. In: Antimicrobial agents and immunity (Eds.: Jeljaszewicz, J. and Pulverer, G.). Academic Press, London, 189-216 (1987).

Veenendaal, D., de Boer, F., and van der Waaij, D.: Effect of selective decontamination of the digestive tract of donor and recipient on the occurrence of murine graft versus host disease. Med. Microbiol. Immunol. 177:133-144 (1988).

Wall, D.A., Hamberg, S.D., Reynolds, D.S., Burakoff, S.J., Abbas, A.K., and Ferrera, J.: Immunodeficiency in graft versus host disease: I. Mechanism of immune suppression. J. Immunol. 140, 2970-2976 (1988).

THE INFLUENCE OF THE MICROFLORA ON GRAFT-VERSUS-HOST DISEASE IN EXPERIMENTAL AND CLINICAL BONE MARROW TRANSPLANTATION

PETER J. HEIDT[1,2] and JAAK M. VOSSEN[2]

[1]Department of Microbiology and Gnotobiology, Radiobiological Institute TNO, Rijswijk, and [2]Department of Paediatrics, University Hospital Leiden, Leiden, The Netherlands

Lecture presented at the 2nd Old Herborn University Seminar on "Interactions between the indigenous microflora and the host immune system" on June 1, 1988

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is currently being used as treatment for many fatal diseases of the haemopoietic system, among them severe aplastic anaemia (*Löwenberg* and *Gale*, 1985) and leukaemia (*Dicke* et al., 1985). Furthermore, patients suffering from fatal hereditary diseases that are associated with a dysfunction of the lymphoid system, like severe combined immunodeficiency (*Dooren* and *Vossen*, 1985) and patients with inherited severe metabolic disorders (*Barranger*, 1984) are being treated with bone marrow grafts. In total about 2500 patients are transplanted annually world-wide in 160 BMT centres.

One of the major complications of allogeneic BMT is graft-versus-host disease (GvHD), which is caused by donor type lymphocytes which react against the recipient's tissues. According to an evaluation of data from 2036 recipients of HLA identical sibling bone marrow transplants reported to the International Bone Marrow Transplant Registry, moderate to severe GvHD occurred in about 45% of these patients. In 48% of them, GvHD was related to their death (*Gale* et al., 1987). The severity of GvHD is influenced by several factors, which include the degree of immunogenic disparity (*Uphoff* and *Law*, 1958), the number of cells grafted (*van Bekkum*, 1964), the number of T-lymphocytes present in the graft (*van Bekkum*, 1964, 1972), the donor's sex (*Gale* et al., 1987) and the age of the recipient (*Gale* et al., 1987).

EXPERIMENTAL BONE MARROW TRANSPLANTATION

Another important factor influencing GvHD is the recipient's gastrointestinal microflora. This was originally observed in gnotobiotic mice. In contrast to the bone marrow of primates, rodent bone marrow contains a low proportion of immunocompetent T-lymphocytes. As a consequence, its GvHD-inducing potential is comparatively low (*van Bekkum* and *de Vries*, 1967). Infusion of 10^7 H-2 incompatible bone marrow cells into lethally irradiated (9.0 Gy X-rays) conventional mice results in a late onset type GvHD which does not give rise to symptoms until about three weeks after BMT. This disease kills the

majority of the recipients during the next two months but those that survive for more than three months seem to have recovered (*van Bekkum* and *de Vries*, 1967; *van Bekkum* et al., 1974). This type of GvHD is called delayed GvHD to distinguish it from the acute GvHD which can be induced in mice by supplementing the allogeneic bone marrow graft with donor derived spleen or lymph node cells. Mortality attributable to delayed GvHD can be completely prevented if the recipients are germfree mice (*Jones* et al., 1971; *van Bekkum* et al., 1974; *Truitt*, 1978; *Veenendaal* et al., 1988), or when they are conventional animals which have been subjected to complete (*Heit* et al., 1973; *Truitt*, 1978) or selective gastrointestinal decontamination (*van Bekkum* et al., 1974) by means of orally administered non-absorbable antibiotics prior to transplantation. In these experiments, selective decontamination resulted in animals which only harboured a strict anaerobic microflora. Mitigation of GvHD is also observed when the recipients of allogeneic H-2 mismatched bone marrow are conventional mice which have been associated with a strictly anaerobic microflora after a period of complete decontamination, or if they are germfree animals which have been associated with this flora (*van Bekkum* et al., 1974). This anaerobic microflora was originally obtained from selectively decontaminated conventional mice and was named CRF (colonisation resistance factor) flora after its capacity to provide animals with resistance against newly colonising microorganisms (*van der Waaij*, 1971). This CRF flora is largely composed of spore forming Gram-positive rods (mainly different *Clostridium* spp.; *Wensinck* and *Ruseler-van Embden*, 1971). The most striking difference between the conventional mice on the one hand, and the selectively decontaminated and the CRF mice on the other hand was the absence of aerobic Gram-negative rods (i.e. *Enterobacteriaceae*) in the second and third group.

If the bone marrow graft of conventional mice is supplemented with 10^7 spleen cells so as to provide a graft composition which is, like primate marrow, rich in immunocompetent T-lymphocytes, the recipients suffer from an early onset GvHD which takes a fatal course within three weeks after transplantation. The absence of a gastrointestinal microflora in the recipients (i.e. germfree or completely decontaminated animals) delays this mortality by two weeks, but does not prevent it (*van Bekkum* et al., 1974).

Jones et al. (1971) showed that conventionalisation of germfree or completely decontaminated recipients of allogeneic H-2 mismatched bone marrow at 150 and 180 days after BMT caused their death within 4 weeks. It is conceivable that this mortality was the result of infection caused by uncontrolled colonisation of the gastrointestinal tract, since *Heit* and colleagues (1973) showed that reconventionalisation of the completely decontaminated chimeras, starting 175 days after BMT, did not produce mortality. We investigated the time period after BMT during which the recipients had to be maintained germfree or decontaminated in order not to loose the mitigating effect of the gnotobiotic state on GvHD. It became clear that reconventionalisation at day 40 or later after BMT did not influence the beneficial effect of the decontaminated or germfree state; also >90% of these chimeras survived after reconventionalisation without showing any signs of GvHD (*van Bekkum* et al., 1974). We also studied the effect of earlier reconventionalisation after BMT (i.e. days 8, 20 and 26 after BMT). Reconventionalisation at days 8 and 20 resulted in a mortality pattern after BMT, which was

identical to that of conventional recipients of allogeneic BMT. Reconventionalisation at day 26 after BMT gave a protective effect in about 50% of the recipients, the remaining animals survived for >200 days after BMT (*van Bekkum*, 1977). The results of the described experiments show that the severity of GvHD is determined by the presence or the absence of (some constituents) of the recipient's microflora. These findings suggested that not only histo-incompatibility is determining the occurrence and severity of GvHD, but that microflora-related factors also are of major importance. The hypothesis was that donor type lymphocytes could be stimulated or activated by antigens of bacteria from the gastrointestinal tract which cross-react with antigens present on the recipient's epithelial tissues, being the main targets in GvHD. A key role in this phenomenon was attributed to the *Enterobacteriaceae*, which were absent in the germfree, selectively or completely decontaminated, and CRF recipients of allogeneic mismatched bone marrow in which GvHD was mitigated, while this group of bacteria was always present in the conventional recipients of which 90% died from severe GvHD.

The hypothesis that GvHD is the result of T-cell stimulation, or of activation by cross-reactive antigens present on enteric bacteria and the recipients epithelium was confirmed by experiments performed by *van Bekkum* and *Knaan* (1977). In (CBA x C57BL) F1 hybrid mice they implanted CBA and F1 foetal gut fragments. After the implants were established (about 15 to 30 days after implantation), the mice were irradiated (9.0 Gy X-rays) and transplanted with 2x 10^6 CBA bone marrow plus 2x 10^6 CBA spleen cells, so that they developed acute GvHD. This was done in conventional as well in completely decontaminated carriers of foetal gut implants. As a control, conventional and completely decontaminated F1 carriers of foetal gut implants were transplanted with similar amounts of F1 bone marrow and spleen cells. Scoring of GvHD in the different groups was done by counting the numbers of degenerated and intact crypts in sections of the implanted F1 or CBA guts. After transplantation of CBA bone marrow and spleen cells in conventional F1 mice, the damage in the F1 foetal gut implant was twice as great as in F1 foetal gut implants of the decontaminated recipients. In conventional recipients of CBA bone marrow and spleen cells, the CBA foetal gut implants which were not in direct contact with any microflora, showed a significant score of GvHD lesions, while in decontaminated chimeras the CBA implant showed no histological lesions. None of the implants of the control animals, which had been treated with isogeneic cells, showed histopathological lesions characteristic for GvHD. It was concluded that the presence of a microflora at a distant site is capable in magnifying GvHD lesions in the germfree F1 gut implant, and is even capable of inducing donor type immune cells to cause GvHD lesions in the germfree CBA implant which is syngeneic to these cells.

The above-summarised observations all clearly indicate an important role of the microflora in the development and severity of GvHD after allogeneic bone marrow transplantation.

To investigate the general applicability of the observations in mice, we studied the effect of gastrointestinal decontamination on GvHD after allogeneic BMT, using beagle dogs as a preclinical model (*Vriesendorp* et al., 1981). The dogs were studied under three different gnotobiotic conditions: conventional, selective gastrointestinal decontamination, and complete gastrointestinal decontamination. Both selective and com-

Table 1: Incidence of lethal GvHD in different gnotobiotic groups of monkeys (different donor/recipient combinations)

Donor/recipient combination			Gnotobiotic state		
RhLA A/B	RhLA D/DR	Family relationship	Complete GID	Selective GID	Clean conventional
=	≠	none	0/4[a]	-	5/9[b]
≠	=	sibling	0/2[c]	0/1[d]	4/5[e]
≠	≠	none	6/8[f]	1/1[g]	5/5[h]

Chi-square test: (a+c+d) vs. (b+e): $p<0.05$
(f+g) vs. h : $p>0.05$ (n.s.)

plete gastrointestinal decontamination were discontinued in surviving animals on day 40 after BMT, since experiments in mice had shown that after this period no extra beneficial effect on GvHD can be expected from the gnotobiotic state of the recipient (*van Bekkum*, 1977). Donors and recipients were typed for the major histocompatibility complex (MHC).

After conditioning with total body irradiation, the animals were given bone marrow cells to which lymph node cells were added to mimic the human situation. Selective gastrointestinal decontamination was found to mitigate acute GvHD in this study with dogs; a small effect on the incidence of GvHD and on the mortality resulting from this disease was observed in the groups in which 10^8 lymph node cells per kg body weight were added to the bone marrow graft. It is to be assumed that complete decontamination gives the same degree of protection if not more. We did not observe any effect of complete GID on the incidence of GvHD and subsequent mortality after grafting of allogeneic bone marrow to which 2x 10^8 lymph node cells per kg body weight were added. Therefore, the impression is gained that the acute GvHD caused by 10^8 donor lymph node cells per kilogram body weight from a MHC identical donor is the maximum severity of GvHD that can be prevented by GID. This is in agreement with experiments in mice, which showed that the effect of the gnotobiotic state on GvHD is limited by the number of T-lymphocytes present in the graft (*Heidt* et al., 1981).

As a final pre-clinical model, we studied the influence of gastrointestinal decontamination on GvHD after (partially) mismatched allogeneic BMT in rhesus monkeys (*Macaca mulatta*). Twenty-five monkeys were either subjected to complete or to selective decontamination, irradiated with a single dose of 8.5 Gy X-rays (n=10) or 2 fractions of 7.0 Gy separated by 3 days (n=15), and transplanted with stem-cell enriched, lymphocyte depleted bone marrow. The donors were either unrelated mismatched (n=12), unrelated A/B matched (n=6), or related D/DR matched (n=7). The cell dose was 5x 10^7/kg body weight.

GvHD could be studied in 16 of the 25 transplanted animals; of the remaining animals, 4 did not establish a take of the donor bone marrow while 5 monkeys were not evaluable due to early death caused by other complications being mostly severe electrolyte imbalance. The incidence of lethal GvHD in the different donor/recipient combinations is given in Table 1. Decontamination of the gastrointestinal tract resulted in the prevention of lethal GvHD in re-

Table 2: Characteristics of 94 evaluable patients

	Bone marrow failure			Haematological malignancy	
	Severe aplastic anaemia	Fanconi's anaemia	Myelodisplastic syndrome	Leukaemia	Non-Hodgkin lymphoma
Selective GID (n=18):	9	2	1	6	0
Complete GID (n=76):	14	4	7	47	4

cipients of partially matched T-lymphocyte depleted allogeneic bone marrow grafts. A possible difference between the two types (complete and selective) gastrointestinal decontamination could not be evaluated due to the small individual groups in this study. Gastrointestinal decontamination showed not to be effective in preventing lethal GvHD after transplantation with partially T-lymphocyte depleted completely mismatched unrelated bone marrow grafts. This failure was ascribed to the comparatively large number of T-lymphocytes which remained present in these grafts after using discontinuous albumin density gradient centrifugation for lymphocyte depletion (*Dicke* and *van Bekkum*, 1971).

CLINICAL BONE MARROW TRANSPLANTATION

In man, the effect of GID on GvHD has been controversial for a long time. Several studies reported a reduction of the incidence of acute GvHD after allogeneic BMT (*Mahmoud* et al., 1984; *Schmeiser* et al., 1984; *Storb* et al., 1983). However, such an effect was not observed in other studies (*Leblond* et al., 1987; *Skinhøj* et al., 1987; *Storb* and *Thomas*, 1985). Recently, we reported on a retrospective evaluation of the efficacy of GID in a protective environment for the prevention of GvHD in 65 children and adolescents, grafted consecutively for either severe bone marrow failure (n=29) or leukaemia (n=36) (*Vossen* et al., 1990). It was concluded that, in contrast to selective gastrointestinal decontamination (Group I, n=21), complete gastrointestinal decontamination (Group II, n=44) in a strict protective environment is a very effective method for preventing acute GvHD in children and adolescents; it resulted in a cumulative frequency of ≥2 grade II acute GvHD of 17.5%, a low transplantation-related mortality of 26% and a good quality of survival in 69% of the graft recipients.

More recently the influence of complete and selective gastrointestinal decontamination in a strict protective environment on acute GvHD after allogeneic BMT was re-evaluated in a larger group of patients, which were transplanted in the Leiden Paediatric BMT Centre over a period of about 20 years. Since the above-mentioned report, 37 more completely decontaminated children have been grafted (Group III). Major differences between the former study groups and group III were the use of methotrexate plus cyclosporin-A for GvHD prophylaxis in 25 graft recipi-

Table 3: Antimicrobial drugs for complete and selective gastrointestinal decontamination (daily dose)

Group I	Group II	Group III
Nalidixic acid (90 mg[a,b])	Neomycin (200 mg)	Gentamicin (800 mg)
Co-trimoxazole (12/60 mg[a,b])	Polymyxin B (2000 mg)	Cephaloridin (2000 mg)
Neomycin (15 mg[a])	Cephaloridin (2000 mg)	Amphotericin B (2000 mg)
Polymixin B (20 mg[a])	Amphotericin B (2000 mg)	
Amphotericin B (2000 mg)		

[a]: per kg body weight.
[b]: used only in a limited number of patients.

ents of group III, and the substitution of systemic (i.v.) antimicrobial prophylaxis for peroral GID early after BMT in recipients of group III with gastrointestinal complaints and bad compliance for oral antimicrobial drugs. Patients in all three groups received full bone marrow grafts from HLA genotypically identical siblings, following the usual pre-treatment regimens. The characteristics of the 94 evaluable patients are given in Table 2.

All patients were nursed in a strict protective environment, i.e., in laminar down flow isolators (*van der Waaij* et al., 1973), using aseptic nursing techniques and sterilisation of food, beverages and all other items brought into the isolator (*Vossen* and *van der Waaij*, 1972).

Antimicrobial drugs and dosages administered orally for selective gastrointestinal decontamination in group I, complete gastrointestinal decontamination of patients in group II, and complete gastrointestinal decontamination in group III are given in Table 3. Young children below the age of 2 years received half of the indicated dosages. The drugs were administered in four divided doses per day, except for co-trimoxazole, which was given twice daily. When during the early period after BMT children were unable to swallow the drugs for complete gastrointestinal decontamination due to nausea and vomiting, the suppression of the gut microflora was continued by i.v. administration of co-trimoxazole and cefamandole in usual therapeutical dosages; this was only done in children of group III. In individual cases of the same group, i.v. 5-flucytosine was added to the oral administration of amphotericin B, when elimination of yeasts from the gut had not been successful. This was done to suppress further growing of these microorganisms.

Decontamination started ≥ one week before the date of BMT and was given for at least a total of 40 days after BMT, based on experimental data (*van Bekkum*, 1977). Both complete and selective GID were considered successful when in the period from 7 days before until 40 days after BMT the target-microorganisms could not be isolated from more than two consecutive faecal samples. GvHD was diagnosed by clinical

Table 4: Composition of the SPF and conventional (HF) microflora

SPF-Flora	Houston-Flora
Anaerobic microflora	Anaerobic microflora
CRF-flora (not defined)	Not defined
Aerobic microflora	Aerobic microflora
Streptococcus faecalis (7173711[a])	Streptococcus faecium (7355510[a]) Streptococcus faecium (7317550[a])
Staphylococcus aureus (6726153[b]) Staphylococcus epidermidis (6706133[b])	Staphylococcus xylosus (6736552[b]) Staphyloccus haemolyticus (6632171[b])
Escherichia coli (5144572[c])	Escherichia coli (5144572[c]) Escherichia coli (5144532[c]) Proteus mirabilis (0536000[c]) Proteus mirabilis (0534000[c])
	Pasteurella pneumotropica (1220000[d])[1]

[a]: Biotype (API 20 Strep; API System, Montalieu-Vercieu, France).
[b]: Biotype (API Staph; API System, Montalieu-Vercieu, France).
[c]: Biotype (API 20 E; API System, Montalieu-Vercieu, France).
[d]: Biotype (API 20 NE; API System, Montalieu-Vercieu, France).
[1]: Isolated from nasal washings only.

symptoms. The severity of acute GvHD was graded according to *Thomas* et al., (1975).

According to the criteria used, GID was successful in 14 out of 18 evaluable children with selective GID in group I (78%); in 11 out of 40 evaluable children with complete GID in group II (27.5%) and in 19 of 36 evaluable children with complete GID in group III (53%).

The occurrence of ≥ 2 grade II acute GvHD was 6/18 (33%) in group I, 7/40 (17%) in group II and 1/36 (3%) in group III. More relevant was the finding that 0/30 successfully completely decontaminated children developed ≥ 2 grade II acute GvHD versus 6/14 (43%) successfully selectively decontaminated children. The latter is not different from the incidence (45%) in 2036 recipients of HLA identical sibling bone marrow transplants analysed by the International Bone Marrow Transplant Registry (*Gale* et al., 1987). From our observations it can be concluded that complete gastrointestinal decontamination is superior to selective gastrointestinal decontamination in preventing ≥ 2 grade II acute GvHD.

THE MECHANISM

To study the mechanism, which underlies the influence of the gastrointestinal microflora on GvHD, H-2 different donor and recipient mice with a SPF and a conventional microflora were employed (*Heidt*, 1989). For this purpose a conventional murine microflora was imported from the M.D. Anderson Cancer Institute, Houston, TX, USA, called "Houston flora" (HF), since in our institute only SPF animals are being bred. The composition of the SPF flora

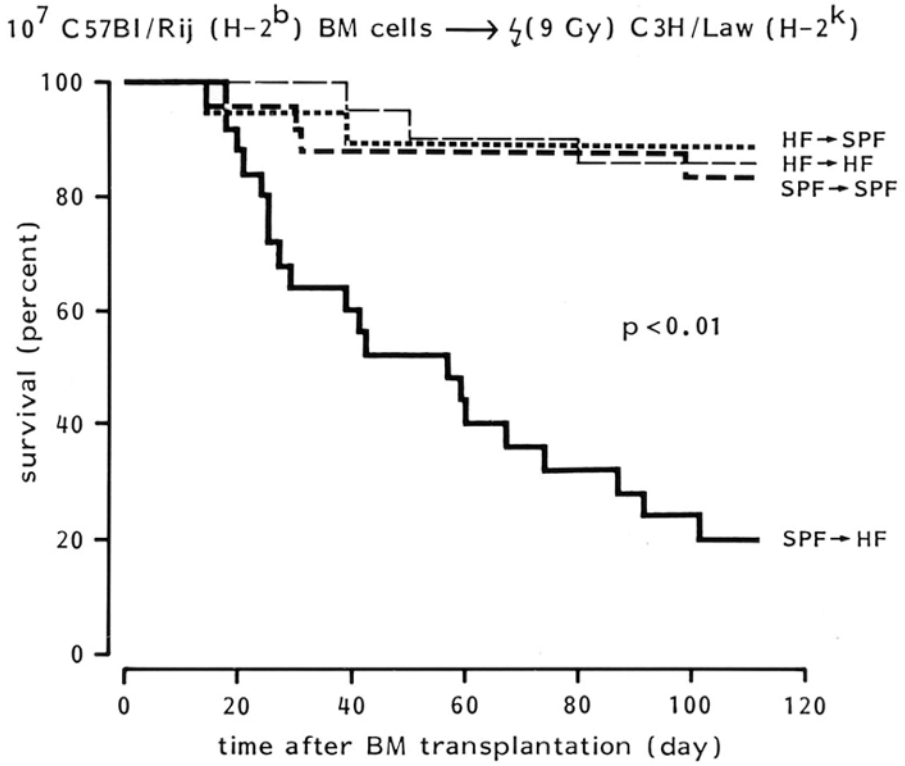

Figure 1: Mortality in the different microbiolically defined donor-recipient combinations.

and this HF is given in Table 4.

Germfree C3H/Law breeding pairs were associated with the conventional flora (HF). HF-bearing C57BL/Rij mice were obtained by foster nursing caesarean derived C57BL/Rij newborns by HF C3H/Law mothers. The HF C57BL/Rij animals obtained in this way were used as breeding animals to produce the experimental animals. Before entering the experiment, all mice were kept in Trexler type plastic film isolators to prevent undue association with any other microorganisms. Beside the above mentioned experiments using donor and recipient mice with the SPF flora or the HF, a second series of experiments was carried out to study the effect of complete and selective GID of HF recipients on GvHD. During both series of experiments, all recipients were housed under conditions of strict reverse isolation in a laminar cross flow isolator to prevent contamination of the animals with any new microorganisms (*van der Waaij* and *Andreas*, 1971). The animals received autoclaved (10 min., 134°C) AM-II food pellets (Hope Farms B.V., Woerden, The Netherlands) and acidified (pH 2.8) sterile drinking water.

The recipients were lethally (9 Gy) irradiated as a conditioning for BMT. The next day, they were injected i.v. with 10^7 bone marrow cells from C57BL/Rij donor mice. Irradiation of the mice and transplantation of the bone marrow cells were also performed under conditions of strict reverse isolation.

According to the microbiological status of the donors and the recipients, there were four different experimental groups in the first series of experiments. They were: HF recipients of

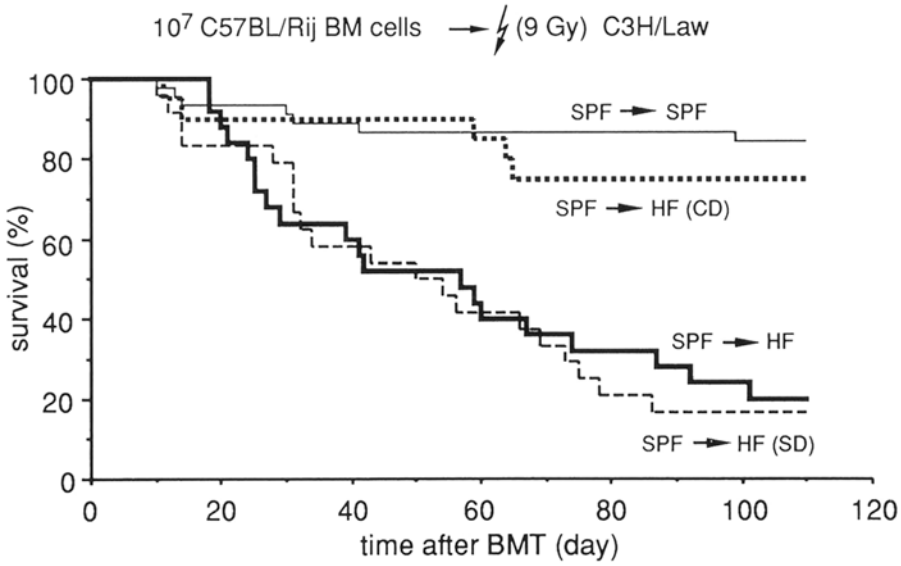

Figure 2: Influence of selective and complete GID on mortality of HF-bearing C3H/Law recipients of SPF-bearing C57BL/Rij bone marrow grafts.

SPF donor bone marrow (SPF to HF), HF recipients of HF donor bone marrow (HF to HF), SPF recipients of SPF donor bone marrow (SPF to SPF), and SPF recipients of HF donor bone marrow (HF to SPF).

No significant mortality from GvHD occurred in HF to HF recipients, SPF to SPF recipients and HF to SPF recipients, but the mortality of SPF to HF recipients was 80% (Figure 1). In the second series of experiments, prevention of GvHD (with a mortality-pattern identical to that in the HF to HF recipients, SP to SPF recipients and HF to SPF recipients) was observed in the completely decontaminated HF C3H/Law recipients of SPF C57BL/Rij bone marrow, while no effect of selective GID of the HF recipients was seen (Figure 2).

In the first series of experiments, lethal GvHD occurred in only one of the four transplanted groups of mice. This was the group of "conventional" (HF) recipients of SPF bone marrow (SPF to HF), in which severe diarrhoea was also observed and which showed the most distinct histopathological lesions. When the donors and recipients of the bone marrow were both HF carrying animals (HF to HF), more than 90% of the recipients survived after BMT. Also in the two other groups in which the recipients were SPF (i.e., SPF to SPF and HF to SPF), more than 90% of the recipients survived. This is comparable with observations made when recipients of allogeneic H-2 mismatched bone marrow are germfree or have been decontaminated (*van Bekkum* et al., 1974). The results show that mortality due to GvHD after allogeneic H-2 mismatched BMT is significantly prevented when there is identity between the microflora of the donor and recipient (HF to HF and SPF to SPF) or when the recipients do not harbour any aerobic microorganisms other than those present in the donors (HF to SPF).

In previous publications it was hypothesised that certain bacteria belonging to the microflora of the recipient may play a role in the induction of de-

layed type GvHD after allogeneic BMT because they carry antigens which are cross-reactive with epithelial antigens. After transplantation of allogeneic bone marrow from a donor which was matched for the major histocompatibility complex (MHC), a mitigation of GvHD was observed in selectively decontaminated dogs (*Vriesendorp* et al., 1981), which confirmed the findings in mice (*van Bekkum* et al., 1974) suggesting that *Enterobacteriaceae* might play a role in the induction and severity of GvHD. The observation that selective GID of HF recipients did not mitigate GvHD, in contrast to complete GID of HF recipients does not support this hypothesis since *Enterobacteriaceae* are being eliminated by selective GID. Therefore, it has to be assumed that other microorganisms than *Enterobacteriaceae* are involved in the induction of GvHD. It is likely that bacteria belonging to the complex anaerobic microflora are responsible for the induction of GvHD. *Foo* and *Lee* (1974) found that antigenic cross-reaction exists between mouse intestine and a *Bacteroides* spp., one of the anaerobic members of the autochthonous microflora of the rodent gastrointestinal tract. The above mentioned conclusion is supported by the earlier mentioned observations that selectively decontaminated human recipients of allogeneic bone marrow developed significantly more ≥ 2 grade II acute GvHD than completely decontaminated patients. The most striking difference between these two groups of patients was the presence of an anaerobic microflora in the first group of recipients (*Vossen* et al., 1990).

HYPOTHESIS

The high mortality due to GvHD in HF recipients of SPF bone marrow in the first series of experiments can be explained by a double mechanism in evoking GvHD after allogeneic BMT. The first mechanism is the reaction of donor type T-lymphocytes against histocompatibility antigens. This graft-versus-host reaction gives rise to minimal intestinal lesions and subsequent limited mortality, as was the case in the completely decontaminated recipients. If however, the flora of the recipient of the allogeneic bone marrow contains anaerobic bacterial species which do not also belong to the indigenous flora of the donor, and which carry antigens that are cross-reactive with tissue antigens of the host, the donor T-lymphocytes are activated by these cross-reactive bacterial antigens and become reactive against host tissues. This results in a clinically and histologically more severe graft-versus-host reaction, leading to the death of the majority of the recipients.

LITERATURE

Barranger, J.A.: Marrow transplantation in genetic disease. N. Engl. J. Med. 311, 1629-1631 (1984).

Dicke, K.A., Zander, A.R., Vellekoop, L., Spitzer, G., and Verma, D.S.: The treatment of leukemia. In: Bone marrow transplantation, biological mechanisms and clinical practice (Eds.: van Bekkum, D.W. and Löwenberg, B.). Marcel Dekker Inc., New York, 435-474 (1985).

Dooren, L.J. and Vossen, J.M.: Severe combined immunodeficiency: Reconstitution of the immune system following bone marrow transplantation. In: Bone marrow transplantation, biological mechanisms and clinical practice. (Eds.: van Bekkum, D.W. and

Löwenberg, B.). Marcel Dekker Inc., New York, 351-381 (1985).
Foo, M.C. and Lee, A.: Antigenic cross-reaction between mouse intestine and a member of the autochthonous microflora. Infect. Immunity 9, 1066-1069 (1974).
Gale, R.P., Bortin, M.M., van Bekkum, D.W., Biggs, J.C., Dicke, K.A., Gluckman, E., Good, R.A., Hoffmann, R.G., Kay, H.E.M., Kersey, J.H., Marmont, A., Masaoka, T., Rimm, A., van Rood, J.J., and Zwaan, F.E.: Risk factors for acute graft-versus-host disease. Brit. J. Haematol. 67, 397-406 (1987).
Heidt, P.J.: Gnotobiotics and bone marrow transplantation: Experimental and clinical studies. Thesis, University of Leiden (1989).
Heit, H., Wilson, R., Fliedner, T.M., and Kohne, E.: Mortality of secondary disease in antibiotic treated mouse radiation chimeras. In: Germfree research: Biological effects of gnotobiotic environment (Ed.: Heneghan, J.B.). Academic Press, New York, 477-483 (1973).
Jones, J.M., Wilson, R., and Bealmear, P.M.: Mortality and gross pathology of secondary disease in germfree mouse radiation chimeras. Radiat. Res. 45, 577-580 (1971).
Leblond, V., Belanger, C., Dreyfus, F., Brunet, F., Gabarre, J., Asselain, B., and Binet, L.: Interest of laminar air flow for prevention of GVHD and infections in BMT for leukaemia and lymphoma. Bone Marrow Transpl. 2 (suppl.1), 181 (1987).
Löwenberg, B. and Gale, R.P.: Aplastic anaemia. In: Bone marrow transplantation, biological mechanisms and clinical practice (Eds.: van Bekkum, D.W. and Löwenberg, B.). Marcel Dekker Inc., New York, 409-433 (1985).
Mahmoud, H.K., Schaefer, U.W., Schuning, F., Schmidt, C.G., Bamberg, M., Haralambie, E,. Linzenmeier, G., Hantschke, D., Grosse-Wilde, H., Luboldt, W., and Richter, H.J.: Laminar air flow versus barrier nursing in marrow transplant recipients. Blut 49, 375-381 (1984).
Schmeiser, T., Kurrle, E., Arnold, R., Heit, W., Krieger, D., Kubanek, B., and Heimpel, H.: Application of antimicrobial prophylactic treatment to the prevention of infection and graft-versus-host disease in allogeneic bone marrow transplantation (BMT). Exp. Haematol. 12 (suppl. 15), 105-106 (1984).
Skinhøj, P., Jacobsen, N., Høiby, N., Faber, V., and the Copenhagen Bone Marrow Transplant Group: Strict protective isolation in allogeneic bone marrow transplantation; effect on infectious complications, fever and graft versus host disease. Scand. J. Infect. Dis. 19, 91-96 (1987).
Storb, R., Prentice, R.L., Buckner, C.D., Clift, R.A., Appelbaum, F., Deeg, J., Doney, K., Hansen, J.A., Mason, M., Sanders, J.E., Singer, J., Sullivan, K.M., Witherspoon, R.P., and Thomas, E.D.: Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. N. Engl. J. Med. 308, 302-307 (1983).
Storb, R. and Thomas, E.D.: Graft-versus-host disease in dog and man: The Seattle experience. Immunol. Rev. 88, 215-238 (1985).
Thomas, E.D., Storb, R., Clift, R.A., Fever, A., Johnson, F.L., Neiman, P.E., Lerner, K.G., Glucksberg, H., and Buckner, C.D.: Bone marrow transplantation. N. Engl. J. Med. 292, 895-902 (1975).
Truitt, R.L.: Application of germfree techniques to the treatment of leukemia in AKR mice by allogeneic bone marrow transplantation. In: The handbook of cancer immunology. Volume 5: Immunotherapy (Ed.: Waters, H.). Garland STPM Press, New York, 431-452 (1978).
Uphoff, D.E. and Law, L.W.: Genetic factors influencing irradiation protection by bone marrow. II. The histocompatibility-2 (H-2) locus. J. Natl. Cancer Inst. 20, 617-624 (1958).
van Bekkum, D.W.: The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures. I. Effect of storage at 4°C. Transplantation 2, 393-404 (1964).
van Bekkum, D.W.: Use and abuse of hemopoietic cell grafts in immune deficiency diseases. Transplant. Rev. 9, 3-53 (1972).
van Bekkum, D.W.: Bone marrow transplantation. Transplant. Proc. 9, 147-154 (1977).
van Bekkum, D.W. and de Vries, M.J.: Radiation Chimeras. Academic Press, New York (1967).
van Bekkum, D.W., Roodenburg, J., Heidt, P.J., and van der Waaij, D.: Mitigation of secondary disease of allogeneic mouse radia-

tion chimeras by modification of the intestinal microflora. J. Natl. Cancer Inst. 52, 401-404 (1974).

van der Waaij, D. and Andreas, A.H.: Prevention of airborne contamination and cross-contamination in germ-free mice by laminar flow. J. Hyg. Camb. 69, 83-89 (1971).

van der Waaij, D., Vossen, J.M., and Korthals-Altes, C.: Patient isolators designed in the Netherlands. In: Germfree research. Biological effect of gnotobiotic environment (Ed.: Heneghan, J.B.). Academic Press, New York, 31-36 (1973).

Vossen, J.M. and van der Waaij, D.: Reverse isolation in bone marrow transplantation: Ultra-clean room compared with laminar flow technique. I. Isolator systems. Eur. J. Clin. Biol. Research 17, 457-461 (1972).

Vossen, J.M., Heidt, P.J., van den Berg, H., Gerritsen, E.J.A., Hermans, J., and Dooren, L.J.: Prevention of infection and graft-versus-host disease by suppression of intestinal microflora in children treated with allogeneic bone marrow transplantation. Eur. J. Clin. Microbiol. Infect. Dis. 9, 14-23 (1990).

Vriesendorp, H.M., Heidt, P.J., and Zurcher, C.: Gastrointestinal decontamination of dogs treated with total body irradiation and bone marrow transplantation. Exp. Hematol. 9, 904-916 (1981).

INTERACTIONS BETWEEN DIGESTIVE TRACT MICROFLORA AND IMMUNE SYSTEM

DIRK VAN DER WAAIJ

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

Lecture presented at the 2nd Old Herborn University Seminar on
"Interactions between the indigenous microflora and the host immune system"
on June 1, 1988

INTRODUCTION

The digestive tract is a very rich source of living (microbial) and dead (food and microbial) antigens. It is therefore conceivable that, where the gastro-intestinal tract is loaded with antigenically foreign material, much if not most of the activity of the local gut associated lymphoid tissue (GALT) is directed to antigens which actively or passively (by pinocytosis; *Walker* et al., 1974a) cross the intestinal epithelium. In the submucosa and the regional lymphatic tissues these antigens "meet" different cells of the immune system. The likelihood of this event is well illustrated by the difference between the state of activity of the gut associated lymphoid tissue (GALT) in germfree and in conventional animals (*Horowitz* et al., 1964; *Bosman* et al., 1964, *Freter* and *Abrams*, 1972). In this paper the fate of living and dead antigens inside the intestinal lumen will be discussed in conjunction with their importance for the host immune system and pathology. The distribution of (bacterial) antigens in the alimentary canal is largely followed by the quantity of lymphoid tissues of the GALT. This suggests at least (re-)activity of the GALT regarding the microbial distribution in the digestive tract. In this respect it is important to mention at this point already that in the presence of a functioning thymus certain microorganisms may exert either enhancing (T-helper) or tolerogenic (T-suppressor) activity. For the latter, microorganisms may have antigens in common with various organs. The antigenic similarity between microorganisms and their host organism could be a clue in the answer to the question how several bacteria can escape from the T-cell control mechanism(s) which normally prevents them from sensitising the host's immune system. In the absence of T-cells, bacteria belonging to the indigenous microflora may ensue hypersensitivity and/or autoimmune disorders.

THE ROLE OF THE THYMUS IN GUT ECOLOGY

The intestinal flora and the thymus

The thymic cortex is a well-known site of intensive (immune) activity. Early studies have indicated already that the majority of the newly formed or instructed thymocytes migrate to other organs (*Joel* et al., 1971, 1974). The magnitude of migration of cells matured in the thymus (T cells) into the GALT has been studied by *Laissue* and coworkers (1976). They found that the

radioactively labelled T-cells were rapidly catabolised in the small intestine. Furthermore, in early periods of postnatal life of mice, the vast majority of the lymphocytes that enter the GALT appeared is of thymic origin (*Joel*, 1972). *Cottier* and co-workers (1975) have made likely that the growth of GALT-cells and therewith the thymic activity, is largely antigen driven in mammals. In germfree mice, Peyer's patches and mesenteric lymphnodes are poorly developed, but increase in size when stimulated via intestinal route. No such reaction was seen when the antigens were administered parenterally (*Pollard* and *Sharon* 1970). These observations indicate that the microflora may play a crucial role in thymus and GALT activity. Conversely, as will be discussed later on, the immune system may also exert bet strong influence on the bacterial colonisation pattern of the digestive tract. Comparison of the relative thymus weight in germfree mice and conventional mice of different ages showed that at all ages (up to l000 days) the relative thymus weight in conventional mice was higher than in the germfree counterpart. The histology of the thymus correlated well with the age of the animals. Oral treatment of conventional mice with broad-spectrum non-absorbable antibiotics, which causes bacteria-freeness of the animal's digestive tract, has been found to cause a decrease of relative thymus weight to what is seen in germfree mice of the same strain and age (*van der Waaij*, 1986). Not only the thymus but also the spleen appears to decrease in size during total gut decontamination in these animals to values normal in germfree control animals (*van der Waaij*, 1969).

Oral induction of tolerance

Andre et al. (1975) reported an important observation which may help to explain the development of tolerance for intestinal indigenous flora components. They found that prolonged oral administration of sheep erythrocytes to mice decreased the level of plaque forming cells in the spleen after systemic challenge with this antigen. A few years later, *Kagnoff* (1978) showed that this relative hyporesponsiveness was due to the induction of suppressor T-cells. These observations have been confirmed since then by several groups and appear to apply to a number of different antigens.

Immunologic tolerance in gut associated lymphoid tissue (GALT)

The great majority of the intestinal microflora comprising of numerous different anaerobic species and some viridans group streptococci does not elicit an immune response in the digestive tract. On microscopic examination of freshly voided faecal flora of mice (*van der Waaij* and *Heidt*, 1978) and humans (*van Saene* and *van der Waaij*, 1979) with fluorescent anti-IgA antibodies, it has been found that the anaerobic bacteria of mice and humans are not covered with IgA. *Enterobacteriaceae* species present in the faeces, on the other hand, appeared frequently to have an "lgA-paint". In this respect it is of importance that parenteral inoculation of anaerobes isolated from an animals own faecal microflora appears to evoke only a very poor immunologic response (*Foo* and *Lee*, 1971).

Such observations of limited or no immune reactivity to indigenous flora upon parenteral injection suggest that as a rule, the immune system is not stimulated by the bulk of the intestinal bacteria. The intestinal mucosa however, does not completely block invasion of (small) numbers of bacteria. Rather an antigen (including bacterial) uptake through the epithelial layer occurs regularly. The specialised structures of Peyer's patches, solitary lymph follicles, and their associated epithelium allow a controlled uptake of antigenic,

among other bacterial, substances. Therefore, the state of specific immunologic tolerance for the residing indigenous microflora may exist. Evidence for antigen induced hypo- or non-responsiveness of GALT may be related to the presence of suppressor T-cells in the Peyer's patches.

Influence of age on GALT immune reactivity

Kenny et al. (1971) have described a study of oral *Escherichia coli*-O127 infection in mice of different ages. Titres of haemagglutinating antibody to *E. coli*-O127 upon the enteric infection showed significant differences among three age groups. In neonatal and infant mice no antibody was detected until the age of three weeks. Immune response in weaning mice was not consistent. Whereas some mice were able to respond upon oral infection with titres of 160, others demonstrated no response. Adult animals all responded with antibodies from the tenth day after infection.

The colonisation pattern of biotypes of *Enterobacteriaceae* species in thymus-bearing and in congenitally athymic mice

The colonisation pattern of the digestive tract is determined by a mechanism called colonisation resistance (CR). The CR of the digestive tract is caused and maintained by a myriad of factors, which are both of host and microflora origin. To investigate to which extend the genetic composition of the host organism is involved in the CR the following investigation was performed:

Thirty-five individually marked inbred conventional C3H mice were maintained in one large cage inside a germfree isolator during a four week observation period (*van der Waaij*, 1982). Germfree type isolation was applied to guarantee that only sources of Gram-negative (aerobic and facultative anaerobic) bacteria could be cagemates and the foodpellets. The total source was thus known and consisted of twelve biotypes of *Enterobacteriaceae* species. A sample of each mouse's faeces was tested by culture and typing twice weekly. Although the animals were inbred, their Gram-negative (aerobic) flora appeared different. Some animals entered the study with an average of five different biotypes in their faeces; others had two or only one. These differences in number of different biotypes between animals persisted during the four-week study period. Animals that started with one biotype had a low turnover of biotypes with an average of 0.8 biotype per sample, whereas mice with five biotypes at admission had a high turnover with an average of 3.2 biotypes per faecal sample.

There are three intriguing points in these observations:

1. Different mice with similar chances of contamination with the biotypes in common source (twelve biotypes) appeared to make their own selection out of that common environmental source. During the four weeks of study, some animals apparently allowed many different biotypes to pass through their alimentary canal and to appear in the faeces in detectable numbers. Other mice in the same cage appeared to be more selective.
2. This selection occurred in inbred mice, i.e. in genetically identical animals. Whether fewer or more biotypes are permitted to survive during transit through the digestive tract depends on the composition of the indigenous flora. This leads to the conclusion that the latter is apparently phenotypically determined.
3. The mice entered the study with different numbers of biotypes of *Enterobacteriaceae* species in their faeces. Therefore, they had acquired their indigenous flora before the experiment. The experiment started a week after weaning, when the mice

were four weeks of age. It is therefore most plausible that they acquired their intestinal flora from their mothers in the breeding unit. In this experiment we could trace the dams from which the animals used in the experiment originated. The mothers showed differences in numbers of biotypes that corresponded with that of their offspring before and during the four-week observation period. This evidence that mice in different litters had been exposed since birth to different flora with respect to the composition of the indigenous anaerobic bacteria associated with intestinal colonisation resistance.

THE ROLE OF INTESTINAL MICROFLORA IN AUTOIMMUNE PHENOMENA AND WASTING SYNDROME

Microflora associated with wasting syndrome in congenitally athymic mice

Investigations in congenitally thymusless mice with and without an intestinal microflora have indicated that these animals develop an abnormal flora several weeks after weaning. This applies to both aerobic and facultative anaerobic Gram-negative bacteria. The abnormality of the faecal flora in athymics is apparent in the great number of different biotypes of *Enterobacteriaceae* species which these animals appear to acquire from environmental sources when maintained under conventional conditions (*van der Waaij*, 1981). An abnormal intestinal population pattern appears just prior to the development of a clinical syndrome called "wasting disease" (*Jutila*, 1973). The thymusless (nude/nude) mice which are selectively decontaminated (SD) (*van der Waaij*, 1988) with antimicrobial drugs after weaning and are maintained thereafter in an *Enterobacteriaceae*-free condition by continuous SD-treatment, do not develop wasting disease (*van der Waaij*, 1981). Totally decontaminated athymic mice which are mono-associated with an *Escherichia coli* strain under isolation circumstances after weaning, however, also have been found to remain free of symptoms of wasting disease regardless the fact that their intestinal flora consisted of excessive numbers of Gram-negative bacteria (*van der Waaij*, 1981). *Wilson* and *Bealmear* (1965) have presented evidence that in mice the presence of a conventional flora is reflected in their relative thymus weight.

The role of T cells in controlling the intestinal flora for the prevention of wasting disease and comparable syndromes

The absence of functional T cells could be of importance in both the development of respectively the wasting syndrome in thymusless mice and in chronic graft-versus-host disease (GvHD) after allogeneic bone marrow transplantation. The mechanism involved however must be quite complex. As mentioned above in this paper, the presence of potentially pathogenic bacteria in the microflora of the thymusless mouse does not imply that wasting disease necessarily will develop. This applies also to chronic GvHD after allogeneic bone marrow transplantation (*Veenendaal*, this monograph). It is known for example, that the nude/nude mouse can remain without signs of autoimmunity, when it is maintained (bacteriologically) isolated under SPF-conditions (*McIntire* and *Sell*, 1964; *Reed* and *Jutila*, 1976). Association with potentially pathogenic bacteria early in life - as even may occur under the hygienic circumstances in SPF-breeding units - does apparently not condition for

wasting- or autoimmune disease. This could possibly be ascribed to the aspecific immune suppression that has been observed to exist in first weeks of life in mice (*Strobel* and *Ferguson*, 1984).

Endotoxin releasing bacteria (or other T-cell independent antigens) may be responsible for the IgM secretion in the nude/nude mice in the first months before the lymphoid tissues involute (*Pritchar* et al., 1973). Heavy oral and, perhaps more importantly, repeated oral infections, which occur in conventionally maintained mice, initially may lead to lymphoproliferation with immune complex disease, which is however, soon followed by exhaustion of the lymphatic tissues. As long as the immunosuppressive substance is in the circulation this event is perhaps prevented, the more oral infections occur there after the shorter the interval of clinical healthiness. Supportive evidence for the importance of unimpaired proliferation of lymphatic cells (predominantly B-cells) as a result of frequent oral infection with immune stimulating bacteria may come from the fact that chronic GvHD is not enhanced in mice associated before transplantation with only one or two Gram-negative rods (*E. coli* or *Klebsiella*) but maintained isolated after bone marrow (BM) engraftment, i.e. in the period of about three weeks post transplantation during which no or insufficient T-cell control of immune reactions has developed (*van Bekkum* et al., 1974).

In the athymic mice, T-cell control can obviously not develop so that massive B-cell proliferation is unavoidable upon repeated oral infection with bacteria which carry B cell polyclonal stimulating substances in their cell wall. The great majority of bacteria indeed carry T-cell independent antigens such as lipopolysaccharides and peptidoglycan. Therefore, B-cell response to many different bacterial antigens is likely to occur in such animals until lymphatic exhaustion. In case bacteria involved in immune stimulation share antigens with the host organism, autoimmmune phenomena may occur. Otherwise immunecomplex-disease may explain the multi-organ autoaggression in the athymic animals prior to the exhaustion; the "wasting syndrome". The same hypothesis could possibly apply to chronic GvHD where B cell proliferation may remain uncontrolled for a long period once it has started in the absence of a GALT T-cell system. Early studies of *Skopinska* (1972) and *Keast* (1973) may support this hypothesis. When they injected mice several days after BM-transplantation severe chronic GvHD occurred; however, the longer they made the interval between BM-transplantation and endotoxin injection the less signs and symptoms of a subsequent GvHD were.

With working hypothesis, it is understandable why a certain turnover of potentially pathogenic and other immunogenic bacteria that can translocate should be prevented during ageing in the congenitally athymic as to minimise autoimmune phenomena.

LITERATURE

Andre, C., Heremans, J.F., Vaerman , J.P., and Cambiaso, C.L.: A mechanism for the induction of immunological tolerance by antigen feeding: Antigen-antibody complexes. J. Exp. Med. 142, 1509-1519 (1975).

Bosma, M.J., Makinodan, T., and Walburg, H.E.: Development of immunologic competence in germfree and conventional mice. J. Immunol. 99, 420-430 (1967).

Cottier, H., Schaffner Th., Chanana, A.D., Joel, D.D., Sorbat, B., Bryant, B., and Hess M.W.: Species differences in the effective-

ness of intestinal barriers against penetration of inert particulates and bacteria. In: Gram-negative bacterial infections and mode of endotoxin actions (Eds.: Urbaschek, B. Urbaschek, R., and Neter, E.). Springer Verlag, Wien, New York, 80-85 (1975).

Foo, M. and Lee, A.: Immunological response of mice to members of the autochthonous intestinal flora. Infect. Immun. 6, 525-532 (1972).

Freter, R. and Abrams, G.D.: Function of various intestinal bacteria by converting germfree mice to the normal state. Infect. Immun. 6, 119-123 (1972).

Horowitz, R.E., Bauer, H., Paronetto, F., Abrams, G.D., Watkins, K.C., and Popper, H.: The response of lymphatic tissue to bacterial antigen. Amer. J. Pathol. 44, 747-761 (1964).

Joel, D.D., Sordat, B., Hess, M.W., and Cottier, H.: Uptake and retention of particles from the intestines by Peyer's patches in mice. Experimentia 26, 694-697 (1970).

Joel, D.D., Hess, M.W., and Cottier, H.: Thymic origin of lymphocytes in developing Peyer's patches of newborn mice. Experimentia 26, 694-697 (1971).

Joel, D.D., Hess, M.W., and Cottier, H.: Magnitude and pattern of thymic lymphocyte migration in neonatal mice. J. Exp. Med. 135, 907-923 (1972).

Kagnoff, M.F.: Effects of antigen-feeding on intestinal and systemic immune response. II. Suppression of delayed hypersensitivity reactions. J. Immunol. 120, 1509-1513 (1978).

Kenny, J.F., Woleslagle, J.A., Gray, R.H., Michaels, P., and Pearson, M.A.: Enteric infection with *E.coli*-O127 in the mouse. I. Characteristics of infection and systemic and local immune responses in mice of different ages. J. Inf. Dis. 121, 528-540 (1970).

Mattingly, J.A. and Waksman, B.H.: Immunologic suppression after oral administration of antigen. I. Specific suppressor cells formed in rat Peyer's patches after oral administration of sheep erythrocytes and their systemic migration. J. Immunol. 121, 1878-1883 (1978).

McIntire, K.R. and Sell, S.: Pathogenesis of the post-neonatal thymectomy wasting syndrome. Nature 204, 151-155 (1967).

Pollard. M.: Virus directed host response. In: Perspectives in Virology (Ed.: Pollard, M.). Academic Press, New York, 96-100 (1967).

Ritchard, H. Riddaway, J., and Micklem, M.S. II: Quantitative studies of serum immunoglobulins. The antibody response to sheep erythrocytes and the effect of thymus allografting. Clin. Exp. Immunol. 13, 125-138 (1973).

Reed, N.D. and Jutila, J.W.: Wasting disease induced with cortisol acetate. J. Immunol. 99, 238-245 (1967).

Strobel, S. and Ferguson, A.: Immune responses to fed protein antigens in mice: 3. Systemic tolerance or priming is related to age at which antigen is first encountered. Pediatric Research, 18, 588-594 (1984).

van Saene, H.K.F. and van der Waaij, D.: A novel technique for detecting IgA-coated potentially pathogenic microorganisms in the human intestine. J. Immunol. Methods 30, 87-96 (1979).

van Bekkum, D.W., Roodenburg, J., Heidt, P.J., and van der Waaij, D.: Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of intestinal microflora. J. Natl. Cancer Inst. 52, 401-404 (1974).

van der Waaij, D.: Similarities between germfree mice and mice with an antibiotic decontaminated digestive tract. In: Germfree Biology (Eds.: Mirand, A. and Back, N.). Plenum Press, New York, 181-189 (1969).

van der Waaij, D. and Heidt, P.J.: Intestinal bacterial ecology in relation to immunological factors and other defense mechanisms. In: Food and immunology (Eds.: Hambraeus, L. Hanson, L.A., and McFarlane, H.). Alqvist and Wiskell Int., Stockholm, 133-141 (1977).

van der Waaij, D.: The composition of the microflora, functional aspects of the intestinal immune system and autoimmune phenomena. In: Recent advances in germfree research (Eds.: Sasaki, S., Ozawa, A., and Hashimoto, K.). Tokai university Press, Tokyo, 387-395 (1981).

van der Waaij, D.: The digestive tract in immunocompromised patients: Importance of maintaining its resistance to colonization, especially in hospital in-patients and those taking antibiotics. Antonie van Leeuwenhoek 50, 745-761 (1984).

van der Waaij, D: The influence of the intestinal microflora on the relative thymus weight. Med. Microbiol. Immunol. 175,

335-340 (1986).
van der Waaij, D.: Selective decontamination of the digestive tract: General principles. Eur. J. Cancer Clin. Oncol. 24, 81-84 (1988).
Walker, W.A., Isselhacher, J. and Bloch, K.J.: Immunologic control of soluble protein absorption from the small intestine: A gut surface phenomenon. Am. J. Clin. Nutr. 27, 1434-1440 (1974).