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, and Dirk van der Waaij.
Herborn Litterae, Herborn-Dill, Germany: 1-6 (2004).

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.