INFECTION AND IMMUNITY, Feb. 1996, p. 535–541 Vol. 64, No. 2
0019-9567/96/$04.00+0

A Th1-Associated Increase in Tumor Necrosis Factor Alpha Expression in the Spleen Correlates with Resistance to Blood-Stage Malaria in Mice

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Received 18 August 1995/Returned for modification 31 October 1995/Accepted 16 November 1995

We investigated the kinetics of tissue-specific mRNA expression and systemic production of tumor necrosis factor alpha (TNF-α) and the kinetics of splenic expression of mRNAs of gamma interferon (IFN-γ) and interleukin-4 (IL-4), cytokines that may regulate TNF-α production, during the early phase of blood-stage infection with *Plasmodium chabaudi* AS. Northern blot analysis revealed that resistant C57BL/6 mice, which clear the infection by 4 weeks, had higher levels of TNF-α mRNA in the spleen and liver early during infection than did susceptible A/J mice, which succumb to the disease 10 days after initiation of infection. Treatment of resistant mice with a polyclonal anti-TNF-α antibody confirmed the protective role of TNF-α early during the course of infection. Furthermore, resistant C57BL/6 mice also expressed high levels of mRNA of IFN-γ (a Th1 marker) and low levels of mRNA of IL-4 (a Th2 marker) in the spleen, whereas susceptible A/J mice had low levels of IFN-γ mRNA but high levels of IL-4 mRNA in the spleen early during infection. On the other hand, susceptible A/J mice expressed high levels of TNF-α mRNA in the liver and had high levels of TNF-α protein in serum, as measured by enzyme-linked immunosorbent assay, later during infection just before death occurred. These results demonstrate that a Th1-associated increase in TNF-α mRNA expression in the spleen early during infection correlates with resistance to *P. chabaudi* AS, whereas increased TNF-α mRNA levels in the liver and excessive levels of the TNF-α protein in serum later during infection correlate with susceptibility. Thus, the role of TNF-α during malaria appears to depend on the timing and site of its expression and the presence of cytokines regulating its production.

Tumor necrosis factor alpha (TNF-α) is a protein produced during many infectious diseases, including malaria (for a review, see reference 2). Blood-stage malaria parasites have been shown to release endotoxin-like antigens which induce the production of TNF-α in vivo (1). High levels of TNF-α have been demonstrated in the blood of malaria patients infected with *Plasmodium falciparum* or *Plasmodium vivax* (18, 19). The biological effects of TNF-α during malaria infection are presumed to be both pathogenic and protective. Treatment of patients with anti-TNF antibody has indicated that TNF-α is involved in the induction of high malarial fever (20). Furthermore, studies using murine malaria models have shown that TNF-α may mediate the two major causes of mortality associated with malaria, anemia (5, 35) and cerebral malaria (9). In contrast, repeated injections of recombinant TNF-α into malaria-infected mice have been shown to reduce parasitemia and protect against lethal infection (36). It is often assumed that the protective versus pathogenic role of TNF-α during malaria depends on the quantity of TNF-α produced, the time period over which its production is sustained, the tissue where it is produced, and the presence of cytokines regulating its production (6, 7). Whereas most studies have focused on the quantity of TNF-α produced (18–20), only one in vivo study has demonstrated that increased expression of TNF-α mRNA in the brain correlated with susceptibility to murine cerebral malaria (7). The same authors also suggested that, in the brain, a Th1-type response may up-regulate, whereas a Th2-type response may down-regulate, TNF-α production (7).

Consistent with the results of Langhorne (21), our laboratory has recently demonstrated that elimination of blood-stage *Plasmodium chabaudi* AS by resistant B6 mice occurs by the sequential activation of $CD4^+$ Th1 cells and Th2 cells (31). In contrast, induction of a strong Th2 response early in infection, as observed in susceptible A mice, has been suggested to lead to fulminant parasitemia and a lethal course of malaria (31). Furthermore, we demonstrated that treatment with human recombinant TNF-α (rHuTNF) early during infection protects susceptible A mice from an otherwise lethal infection (29). Thus, A mice treated with rHuTNF-α experience a course of infection similar to that of resistant B6 mice, which is characterized by moderate levels of peak parasitemia on days 7 to 10 postinfection and clearance of infection by 4 weeks. Treatment of resistant B6 mice with the same dose of rHuTNF-α, however, had no effect, suggesting that this mouse strain produces sufficient amounts of TNF-α. Treatment with a fivefold-higher dose of rHuTNF-α resulted in the death of B6 mice within hours. We also demonstrated that an intact spleen is required to resolve *P. chabaudi* AS malaria (32). Others have suggested that the liver, in addition to the spleen, may have a protective role during blood-stage malaria (8).

In the present study, we investigated the kinetics of tissue-specific and systemic production of TNF-α and the kinetics of Th1 and Th2 cytokines that may regulate its production in vivo. More specifically, we determined the following in both mouse strains during the first 2 weeks postinfection: (i) TNF-α mRNA levels in the spleen and liver, (ii) levels of TNF-α protein in serum, and (iii) mRNA levels of gamma interferon (IFN-γ) (a Th1 marker) and interleukin-4 (IL-4) (a Th2 marker) in the

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spleen. In addition, we investigated the protective role of TNF-α in resistant B6 hosts by treating this mouse strain with a polyclonal anti-TNF antibody. Our results confirm that TNF-α has a protective role and indicate that increased levels of TNF-α in the spleen and the liver early during infection are required in order to survive infection with *P. chabaudi* AS. In contrast, increased TNF-α levels in the liver and in serum later during infection appear to have a deleterious effect on the outcome of this disease. Furthermore, a Th1 response appears to sustain a high level of expression of TNF-α in the spleen, whereas a Th2 response appears to down-regulate the expression of TNF-α.

MATERIALS AND METHODS

Mice, parasite, and experimental infections. B6 and A strain mice, 8 to 14 weeks old, were age and sex matched for all experiments. Mice were bred at the animal facility of the Montreal General Hospital Research Institute from breeding pairs obtained from Jackson Laboratories (Bar Harbor, Maine). *P. chabaudi* AS was maintained as previously described (24). Mice were infected intraperitoneally with 10^6 *P. chabaudi* AS-parasitized erythrocytes (PRBC), and parasitemia during the subsequent course of infection was monitored by previously described procedures (31).

Anti-TNF-α antibody treatment. Rabbit anti-recombinant murine TNF-α immunoglobulin G was kindly provided by E. A. Havell (Trudeau Institute, Saranac Lake, N.Y.). The production and purification of these antibodies and their capacity to specifically neutralize TNF-α have been previously described (12). Resistant B6 mice were treated intraperitoneally with 0.2 ml of pyrogen-free saline containing 10^4 neutralizing units of anti-TNF-α antibody (protein content, 2.4 mg) 6 h prior to and 4 days after *P. chabaudi* AS infection. Control mice were similarly treated with 0.2 ml of pyrogen-free saline containing an equivalent amount of normal rabbit serum immunoglobulin G which was prepared in the same way as the anti-TNF-α antibodies.

mRNA levels in spleen and liver. Spleens and livers of B6 and A mice, both normal and infected, were collected aseptically and immediately frozen in liquid nitrogen. Total RNA was isolated by a modification of the guanidine thiocyanate-CsCl method as described by Chirgwin et al. (4). The RNA yield of each sample was determined spectrophotometrically on the basis of the A_{260}, and the purity was assessed by the A_{260}/A_{280} ratio. The purities of our samples ranged from 1.5 to 1.8. mRNA levels were determined by Northern blot analysis (26). Briefly, total-RNA samples (20 μg) were separated on a 1.2% agarose gel containing 2.2 M formaldehyde, and the blots were transferred to a nylon membrane (Schleicher & Schuell, Keene, N.H.) and UV cross-linked (UV Stratalinker 1800; Stratagene, La Jolla, Calif.). The blots were prehybridized for 8 h, hybridized overnight with a specific DNA probe, labeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, Ill.) (specific activity, 10^9 cpm/μg of DNA), and washed. Hybridization was detected by autoradiography with X-Omat-AR film (Kodak, Rochester, N.Y.). mRNA levels were quantified by high-resolution optical densitometry (SciScan 5000; United States Biochemical, Cleveland, Ohio) and normalized to 18S rRNA. A 1.9-kb fragment of rRNA, which recognized 18S rRNA, was kindly provided by N. Arnheim and has been previously described in detail (25). A 1.3-kb fragment of the murine TNF-α gene was kindly provided by A. Cerami (Picower Institute for Medical Research, Manhasset, N.Y.). Fragments of the murine IFN-γ (1.4 kb) and IL-4 (0.6 kb) genes were kindly provided by Ken-Ichi Arai (DNAX, Palo Alto, Calif.). TNF-α, IFN-γ, and IL-4 mRNA and 18S rRNA migrate with mobilities of 1.3, 1.4, 0.6, and 1.9 kb, respectively. All spleen or liver samples from mice of the two strains were run on the same gels. The 18S and TNF-α, IFN-γ, and IL-4 probes were used sequentially on all blots. The autoradiographs with the TNF-α probe were developed for 48 h for the spleen samples and 96 h for the liver samples. The autoradiographs with the IFN-γ and IL-4 probes were developed for 24 and 96 h, respectively. The autoradiographs with the 18S probe were developed for 10 min for all blots.

TNF-α protein in serum. Blood from experimental mice was obtained by cardiac puncture, allowed to clot for 30 min at 4°C, and centrifuged at 13,800 × *g* for 3 min. Sera were collected and stored at −20°C until use. A modification of a double-sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of TNF-α protein in serum. Briefly, 96-well plates (Immunolon II; Dynatech, Chantilly, Va.) were coated overnight at 4°C with 50 μl of hamster anti-murine TNF-α monoclonal antibody (28) (2 μg/ml) (Genzyme, Boston, Mass.) per well. The wells were washed and incubated overnight at 4°C with either 50-μl serum samples or, as a positive control and standard, 50 μl of murine recombinant TNF-α (Genzyme) diluted in 10% normal mouse serum (twofold serial dilution ranging from 1.6 ng/ml to 12.5 pg/ml). After being washed, the wells were coated with 150 μl of skim milk powder (5% wt/vol) for 20 min at room temperature. Subsequently, the wells were washed and incubated for 2 h at room temperature with 50 μl of rabbit polyclonal anti-murine TNF-α (1:1,800 dilution) which was prepared and purified by standard procedures (30). After being washed, the wells were incubated for 1 h with 50 μl of goat anti-rabbit immunoglobulin G (heavy plus light chains) conjugated to horseradish peroxidase (1:3,000 dilution) (Bio-Rad, Richmond, Calif.) and washed, and 50 μl of enzyme substrate (ABTS; Boehringer Mannheim, Laval, Quebec, Canada) was added for 10 to 20 min. The A_{405} was determined with an ELISA reader. The levels of TNF-α in the serum samples (picograms per milliliter) were calculated from the standard curve.

Statistical analysis. The statistical significance of differences in mortality between control and experimental groups receiving anti-TNF-α antibody was determined by using a nonparametric Kruskal-Wallis test. The statistical significance of differences in TNF-α, IFN-γ, and IL-4 mRNA levels, and in serum TNF-α protein levels, between normal and infected animals as well as between B6 and A mice was determined by Student's *t* test. A probability of less than 0.05 was considered significant.

RESULTS

Anti-TNF-α antibody treatment of resistant mice. We have previously demonstrated that treatment with rHuTNF-α could protect susceptible A mice against an otherwise lethal infection with *P. chabaudi* AS (29). In order to confirm the protective role of TNF-α during infection, we treated resistant B6 mice with anti-TNF-α antibody 6 h prior to and 4 days after infection, and parasitemia and survival were monitored. A similar treatment schedule with the same antibodies has previously been shown to eliminate the resistance of B6 mice to infection with *Toxoplasma gondii* (16). As a control, infected B6 mice were treated with normal rabbit serum. Figure 1A shows that 50% (5 of 10) of the mice treated with anti-TNF-α antibody died at, or 1 to 2 days later than, the peak of parasitemia, which occurred on day 7 postinfection. In contrast, 100% (10 of 10) of the control mice treated with normal rabbit serum survived. Although depletion of TNF-α did not significantly alter peak parasitemia level (Fig. 1B), it did delay it by 1 day. The mean peak parasitemia levels (± standard error of the mean), determined as percent PRBC, were 46.2% ± 6.0% for the experimental mice and 47.3% ± 2.5% for control mice. These data demonstrate that treatment of resistant B6 mice with anti-TNF-α antibody results in significantly increased mortality but does not alter the peak parasitemia level. These observations thus confirm that endogenous TNF-α has a protective role during *P. chabaudi* AS malaria and suggest that TNF-α is not involved in controlling the course of parasitemia.

TNF-α mRNA kinetics in spleen and liver. To determine if differences in TNF-α expression in the tissues during infection with blood-stage *P. chabaudi* AS contribute to resistance, total RNA was extracted from spleens and livers harvested from B6 and A mice at various times during the first 2 weeks postinfection, and TNF-α mRNA levels were determined by Northern blot analysis. Expression of TNF-α mRNA in tissue was also examined in uninfected control mice of each strain. As shown in Fig. 2, significantly increased levels of TNF-α mRNA were found in the spleens of resistant B6 mice as early as day 3 postinfection compared with basal levels in the spleens of uninfected control mice on day 0. TNF-α mRNA levels remained elevated through day 7 postinfection, when parasitemia ranged from 20 to 30% PRBC. TNF-α mRNA levels returned to basal levels on day 9 postinfection, when the peak of parasitemia (40% PRBC) occurred, and remained at this level until day 14 postinfection, when the experiment was terminated. In contrast, TNF-α mRNA expression in the spleens of susceptible A mice was not significantly increased over that of the basal level in the spleens of normal control animals until day 5 postinfection, when parasitemia was approximately 10% PRBC. TNF-α mRNA levels returned to basal levels on day 7 in susceptible hosts and remained at this level through day 9 postinfection, when peak parasitemia levels of >50% PRBC occurred. As previously reported (29), death of 100% of susceptible A littermates occurred between days 9 and 12 postinfection. Similar to TNF-α mRNA expression in the spleens of

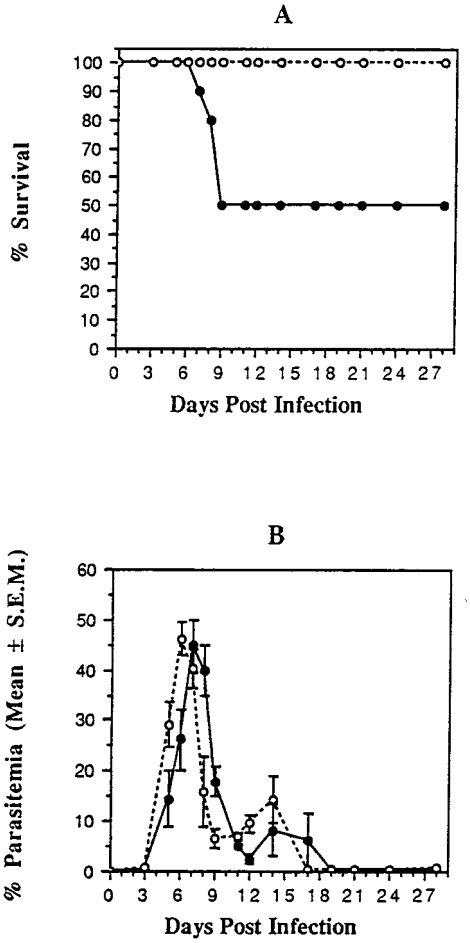

FIG. 1. Effect of treatment with polyclonal anti-TNF-α antibody on host response to *P. chabaudi* AS. Groups of five mice each (resistant B6 strain) were treated intraperitoneally with 10^4 neutralizing units of rabbit anti-murine TNF-α antibody (protein content, 2.4 mg) 6 h prior to and 4 days after *P. chabaudi* AS infection, and survival (A) and parasitemia (B) were monitored. Control mice ($n = 5$) were similarly treated with 2.4 mg of normal rabbit serum proteins. The data shown are pooled from two replicate experiments. The difference in the survival rates between the experimental and control groups was significant ($P < 0.02$). Symbols: ●, anti-TNF-α antibody; ○, control serum. S.E.M., standard error of the mean.

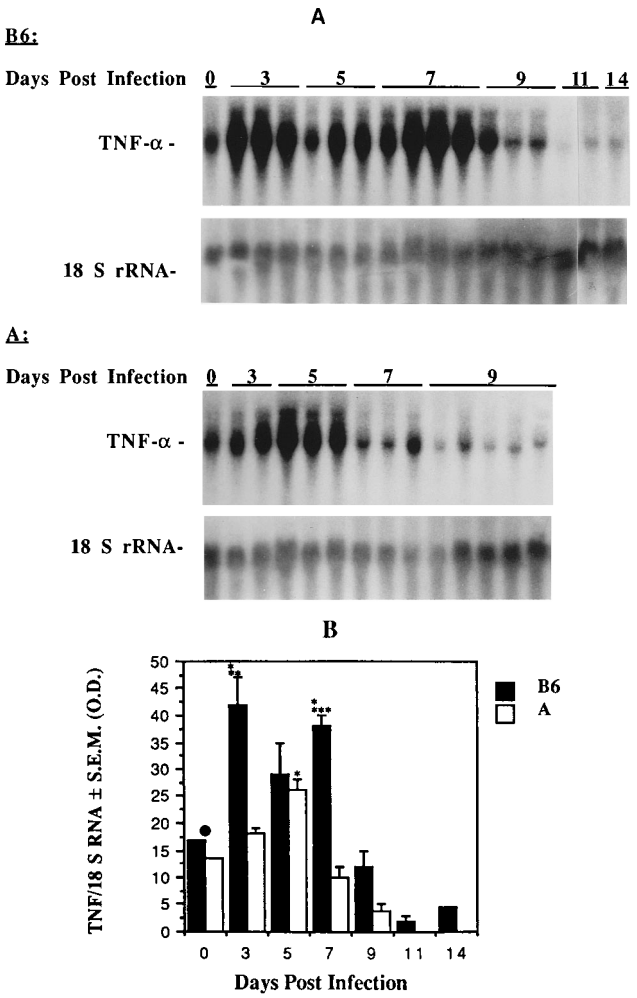

FIG. 2. Kinetics of TNF-α mRNA levels in the spleens of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. (A) Total RNA was purified from spleens recovered from one to four mice per time point, and the levels of TNF-α mRNA expression in individual spleens were determined by Northern blot analysis. 18S rRNA levels were also determined by Northern blot analysis for each sample. The data shown are representative of two replicate experiments. (B) Scanning densitometry of the autoradiographs shown in panel A. The densities of the bands corresponding to TNF-α mRNA expression were determined and normalized to the levels of 18S rRNA expression. O.D., optical density; S.E.M., standard error of the mean. Symbols: *, $P < 0.04$ in comparison with value for control mice on day 0; **, $P < 0.04$ in comparison with value for A mice on day 3 postinfection; ***, $P < 0.01$ in comparison with value for A mice on day 7 postinfection; ●, control mice (results were pooled for statistical analysis).

B6 mice, TNF-α mRNA levels in the livers of B6 mice were significantly increased in comparison with basal levels in control mice early during infection, that is, on days 5 and 7 postinfection, and returned to basal levels later on during infection (Fig. 3). In contrast to the moderate increase in TNF-α mRNA expression in the spleens of A mice on day 5 postinfection, liver TNF-α mRNA levels in susceptible A mice were significantly increased in comparison with basal levels in control mice later during infection, that is, reaching their highest level on day 9 postinfection, coincident with the peak of parasitemia (Fig. 3). Although the exact time course varied from experiment to experiment, significant differences in the level as well as the timing of TNF-α expression in spleens and livers of resistant B6 versus susceptible A mice were consistently observed. These results thus demonstrate that early during acute infection, that is, during the ascending phase of parasitemia, resistant B6 mice have much higher peak levels of TNF-α mRNA in the spleen and liver than susceptible A mice. In contrast, susceptible A mice have significantly increased peak levels of TNF-α mRNA in the liver later during infection at the time of peak parasitemia, just before death occurs. This suggests that tissue-specific production of TNF-α in the spleen and liver early during infection confers protection, whereas production of TNF-α in the liver later during infection has a deleterious effect on the outcome of the disease.

TNF-α protein kinetics in serum. In order to determine the level of in vivo systemic production of TNF-α during infection with *P. chabaudi* AS, levels of TNF-α were determined by ELISA of sera collected from B6 and A mice at different times during infection. TNF-α was not detectable in the sera collected from uninfected mice of either strain on day 0 or infected mice of either strain on day 3 postinfection (Table 1). Low levels of TNF-α (≤20 pg/ml) were found in only 33% of

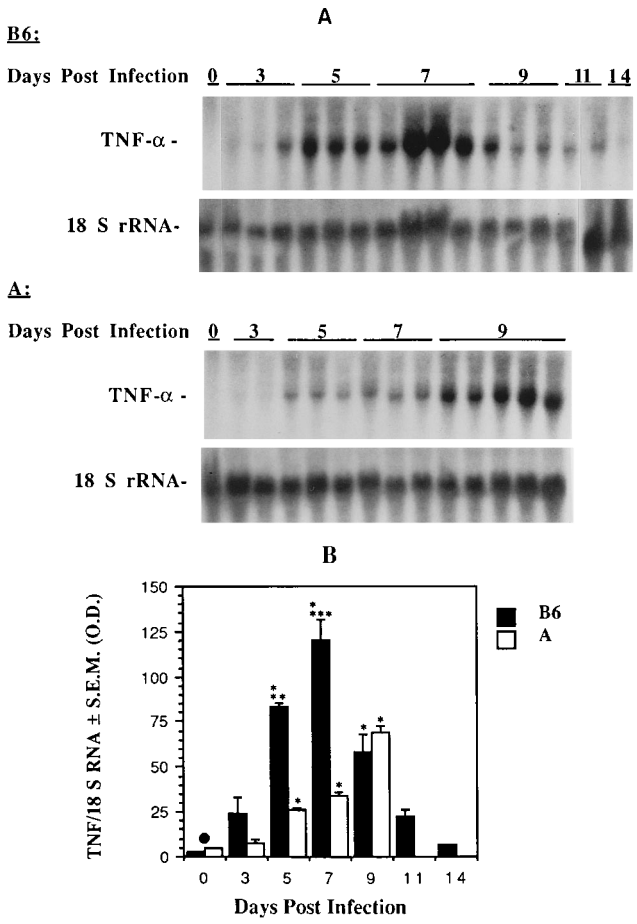

FIG. 3. Kinetics of TNF-α mRNA levels in the livers of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. (A) Total RNA was purified from livers recovered from one to five mice per time point, and the levels of TNF-α mRNA expression in individual livers were determined by Northern blot analysis. 18S rRNA levels were also determined by Northern blot analysis for each sample. The data shown are representative of two replicate experiments. (B) Scanning densitometry of the autoradiographs shown in panel A. The densities of the bands corresponding to TNF-α mRNA expression were determined and normalized to the levels of 18S rRNA expression. O.D., optical density; S.E.M., standard error of the mean. Symbols: *, $P < 0.04$ in comparison with value for control mice on day 0; **, $P < 0.01$ in comparison with value for A mice on day 5 postinfection; ***, $P < 0.01$ in comparison with value for A mice on day 7 postinfection; ●, control mice (results were pooled for statistical analysis).

mice of either strain on days 5 to 6 postinfection, when parasitemia ranged from 16 to 34% PRBC. The highest level of TNF-α in the serum of either mouse strain was apparent on day 7 to 10 postinfection, coincident with the peak of parasitemia. More important, however, is that during this period, more A than B6 mice (80 versus 44%, respectively) had detectable TNF-α levels in serum and that the average TNF-α level detected in A mice was three times higher than the level in B6 mice (117 versus 38 pg/ml, respectively). As described above, 100% of susceptible A mice died between days 9 and 12 postinfection. Low levels of TNF-α (≤20 pg/ml) could be detected in the sera of B6 mice on day 14 postinfection, when only 1% of peripheral RBC were infected. These data thus indicate that, at peak parasitemia, higher levels of TNF-α are present in the sera of susceptible A mice than in the sera of resistant B6 mice. This suggests that a high level of systemic production of TNF-α later during infection may have a deleterious effect on the outcome of *P. chabaudi* AS malaria.

TABLE 1. Kinetics of TNF-α protein in sera of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS[a]

Day(s) postinfection	Parasitemia (mean % PRBC ± SEM)		Prevalence of serum-TNF-α-positive mice (%)		TNF-α level (mean pg/ml ± SEM)	
	B6	A	B6	A	B6	A
0	0	0	0	0	0	0
3	4 ± 4	5 ± 2	0	0	0	0
5–6	16 ± 1	34 ± 0	33	33	≤20	≤20
7–10	38 ± 4	45 ± 3	44	80	38 ± 18	117 ± 32[b]
14	1 ± 0	ND[c]	33	ND	≤20	ND

[a] Blood from groups of three to eight A and B6 mice was obtained by cardiac puncture at different times postinfection, and the level of TNF-α protein was determined by a modification of a double-sandwich ELISA. The data shown are representative of two replicate experiments.
[b] $P < 0.04$ in comparison with value for B6 mice on days 7 to 10 postinfection.
[c] ND, not determined.

IFN-γ (Th1) versus IL-4 (Th2) mRNA kinetics in spleen. Previous results from our laboratory have shown that ex vivo, in response to malaria antigen within 1 week of infection, spleen cells isolated from resistant B6 mice produce high levels of IFN-γ (a Th1 cytokine) and low levels of IL-5 (a Th2 cytokine) (31). In contrast, spleen cells from susceptible A mice produce high levels of IL-5 and low levels of IFN-γ within the first week of infection (31). Others have shown that increased levels of TNF-α mRNA in the brains of *Plasmodium berghei* ANKA-infected mice are associated with a Th1-like response (7). To determine if, in the spleen, a Th1 versus a Th2 response correlates with a high versus a low level of expression of TNF-α, respectively, we determined by Northern blot analysis the kinetics of in vivo expression of mRNAs of IFN-γ and of the Th2 cytokine IL-4 in the spleens of both B6 and A mice during the first 2 weeks postinfection. Expression of IFN-γ and IL-4 in tissue was also examined in uninfected control mice of each strain. Figure 4 shows that significantly increased levels of IFN-γ mRNA were found in the spleens of resistant B6 mice from day 3 through day 9 postinfection compared with basal levels in the spleens of uninfected control mice on day 0. A maximal increase in IFN-γ expression occurred several days before peak parasitemia, that is, on day 7 postinfection, when parasitemia ranged from 20 to 30% PRBC. IFN-γ mRNA levels returned to basal levels on day 14 postinfection, when the experiment was terminated. In contrast, a significant increase in IFN-γ mRNA expression in the spleens of susceptible A mice compared with the basal level in the spleens of normal control animals only occurred on day 5 postinfection, when parasitemia was approximately 10% PRBC. Moreover, the maximal IFN-γ mRNA level in the spleens of susceptible A mice on day 5 postinfection was significantly lower than the IFN-γ mRNA levels in the spleens of resistant B6 mice on days 5 and 7 postinfection. In contrast to IFN-γ mRNA expression, the highest levels of IL-4 mRNA were found in the spleens of A mice (Fig. 5). Significantly increased levels of IL-4 mRNA in the spleens of A mice were found on days 5 and 7 postinfection compared with basal levels in the spleens of control mice. A significant increase in IL-4 expression in the spleens of B6 mice was found on day 7 postinfection compared with basal levels in the spleens of control mice. However, the maximal expression of IL-4 in the spleens of resistant B6 mice on day 7 postinfection was two times lower than the maximal expression of IL-4 in the spleens of susceptible A mice on day 5 postinfection. These data indicate that, early during infection, resistant B6

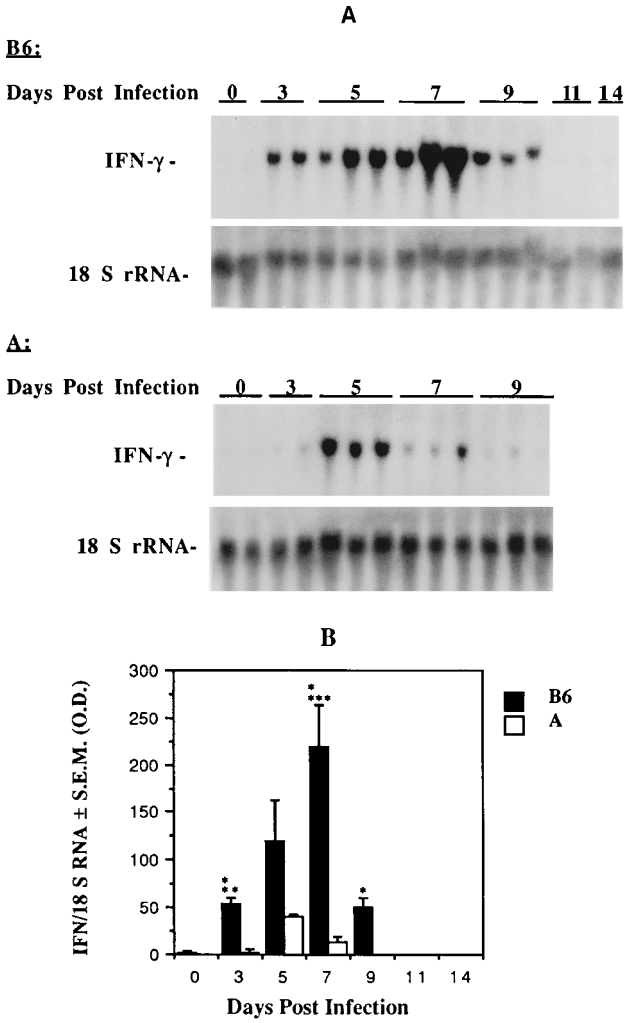

FIG. 4. Kinetics of IFN-γ mRNA levels in the spleens of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. (A) Total RNA was purified from spleens recovered from one to three mice per time point, and the levels of IFN-γ mRNA expression in individual spleens were determined by Northern blot analysis. 18S rRNA levels were also determined by Northern blot analysis for each sample. (B) Scanning densitometry of the autoradiographs shown in panel A. The densities of the bands corresponding to IFN-γ mRNA expression were determined and normalized to the levels of 18S rRNA expression. O.D., optical density; S.E.M., standard error of the mean. Symbols: *, $P < 0.04$ in comparison with value for control mice on day 0; **, $P < 0.03$ in comparison with value for A mice on day 3 postinfection; ***, $P < 0.05$ in comparison with value for A mice on day 7 postinfection.

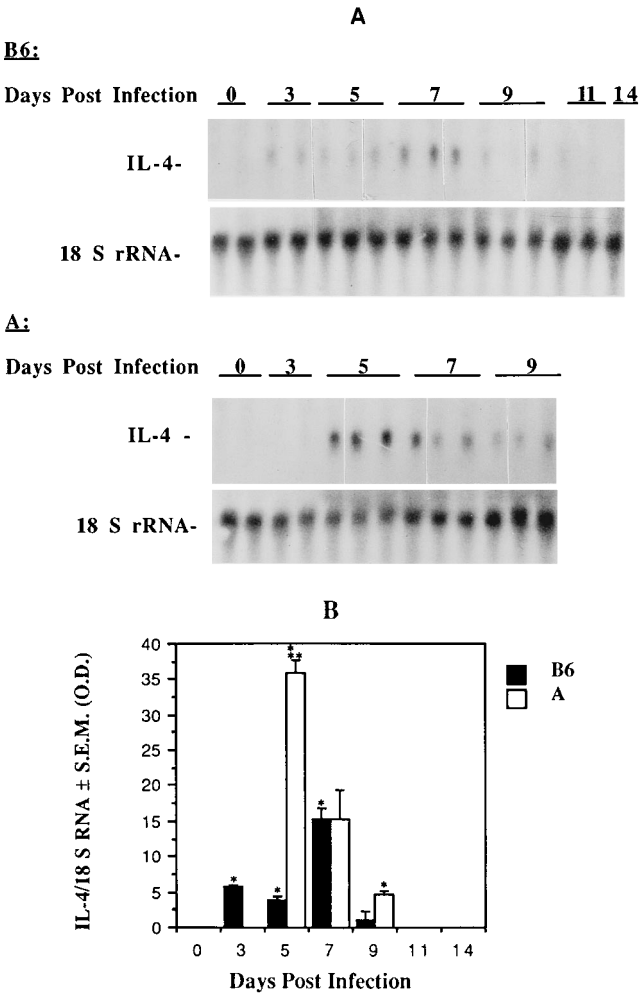

FIG. 5. Kinetics of IL-4 mRNA levels in the spleens of resistant B6 and susceptible A mice during infection with *P. chabaudi* AS. (A) Total RNA was purified from spleens recovered from one to three mice per time point, and the levels of IL-4 mRNA expression in individual spleens were determined by Northern blot analysis. 18S rRNA levels were also determined by Northern blot analysis for each sample. (B) Scanning densitometry of the autoradiographs shown in panel A. The densities of the bands corresponding to IL-4 mRNA expression were determined and normalized to the levels of 18S rRNA expression. O.D., optical density; S.E.M., standard error of the mean. Symbols: *, $P < 0.02$ in comparison with value for control mice on day 0; **, $P < 0.01$ in comparison with value for B6 mice on day 5 postinfection.

mice have high levels of IFN-γ mRNA and low levels of IL-4 mRNA, whereas susceptible A mice have low levels of IFN-γ mRNA but high levels of IL-4 mRNA. These results suggest that, in the spleen, a Th1 response may sustain a high level of expression of TNF-α, whereas a Th2 response may down-regulate the expression of TNF-α.

DISCUSSION

We report here that a Th1-associated increase in TNF-α in the spleen early during infection correlates with protection to blood-stage *P. chabaudi* AS malaria. Indeed, we found significantly higher levels of mRNA of IFN-γ (a Th1 marker) and TNF-α in the spleens of resistant B6 mice than in the spleens of susceptible A mice within the first week postinfection. In contrast, higher levels of mRNA of IL-4 (a Th2 marker) were found in the spleens of susceptible A mice than in the spleens of resistant B6 mice during the same period. A similar correlation among increased TNF-α mRNA levels, high-level IFN-γ expression and low-level IL-4 expression in the brains of *P. berghei* ANKA-infected mice has recently been demonstrated (7). Our results are also in agreement with previous results from our laboratory indicating that spleen cells recovered from resistant B6 mice early during infection and stimulated with malaria antigen released high levels of IFN-γ and low levels of IL-5 (a Th2 marker), whereas similarly stimulated cells in the spleens recovered from susceptible A mice produced high levels of IL-5 and low levels of IFN-γ (31). Furthermore, studies using a model of sepsis with mice lacking the IFN-γ receptor have shown that IFN-γ has an important role in the up-regulation of TNF-α production in vivo (17). In contrast, IL-4 has been demonstrated to down-regulate TNF-α mRNA levels in

human monocytes in vitro (11). Our results thus suggest that parasite antigen-stimulated cells in the spleens of resistant B6 mice produce high levels of IFN-γ which may up-regulate the production of TNF-α, whereas T cells in the spleens of susceptible A mice produce high levels of IL-4 which may inhibit the production of TNF-α. We do not yet know the reason why a Th1 versus Th2 type of response develops in the spleens of B6 versus A mice, respectively. We are currently investigating if there is differential production of IL-12, a cytokine with the ability to induce the development of Th1 cells (13), since we recently demonstrated that treatment with murine rIL-12 protects A mice against *P. chabaudi* AS (33).

Macrophages in the spleens of B6 mice may be the major TNF-α-producing cells in vivo. Previous studies in our laboratory have shown that macrophages recovered from the spleens of B6 mice during the first week postinfection and stimulated with malaria antigen or PRBC are able to produce large quantities of TNF-α protein in vitro (30). Moreover, similarly stimulated macrophages recovered from the spleens of susceptible A mice also produce high levels of TNF-α (30). These results and our present findings indicate, first, that macrophages recovered from the spleens of A mice and separated from a suppressive, Th2-type splenic environment are capable of producing large amounts of TNF-α and, second, that the observed differences in TNF-α expression in vivo between B6 and A mice are probably due to immunoregulatory cytokines and not to the direct TNF-α-inducing effect of malaria antigens on macrophages (1).

We also report here that treatment of resistant B6 mice early during infection with anti-TNF-α antibody results in 50% mortality but has no effect on the course of parasitemia. It is possible that higher doses of anti-TNF-α polyclonal antibodies would have resulted in 100% mortality of these mice, but we were unable to perform this experiment because of the limited amount of antibody in our possession. Furthermore, this result is partially in agreement with previous results from our laboratory showing that treatment of A mice during the early course of infection with rHuTNF-α results in 100% survival but decreased parasitemia (29). It thus appears that TNF-α has a protective role early during infection which involves more than the inhibition of parasite growth. TNF-α has no direct parasiticidal effect in vitro (36) but has a crucial role in modulating the initial phase of the cell-mediated immune response in vivo (3). In this respect, we have recently observed that the protective role of TNF-α in the spleen in vivo involves the induction of nitric oxide, a molecule produced during cell-mediated immune responses (27), and suggested that NO is not involved in parasite killing but may protect the host against oxygen radical-mediated tissue damage during blood-stage malaria (14, 15). In addition, we report here that treatment of resistant B6 mice with anti-TNF-α antibody results in a delay of 1 day in the peak of parasitemia. However, we do not know the significance of this finding.

Our present results also demonstrate that TNF-α expression is increased not only in the spleens but also in the livers of resistant B6 mice early during infection. Liver macrophages recovered from mice during acute malaria have been shown to have an increased capacity to secrete TNF-α in vitro (34). The liver, in addition to the spleen, may thus have a protective role during malaria infection, as has been suggested previously (8). However, we could not demonstrate an increase in NO production in the livers of resistant B6 mice (14). This observation may suggest that, in contrast to what has been suggested for liver-stage malaria (23), the protective role of TNF-α in the liver during blood-stage malaria may not involve the induction of nitric oxide. TNF-α in the liver may induce the production of acute-phase proteins (37). Little is known, however, about the precise role of these proteins during malaria (37), and previous results from our laboratory have shown that, early during infection, susceptible A mice have higher levels of serum amyloid P component, a major acute-phase protein in mice, than resistant B6 mice (22). Furthermore, the data presented here also demonstrate that the combination of increased TNF-α expression in the liver and high TNF-α levels in serum is detectable in susceptible A mice, but not in resistant B6 mice, later during infection, i.e., coincident with the time A mice begin to succumb to infection. Thus, this high level of systemic production of TNF-α by A mice later during infection seems to correlate with pathology and death due to malaria. It should be noted that the levels of TNF-α mRNA in the livers of B6 mice, which are nearly equal to those in A mice, late during infection, are not accompanied by high TNF-α protein levels in serum and do not correlate with pathology but correspond to the late, descending phase of the earlier peak production of TNF-α mRNA in this organ. Moreover, it has been suggested that TNF-α induces liver damage in mice during *P. berghei* malaria (38) and endotoxemia in vivo (10). Furthermore, large amounts of TNF-α in the sera of malaria patients correlate with severe and fatal disease (19). It has been suggested that this excessive and harmful production of TNF-α is the result of a massive release of TNF-α-inducing malaria antigens during schizogony (6, 37). The data presented here suggest that excessive production of TNF-α, as seen only in susceptible A mice, may be a consequence of the host's initial Th2 immune response; this response is unable to limit parasite multiplication, as demonstrated by a high peak parasitemia, resulting in a massive release of malaria antigens later on during infection.

Taken together, our results suggest that TNF-α has a protective role during blood-stage *P. chabaudi* AS malaria when it is produced in the spleen and the liver early during infection. Moreover, a Th1-type response in the spleen correlates with high-level expression of TNF-α, whereas a Th2 response correlates with low-level TNF-α expression. In contrast, increased TNF-α mRNA levels in the liver and excessive levels of the protein in serum later during infection appear to have a deleterious effect on the outcome of this disease. In conclusion, the paradoxical role of TNF-α, that is, protection versus pathology during malaria, may thus depend not only on the amount of TNF-α released but also on the timing and site of its expression and the presence of other cytokines regulating its production. A better understanding of the kinetics and site of TNF-α production in vivo will be necessary before TNF-α-modulating agents are considered for treatment of malaria.

ACKNOWLEDGMENTS

We thank Luis Barrera for his advice on Northern blot analysis. The expert technical assistance of Mifong Tam is gratefully acknowledged.

This work was supported by a grant to M.M.S. from the Medical Research Council of Canada (grant MT-12638). P.J. is supported by a bursary from the Quebec Ministry of Education (Programme Québécois de Bourses d'Excellence).

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Editor: J. M. Mansfield