Metabolic-cytokine responses to a second immunological challenge with LPS in mice with T. gondii infection


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Arsenijevic, D., L. Girardier, J. Seydoux, J. C. Pechere, I. Garcia, R. Lucas, H. R. Chang, and A. G. Dulloo. Metabolic-cytokine responses to a second immunological challenge with LPS in mice with T. gondii infection. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E439–E445, 1998.—Injection of 10 cysts of Toxoplasma gondii (Me49 strain) into Swiss Webster mice results in 1) an acute phase of infection lasting for 2–3 wk, characterized by weight loss, and 2) a chronic phase in which surviving mice show either partial weight recovery (Gainers) or persistent, although stable, cachexia (Nongainers). In response to a second immunological stimulation with lipopolysaccharide (LPS) in the chronic phase of the infection, it is shown that 1) the increase in energy expenditure was more prolonged in both groups of infected mice than in controls, 2) the intensity and duration of hypophagia were also differentially affected with Nongainers > Gainers > controls, and 3) the infected mice had higher serum levels of tumor necrosis factor-a (TNF-a) and interleukin (IL)-10 and a lower ratio of IL-10 to TNF-a than controls. In contrast, serum IL-4 increased to the same level in all three groups. Evaluation of the permeability of the blood-brain barrier by intravenous injection of Evans blue revealed a marked staining in the brain of only the infected Nongainers. Taken together, these results indicate that, in mice with chronic toxoplasmosis, a second nonspecific challenge (with LPS) exacerbates the hypophagic and hypermetabolic states, the latter being associated with hyperresponsiveness in TNF-a and IL-10 production. Furthermore, the greater exacerbation of the hypophagic state in mice showing persistent cachexia may be due to a preexisting higher permeability of the blood-brain barrier, which would allow a greater access of plasma-borne cytokines and/or other neuroimmunologically active substances to the central nervous system.

cachexia; thermogenesis; anorexia; starvation

INFECTION can lead to cachexia, and this has been associated with an unfavorable outcome in terms of survival, particularly when there is a drastic loss of body weight. A role for cytokines in inducing cachexia has been suggested by their ability to reduce appetite (15) and/or to increase energy expenditure (17), thus leading to a negative energy balance. In most published metabolic studies, however, injections of a single cytokine were performed, and it is difficult to separate pharmacological from physiological effects.

To gain insights into the physiological role of in vivo cytokine levels and their interactions in infection-induced cachexia, we have recently described an experimental murine model of chronic infection with Toxoplasma gondii to study the consequences of infection on long-term energy balance (1). Injection (ip) of 10 cysts of T. gondii (Me49 strain) into female Swiss Webster mice was shown to induce a progressive and substantial loss in body weight within 2–3 wk, and this was associated with a mortality rate of <25% during this acute phase of cachexia. In the chronic phase of the infection, about one-half of the surviving mice had persistent, although stable, cachexia (Nongainers), whereas the other half showed a partial weight regain (Gainers). This evolution into two distinct groups of surviving mice is a reproducible phenomenon, since it was regularly observed. The Nongainers were characterized by hypermetabolism, hypophagia, and elevated mRNA levels of spleen and brain cytokines, particularly those of the TH2 profile, i.e., those which are primarily involved in humoral immunity [e.g., interleukin (IL)-4, IL-5, IL-10] rather than in cellular immunity [e.g., interferon (IFN)-

Y]. The Gainers, on the other hand, although still hypophagic, were no longer hypermetabolic, and their cytokine mRNA levels were no longer elevated, except for tumor necrosis factor-a (TNF-a) and IL-10.

In this context of chronic infection, it was of interest to investigate whether a second immunological challenge, such as intraperitoneal injection of bacterial lipopolysaccharide (LPS) to Gainers and Nongainers mice, would induce further changes in energy balance and to compare their immune response by the determination of TNF-a, IL-4, and IL-10 production, three cytokines which have previously been found to be highly expressed in the spleen and brain of these T. gondii chronically infected mice.

MATERIALS AND METHODS

Mice and diets. Female Swiss Webster mice (BRL, Fullinsdorf, Switzerland) of ~1 mo of age were used and kept at room temperature with a 12:12-h light-dark cycle. The mice were housed in polycarbonate cages (4 mice/cage) and had free access to tap water and standard laboratory chow diet. These mice were injected intraperitoneally with 10 cysts of the Me49 strain of T. gondii, and after 7–10 days all lost weight. Subsequently, the infected mice were classified as Gainers if they showed a regain in body weight after infection-induced cachexia or Nongainers if they showed no regain after the acute phase of infection (Fig. 1). On day 90 postinfection, when both groups of infected mice had stable body weight and food intake, they were compared with age-matched noninfected mice fed ad libitum (the control group). All four separate experiments reported here used identical design as described above.

Experiment 1: Food intake and energy expenditure. In the three groups (n = 12–15), food intake was determined from the difference between the amount of food given and that removed from the cage, after the amount of any food spilled was taken into account. Energy expenditure (EE) was mea-
were measured by the murine IL-4 and IL-10 Immunoassay kits (Quantikine M from R&D Systems; UK) using their recommended protocol. All assays were conducted in duplicate.

Experiment 3: Determination of mRNA expression and tissue and serum levels of cytokines. An additional experiment in our model of chronic toxoplasmosis was conducted to assess, in the same study, these three cytokines at the levels of 1) tissue mRNA expression, 2) tissue protein, and 3) serum protein, in response to PBS or to LPS in each of the three groups (n = 3).

Blood was obtained by retroorbital sinus puncture from animals at 90 min after injection of LPS or PBS, and the animals were then immediately killed in order to remove spleen and brain. The cytokine mRNA expression in spleen and brain in all three groups was determined by Northern blot as detailed previously (1), the tissue cytokine proteins were extracted from the whole organ in 2 ml of 1% 3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate in RPMI 1640 medium (Boehringer Mannheim) according to the method described by Nakane et al. (12), and both tissue and serum proteins were assayed using the immunoassay kits (Quantikine M from R&D Systems, UK) for IL-4 and IL-10, or the InnoBios Basic ELISA kit (Innogenetics, Ghent, Belgium) for TNF-α, using the protocol described by Lucas et al. (11). All assays were conducted in duplicate.

Experiment 4: Blood-brain barrier permeability. To evaluate the permeability of the blood-brain barrier, mice from the control, Gainer, and Nongainer groups (n = 3) were injected with 200 µl of 1% Evans blue dye (10) in normal saline in the retro-orbital sinus. Animals were killed 120 min after injection, the organs were washed by infusion of saline into the left heart ventricle, and the brains were excised and rapidly put in Formalin 10%.

Data analysis. All data are presented as means ± SE. Statistical analysis was performed using one-way analysis of variance (ANOVA), with a post hoc Newman-Keuls multiple comparison test for pairwise comparison after ANOVA established significant differences among the groups.

RESULTS

Experiment 1: Body weight, food intake and EE. The profile of weight changes after the infection is presented in Fig. 1. After a delay of 9–11 days, infection with T. gondii resulted in substantial weight loss, after which the surviving mice could be differentiated into Gainers (i.e., those showing partial regain in body weight) or Nongainers (those showing no weight regain). The body weight of controls (n = 12), Gainers (n = 12), and Nongainers (n = 15) on day 90 after infection were 49.1 ± 3.5, 37.0 ± 2.9, and 24.8 ± 1.4 g, respectively, and 2 days after the second challenge with LPS, the loss in body weight in Nongainers (−2.48 g) was significantly greater (P < 0.02) than in Gainers (−0.93 g) and controls (−1.22 g). Food intake after LPS injection was compared with the average food intake before LPS. It was found that both the duration and the magnitude of hypophagia differed among groups in the following order: Nongainers > Gainers > controls (Fig. 2). Three days after LPS injection, the food intake of the controls and Gainers was ~70% of the level before LPS, whereas the food intake of Nongainers was still mark-
edly reduced, amounting to only 20% of that required for their preinjection energy balance. Chronic infection with *T. gondii* therefore is associated with an increased hypophagic response to LPS both in duration and intensity. After LPS injection, an increase in the absolute level of EE was observed in the three groups of mice (Fig. 3A), which peaked 1–2 h postinjection; however, the increase in EE was sustained only in the infected groups. This differential thermogenic response between the infected groups and the noninfected controls is clearly evident when EE is expressed in watts per kilogram to the power of 0.75 and as the ratio of postinjection to preinjection values (Fig. 3B). It is found that at 1 h after LPS, the increases in EE were not significantly different among controls, Gainers, and Nongainers, but they were subsequently higher and more sustained in both groups of infected mice than in controls. It is to be noted that on day 2 postinjection, two out of the 12 Nongainers mice died, whereas no death occurred in the other two groups.

**Experiment 2: Circulating cytokines.** The cytokine levels were determined 90 min after LPS injection, since it was shown in previous studies (3) that TNF-α concentration was maximal at this time point. In each group, mice that were injected with the vehicle buffer PBS had no detectable circulating cytokine levels. After LPS injection, serum TNF-α levels increased to a greater extent in the infected mice than in controls (Fig. 4); infected Gainers and Nongainers had about the same level of circulating TNF-α. Hence, chronic infection enhanced serum TNF-α level in response to LPS. Serum IL-4 concentrations increased to a similar level in the three groups, indicating that chronic infection did not significantly modify the IL-4 response. By contrast, IL-10 response was significantly greater in infected mice, and, in addition, the Gainers had a higher concentration of IL-10 than Nongainers (P < 0.001). The ratio of IL-10 to TNF-α was found to be significantly lower (P < 0.001) in the infected mice (Nongainers: 0.06 and Gainers: 0.08) compared with the controls (0.31), whereas these ratios for both groups of infected mice were not statistically different.

**Experiment 3: mRNA expression and tissue and serum levels of cytokines.** In our analysis of the data on circulating cytokines in the above experiment, it became clear that in the absence of LPS few or no serum levels of TNF-α, IL-10, and IL-4 were detectable in mice chronically infected with *T. gondii*. These observations contrast with our previously reported findings in this same model that mRNA expressions of these cytokines were markedly upregulated in spleen and brain (1). Because of this apparent inconsistency between the cytokine mRNA levels in our previous study and the circulating levels in the present study, we have conducted an additional experiment in our model of chronic...
toxoplasmosis in which we have assessed, in the same study, these three cytokines at the levels of 1) tissue mRNA expression, 2) tissue protein, and 3) serum protein, in response to PBS or to LPS in each of the three groups. The results are presented in Fig. 5. First, they confirm the above-mentioned discrepancy that, in the absence of LPS (i.e., in PBS-injected mice), serum levels of TNF-α and IL-10 were significantly greater in infected groups than in the control group, whereas in the case of serum IL-4, no difference was found among the 3 groups.

Second, the results presented in Fig. 5 show that in response to LPS the upregulation of mRNA and tissue protein levels for TNF-α and IL-10 were enhanced in all groups, and serum level of these two cytokines was found to be more elevated in the infected groups than in the control group. The ratio of serum IL-10 to TNF-α was, however, found (as in experiment 2) to be significantly lower (P < 0.001) in the infected mice (Nongainers: 0.02 and Gainers: 0.02) than in the controls (0.58). In the case of IL-4, serum levels of this cytokine were also found to be markedly increased in all groups in response to LPS, and no significant differences were observed across the three groups. IL-4 mRNA and protein levels in both spleen and brain were elevated after LPS in all groups, and these values after LPS tended to be greater than those after PBS in controls and Gainers. In the Nongainers, however, it is of interest to confirm here our previous findings (1) of the marked upregulation of mRNA expression, as well as tissue protein of this TH2-type cytokine, and that the administration of LPS produced few or no further increases.

Experiment 4: Blood-brain barrier permeability. As can be seen in Fig. 6, only Nongainers showed a marked Evans blue accumulation in their brains (reflected by being darker), thereby indicating that the permeability of the blood-brain barrier of these mice was clearly increased, in contrast to mice in the control or Gainer groups.

DISCUSSION

Using this murine model of infection with T. gondii, we have previously shown that, during the chronic phase of infection, mice from the Gainers group were no longer hypermetabolic, since their 24-h EE/kg^0.75 was similar to that of controls, and that their lower body weight could be quantitatively accounted for by their lower food intake (1). In contrast, the Nongainers showed not only a reduction in food intake but also an increase in EE, as judged by their higher 24-h EE/kg^0.75 relative to ad libitum-fed controls or by their higher absolute 24-h EE (i.e., per mouse) relative to underfed weight-matched controls, i.e., noninfected mice underfed to lose the same amount of weight as the infected mice. Marked differences between infected Gainers and Nongainers were also observed in cytokine mRNA expressions in brain and spleen; namely, only TNF-α and IL-10 mRNA were expressed in Gainers, whereas in the Nongainers all cytokines measured IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, TNF-α, and IFN-γ mRNA were expressed, with values for IL-4, IL-5, IL-6, and IL-10 being particularly elevated (1). Moreover, the Nongainers had increased serum immunoglobulins and glomerulonephritis, which are indications of an autoimmune state. These findings suggested that the chronically cachexic mice (in the Nongainer group) showed a reduced capability to resist T. gondii infection, with a predominant TH2-type cytokine expression pattern (i.e., primarily involved in humoral immunity), and had a greater number of cysts in their brains (1).
The study described here further demonstrates that chronic infection with *T. gondii* modifies the response to an LPS challenge: 1) by prolonging the hypermetabolic response and potentiating the hypophagic phase and 2) by enhancing the concentration of circulating TNF-α and IL-10, which, unlike serum IL-4, were found to be greater in the infected groups than in the control group in response to LPS. To what extent other cytokines (e.g., IL-1, IL-2, IL-5, IL-6, and so forth) might also be implicated in the hyperresponsiveness of the infected animals to a second challenge cannot be disregarded and would need to be addressed in future studies, but it is clear from the data presented here that at least two cytokines (IL-10 and TNF-α) can be associated with the anorectic and thermogenic hyperresponsiveness after the second challenge with LPS.

It is to be noted that, despite the elevated cytokine mRNA level previously observed on day 90 in the Nongainers, no detectable circulating TNF-α, IL-4, and IL-10 were found before LPS injection in this group. This again brought into focus the problems raised in our previous work (1) in the attribution of specific cytokines to metabolic responses and prompted us to conduct an additional experiment in which these three cytokines were examined at the serum level as well as at the levels of their mRNA expression and their protein product in the spleen and in the brain. The results of this experiment confirm the existence of this inconsistency between the upregulated cytokine mRNA levels and the virtually undetectable serum levels of their protein products but also reveal that the marked mRNA expression of these cytokines in the spleen and brain of the chronically infected mice was associated with parallel increases in the tissue level of their respective protein products. On the basis of these findings, it is therefore reasonable to attribute the inconsistency between mRNA expression and lack of elevation in circulating cytokines to the regulation of...
the secretory processes that underlie the release of cytokines from the tissues into the circulation rather than to the regulation of cytokines at the translational level. Because marked increases in circulatory levels of these cytokines occurred only when both the mRNA expression and tissue protein levels were enhanced in response to LPS, the possibility arises that either an acute stimulus is essential and/or a critical threshold in tissue cytokine level needs to be reached before it can be released into the circulation. There is clearly a need for further studies that would address the mechanisms underlying cytokine secretion from the tissues into the circulation, on the one hand, and the relationship between tissue content of a given cytokine and its autocrine effect (particularly in the brain) on the other.

It is nonetheless clear from the present study that, in response to a second immunological challenge (with LPS), the chronically T. gondii-infected mice (the Nongainers) show a greater increase in serum levels of TNF-α and IL-10 than the controls. An increase in TNF-α response to LPS injection during chronic infection has previously been reported in Swiss Webster (4) and in C57BL/J (5) mice infected with T. gondii (C56 strain). The same finding has also been reported in hamsters infected with Leishmania donovani, in which their macrophages showed hyperproduction of TNF-α in the chronic phase (8th wk) compared with the acute phase (2nd wk) of the infection (13). IL-10 has been shown to have antagonistic effects on the pathophysiological action of TNF-α in vivo (2). In support of this observation, we have presently found that the severity of LPS-induced hypophagia in the infected Nongainers and Gainers is associated with a lower ratio of serum IL-10 to TNF-α than in control mice. However, little or no difference in this ratio was observed between Gainers and Nongainers, and hence cannot be invoked for the greater severity of the hypophagic response in the Nongainers. A possible explanation for the prolonged duration of LPS-induced hypophagia in Nongainers may reside from a greater permeability of their blood-brain barrier, thus allowing a greater access to the central nervous system for LPS-induced circulating cytokines such as TNF-α and/or LPS itself, where they would be acting locally (16). Furthermore, an autocrine role for brain cytokines underlying the prolonged suppression of appetite is also a plausible explanation, particularly in light of our findings presented here and those of Chang et al. (5) in chronic murine T. gondii encephalitis, that brain cytokines are found to be markedly elevated in the absence of any detectable increase in circulating (systemic) cytokine. To what extent cytokine induction of leptin, a protein that has been demonstrated to produce anorexia and hypermetabolism (8, 14), also plays a role in this exaggerated hypophagic and thermogenic response to LPS in chronic toxoplasmosis is currently unknown. This is certainly an interesting future line of investigation, particularly in light of a recent report (18) showing that 1) administration of LPS to mice increased leptin gene expression and circulating leptin levels and 2) the administration of TNF-α and IL-1 produced a dose-dependent increase in serum leptin levels.

In conclusion, the negative energy balance induced by an immunological stress such as LPS injection is worsened in mice chronically infected with T. gondii compared with controls. The more severe anorectic state of the chronically infected mice is associated with a higher LPS-induced rise in the concentration of circulating TNF-α and IL-10 and a marked reduction in the ratio of IL-10 to TNF-α. However, although a causative link between anorexia and these cytokines cannot be ascertained, the severity of the hypophagic response seems to depend on preexisting lesions of the blood-brain barrier, associated with signs of autoimmunity. Given the importance of secondary infections in the exacerbation of cachexia in many pathological conditions, further studies are warranted to better define the role of cytokines in the exacerbation of anorexia and hypermetabolism. In this context, the murine model of chronic toxoplasmosis described here could be of value, particularly since its metabolic responses to LPS reflect those observed in patients with secondary infections. The enhanced thermogenic and anorectic effects from secondary challenge in this murine model are reminiscent of the marked anorexia, high resting EE, and rapid weight loss of acquired immune deficiency syndrome (AIDS) patients with active secondary opportunistic or bacterial infections such as Mycobacterium avium complex (6, 7). Furthermore, as in this model of chronic toxoplasmosis, in humans with chronic infections associated with cachexia, serum cytokines are generally not found to be higher than in controls (healthy individuals), but only in response to secondary challenge, such as during sepsis or acute parasitic infections (6). In the case of human immunodeficiency virus infection, for example, elevated levels of serum TNF-α in AIDS were predominantly found in patients with secondary infections (6, 9).

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