Brazilian Journal of Medical and Biological Research (1998) 31: 105-110
ISSN 0100-879X

Parasitic infections in germfree animals

L.Q. Vieira[1], M.R. Oliveira[2], E. Neumann[1], J.R. Nicoli[3] and E.C. Vieira[1]

Departamentos de [1]Bioquímica e Imunologia, [2]Parasitologia and [3]Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Correspondence
L.Q. Vieira
Departamento de Bioquímica e Imunologia, ICB, UFMG
Caixa Postal 486
30161-970 Belo Horizonte, MG
Brasil
Fax: 55 (031) 441-5963
E-mail: lqvieira@icb.ufmg.br

Presented at the International Meeting on Cytokines, Angra dos Reis, RJ, Brasil, November 24-28, 1996.

Research supported by CNPq, CAPES and FINEP. L.Q. Vieira is the recipient of a CNPq fellowship.

Received September 24, 1997
Accepted September 30, 1997

Abstract

The association of vertebrate hosts with the indigenous microbiota and its effect on the response to infections has long been a subject of scientific curiosity. From the first theory supported by Louis Pasteur that life would be impossible in the absence of associated microorganisms to the development of germfree mammals for research, a lot was learned about how the normal microbiota influences the environment in which pathogens may find themselves. In the present review, we attempt to summarize the more recent results from our group and others on the influence of the normal microbiota on the outcome of parasitic infections. Our results and those of others point to a complex relationship between the mammalian system and its indigenous microbiota, leading to greater resistance to some infections and enhanced susceptibility to others.

Key words
• Germfree
• Microbiota
• Gnotobiotic
• Infection
• Leishmania
• Trypanosoma

Animals are associated with their normal microbiota from the early days of life. The number of bacteria associated with humans is estimated to be 10^{14} per individual, which makes microorganisms ten times more numerous than the actual cells of the human body. A major part of the microbiota is located in the gut (1,2). These associated microorganisms should influence the host homeostasis, and several studies have investigated the host-microbiota relationship. Germfree animals have been a major tool in these studies. These animals are, in a way, an extension of the microbiologist's pure culture concept, allowing the study of the interaction of the host with pathogens without the interference of other associated organisms.

Germfree animals, when infected with a pathogen, have been shown to be sometimes more resistant and other times more susceptible to the invading organism. Several investigators have reported that the indigenous microbiota affects the development of the host's immune response (reviewed in Ref. 2). A few studies have been performed to investigate the outcome of infection with several parasites in germfree animals, in comparison with conventionally reared controls.

An obvious question, addressed by several authors, is the effect of the associated microbiota on infection with intestinal pathogens. The intestinal mucosa of germfree animals is characterized by smaller numbers of intraepithelial lymphocytes (IEL) and lamina propria lymphocytes. Furthermore, Peyer's patches are less developed and lack a fully developed germinal center (2). The intestinal microbiota has also been shown to influence the development of CD4+ CD8+ alpha+ IEL through an extrathymic pathway (3). Moreover, both alpha-beta and gamma-delta IEL from germfree mice showed lower in vivo incorporation of 5-bromo-2'-deoxyuridine, reflecting a lower proliferation rate when compared to conventional animals (4). Therefore, it would not be surprising to expect

differences in the outcome of intestinal infections in germfree animals when compared to their conventional counterparts. The pioneering work by Phillips and Wolfe (5) showed that the intestinal microbiota is essential for the establishment of infection with Entamoeba histolytica. Similarly, pathogenicity by Giardia lamblia in mice only occurred in the presence of the microbiota, as revealed by an intense mononuclear infiltration in the lamina propria and high reactional hyperplasia of the lymphoid tissues in conventional animals. Although the parasite could multiply normally in the gut of germfree mice, no pathology of the gut was observed (6). Both total and G. lamblia-specific IgA levels were higher in the gut contents of infected conventional mice when compared with germfree animals (7). Taken together, these results indicate that some gut parasites will only invade tissues in the presence of the normal microbiota and that, in its absence, these parasites are not pathogenic.

Several systemic effects of the associated microbiota have been described, but few studies have been conducted on the effect of the microbiota on cytokine production. Peritoneal macrophages from conventionalized mice produced higher levels of IL-1, IL-6 and TNF-α than germfree animals. Monoassociation with Escherichia coli (a Gram-negative bacterium) also stimulated production of high levels of these cytokines, whereas the association with Bifidobacterium bifidum (a Gram-positive bacterium) was ineffective in raising the production of cytokines by peritoneal macrophages (8). The same kind of effects were reported later for bone marrow macrophages (9) and serum (10), which implicates the microbiota in the regulation of cytokine production in an even more systemic way. Accordingly, IL-12 and TNF-α serum levels in response to intravenous injection of E. coli are higher in conventional and Lactobacillus acidophilus-monoassociated mice than in germfree mice (our unpublished observation). In a model for intestinal and joint inflammation, it was shown that chronic granulomatous inflammation mediated by T lymphocytes, TNF-α and IL-1 was prevented when the resident normal enteric flora was removed by treatment of conventional rats with metronidazole (11). However, TNF-α production and consequent acute pancreatitis in response to artificial bile (endotoxin-free) were similar in conventional and germfree rats (12). Taken together, these data suggest that monokine production is boosted by products of the normal microbiota.

Systemic effects of the microbiota were also found when germfree mice were associated with non-intestinal parasites. The germfree environment favored a partial escape from the normal attrition that Schistosoma mansoni, a blood fluke, suffers when infecting conventional mice. Moreover, there was greater proliferation in the granulomas around S. mansoni eggs in livers from germfree animals (13). However, granulomas from germfree and conventional animals had the same general aspect and size (14) and oviposition was also similar in germfree and conventional mice (13,14). TNF-α has been implicated as a required factor for optimum oviposition and granuloma formation in conventional mice (15). Therefore, although no cytokine production data are available for germfree animals infected with S. mansoni, it is reasonable to conclude that, although basal production of TNF-α is lower in germfree mice (8-10, our unpublished observations), TNF-α production is normal in animals monoassociated with S. mansoni.

Infection with the intracellular parasite Trypanosoma cruzi is more severe in germfree animals, as shown by a higher mortality when compared with conventional controls (16). Germfree mice also displayed a more precocious and higher parasitemia than conventional controls. Moreover, tissues from germfree mice were more intensively parasitized and presented a more aggressive inflammatory response (16,17). Germfree mice infected with T. cruzi presented a stronger local reaction to subcutaneous injection of formalin-killed parasites as determined by footpad swelling than conventional animals

(17). In addition, germfree mice infected with T. cruzi did not survive the subcutaneous injection of antigen but died within 24 h, apparently of shock (18). Taken together, these data suggest that T. cruzi triggered a stronger cellular (type 1?) response in germfree animals than in their conventional counterparts. Unfortunately, cytokine data are not available to date. Interestingly, germfree-reared mice were even more susceptible to infection with T. cruzi when associated with single components of the normal microbiota (18). Although the reason for this enhanced susceptibility is unknown, it is tempting to speculate that antigen cross-reactivity between components of the microbiota and T. cruzi may play a role. Such cross-reactivity has been described not only for T. cruzi (17) but also for the African trypanosome T. brucei (19). Surprisingly, infection with Leishmania amazonensis, a Kinetoplastidae somewhat related to T. cruzi, was almost innocuous in germfree animals. Only one out of twelve L. amazonensis-injected mice had a small lesion when infected at the base of the tail, while all conventional controls showed large ulcerative lesions. Nevertheless, when the tissue was examined for parasites, it was found that a large number of macrophages were infected with L. amazonensis both in germfree and conventional animals. However, conventional animal tissues presented a large inflammatory response and necrosis, contrary to germfree animals in which this inflammatory response was almost absent (20).

Different effector mechanisms can be used by the immune system to overcome the invasion of pathogenic organisms such as intracellular parasites. Perhaps the best understood system is the conventional murine model of infection with Leishmania major. In this model, resistance to infection correlates with IL-12 production which directs the development of CD4+ T lymphocytes able to produce high levels of IFN-γ(21-24). Susceptibility in these animals has been correlated with a predominance of IL-4-producing T cells (25). These data have supported the paradigm suggested by Mosmann and

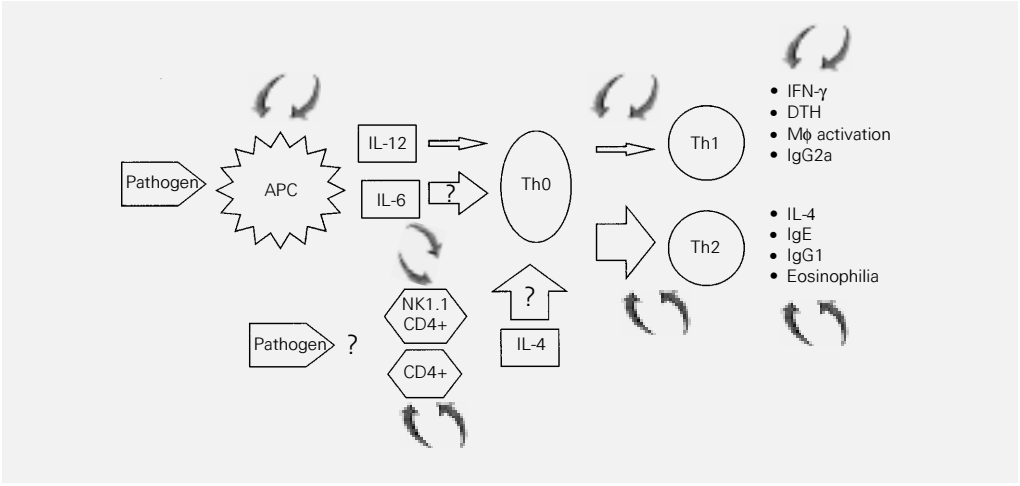

Figure 1 - Diagrammatic representation of the influence of cytokines on the innate or acquired immune response to parasites. Pathogens, in contact with antigen-presenting cells (APC), dendritic cells, macrophages, etc., will trigger either IL-12 or IL-6 production. IL-12 will induce Th0 cells to differentiate into the Th1 subset, resulting in interferon-γ (IFN-γ) production, delayed-type hypersensitivity (DTH), macrophage activation (Mϕ activation) and IgG2a. Alternatively, IL-6 from APC and IL-4 from NK1.1 CD4+ cells or CD4+ T cells will induce Th0 cells to differentiate into the Th2 subset, resulting in the production of IL-4, IgE, IgG1 and eosinophilia (based on References 21-28,38). Filled arrows indicate the points at which the normal microbiota may influence the immune response: 1) at the first interaction of APC with pathogens and the resulting production of cytokines, 2) at the putative interaction of pathogens with IL-4-producing cells, 3) at the differentiation of Th0 cells into Th1 or Th2 and 4) at the effector mechanisms.

Coffman (26) according to which the immune response to an infection could develop either towards a Th1 phenotype, characterized by high production of IFN-γ and subsequent macrophage activation, delayed-type hypersensitivity (DTH) response and production of IgG2a in mice, or towards a Th2 phenotype, characterized by production of high levels of IL-4 and IL-5 and subsequent high IgE and IgG1 and eosinophilia (Figure 1).

Many factors could influence the type of immune response mounted by the host. By far, cytokines secreted early in infection are best understood and explored (21-28). In addition to cytokines, the antigen dose has historically been related to the development of cell-mediated or humoral immune responses. Thus, when animals were immunized with a low dose of antigen, they preferentially developed a DTH response, while this response was abrogated when animals were immunized with higher doses of antigen. Conversely, antigen doses that favored DTH did not induce an antibody response, although at higher doses of antigen antibodies could be detected in the system (29). More recently, similar experiments were performed using infection with L. major, and similar results were obtained: infection with low numbers of L. major resulted in a resistance phenotype in an ordinarily susceptible strain of mice (30,31). The effect of the normal microbiota on the kind of response an animal will mount is not completely understood. MacDonald and Carter (32) addressed this issue using a model similar to that described by Parish (29). These authors immunized germfree and conventional mice with different doses of sheep red blood cells and showed that, while conventional mice developed a DTH response to low doses of antigen and a predominantly humoral response to higher doses, germfree animals did not efficiently develop a cell-mediated response. The antibody response was comparable to that of conventional animals. Upon association with a Gram-negative microorganism or conventionalization, former germfree mice reacted as conventional animals. One possible explanation for these results could be given in the light of experiments performed by Hooper et al. (33). These authors showed that antigen-presenting cells from antigen-free and germfree mice were more efficient in priming T cells than antigen-presenting cells from conventional animals, perhaps because of a higher density of antigen on their surface. We decided to verify if germfree mice could respond to antigens with a typical Th1-type response by infecting germfree mice with L. major and investigating the cytokine profile and the outcome of infection. Conventional Swiss mice developed a small lesion that was completely healed by 13 weeks of infection. Germfree mice, on the other hand, had not healed at 13 weeks. Histological analysis of tissues revealed that parasite growth was controlled by conventional mice, whereas germfree mice seemed to be permissive to growth. However, while conventional mice showed the expected cytokine profile, with high levels of IFN-γ and low levels of IL-4 being produced, the cytokine profile displayed by germfree mice was indistinguishable from that of conventional animals (our unpublished observations). These observations differ from the conventional model where high levels of IFN-γ production have been correlated with resistance to infection (21,25). IL-12 and early (day 2) IFN-γ production were also similar for the two groups, suggesting that the innate response to L. major was also similar for conventional and germfree animals (our unpublished observations), ruling out the possibility that a defect in the early response is the cause of the higher susceptibility of germfree mice to L. major (24,31,34). Our results may reflect a more intricate balance in cytokine production in germfree animals. Analysis of the involvement of other cytokines, such as IL-10 and TGF-ß, not only regarding their concentrations but also the kinetics of their production, should help to clarify this issue. Peritoneal cells from germfree mice activated with IFN-γ and infected with L. major produced nitric oxide

(NO) levels similar to those observed for conventional animals. However, parasitized macrophages were not able to destroy the parasites in vivo, as shown by histopathological analysis of the lesions. In the conventional murine model, the production of NO by activated macrophages is crucial for the killing of parasites (35,36). Our data suggest that macrophages from germfree mice are capable of producing NO in response to infection with Leishmania. However, it is not clear why the germfree mice do not resolve lesions as efficiently as their conventional counterparts. Both IL-10 and TGF-ß are known to interfere in macrophage killing of pathogens (37), and the later production of these cytokines by macrophages from germfree animals in vivo may explain our results.

Conventionalization of germfree mice caused mice to develop slightly larger lesions than conventional mice, if performed early in the course of infection with L. major (up to three weeks after infection, our unpublished observations). However, conventionalization after 8 weeks of infection did not allow mice to heal, and 10 out of 10 mice had non-healing lesions 17 weeks after infection, while conventional mice were healed by 9 weeks (our unpublished observations). Cytokine production did not differ between the conventional and conventionalized groups. It is clear from the above data that in the absence of the normal microbiota mice can mount a classic Th1-type response to infection with L. major. However, this response is not sufficient for the resolution of infection, as previously supposed (21,34).

Conclusions

Parasites, when associated with the host in the absence of the normal indigenous microbiota, can either be less pathogenic or more pathogenic. Infection with L. major was used as a model to establish a correlation between resistance or susceptibility and the cytokine profile displayed by the host. In our studies, however, we have found a picture of greater susceptibility in the presence of high levels of IFN-γ and in the absence of significant levels of IL-4. We propose that, in addition to IFN-γ, other factors contribute to resistance to L. major.

Acknowledgments

We are grateful to Drs. Luis Carlos C. Afonso and Ricardo Gazzinelli for a critical review of this manuscript.

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