Polymorphisms in the Tumor Necrosis Factor α (TNF-α) Gene Correlate with Murine Resistance to Development of Toxoplasmic Encephalitis and with Levels of TNF-α mRNA in Infected Brain Tissue

By Yvonne R. Freund,* Gregory Sgarlato,* Chaim O. Jacob,† Yasuhiro Suzuki,§ and Jack S. Remington*‖

From the *Department of Immunology and Infectious Diseases, Research Institute, Palo Alto Medical Foundation, Palo Alto, California 94301; †Syntex Research, Palo Alto, California 94303, the ‡Department of Parasitology, Jikei University School of Medicine, Tokyo, Japan; and the §Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

Summary

Murine resistance to development of toxoplasmic encephalitis (TE) has recently been mapped to the D region of the major histocompatibility complex (H-2). Since the gene for tumor necrosis factor α (TNF-α) is located 5' of the D region and TNF-α has been implicated as playing a role in neurological diseases, we were interested in determining the relationship of TNF-α production to TE resistance. We have demonstrated that resistance to TE in inbred mice can be correlated with specific restriction fragment length polymorphisms and microsatellite variants in the TNF-α gene. Mice that are susceptible to TE express elevated levels of TNF-α mRNA in brain tissue 6 wk after infection with the ME49 strain of Toxoplasma gondii. Resistant mice and all mice that are uninfected show no detectable TNF-α mRNA expression in brain tissue. Differences in the TNF-α gene between susceptible and resistant mice have been localized to the first intron, the promoter, and the 3' end of the TNF-α gene. These data implicate differences in regulation of TNF-α production in brain tissue as contributing to differences in susceptibility to development of TE.

The cytokine TNF-α plays a fundamental role in immunological responses of host tissue to invading organisms and has been implicated in the pathogenesis of neurological diseases (1–3). Produced by astrocytes in brain tissue, TNF-α has been shown to cause demyelination of nerve fibers (4) in vitro and to exert cytotoxic effects on myelin-producing oligodendrocytes (5). In mice infected with Plasmodium berghei, TNF-α appeared to play a role in the pathogenesis of cerebral malaria, since antibodies to TNF-α fully protected mice against neurological manifestations and mortality (6). In vitro studies demonstrated that treatment of macrophages with TNF-α had no significant effect on inhibiting intracellular replication of Toxoplasma gondii (7). Analyses of the in vivo role of TNF-α during acute infection with T. gondii have yielded conflicting results. Repeated experiments in our laboratory have revealed earlier mortality after TNF-α treatment in Swiss Webster and BALB/c mice (8; Orellana, M., and J. S. Remington, unpublished observations), whereas Chang et al. (9) have found protection in Swiss Webster mice.

The importance of toxoplasmic encephalitis (TE)† among AIDS patients has served as an impetus for studying the pathogenesis of TE. Suzuki et al. (10) have analyzed resistance to development of TE in a group of inbred mouse strains and localized genes that contribute to differences in resistance to the D region of the mouse MHC (H-2) (Table 1, first four columns). These authors and Brown and McLeod (11) also have reported on a correlation of the numbers of T. gondii cysts in brains of inbred strains of mice with the d haplotype at the D and L loci, respectively. Although it is not known whether the numbers of T. gondii cysts in the brain are related to development of TE, a number of investigators have implicated the cyst form in recrudescence of latent infection in the central nervous system (12, 13). The D region of the H-2 complex contains the genes for TNF-α and TNF-β, at a distance of ~70 kb from the D locus (14). Since RFLPs have

† Abbreviations used in this paper: TBE, Tris-borate-EDTA; TE, toxoplasmic encephalitis.
been identified in the TNF-α gene (15, 16) and are found to be associated with differences in levels of expression of TNF-α protein (15), we investigated polymorphisms in this gene to determine whether these polymorphisms were associated with susceptibility or resistance to development of TE.

Materials and Methods

Mice. BALB/c mice were obtained from Simonsen Laboratories (Gilroy, CA), and CBA/Ca mice from Charles River Breeding Colony (Cambridge, MA) or from The Jackson Laboratory (Bar Harbor, ME). C57BL/10 and A/J mice were obtained from The Jackson Laboratory and the B10.A recombinant congenic mice were obtained through the courtesy of Dr. Timothy Poole of the Fox Chase Cancer Center (Philadelphia, PA).

Southern Blot Analysis. Genomic DNA was isolated from spleens of seven inbred mouse strains and Southern blotting was carried out according to standard protocols (17). DNA was digested to completion with restriction enzymes, according to supplier's recommendations. 10 μg of digested DNA was loaded per lane onto a 0.8% agarose gel and subjected to electrophoresis in TBE. Electrophoresis was carried out for various lengths of time to optimize separation of RFLP fragments. DNA was transferred via southern blotting for 18 h to Nytran membranes (Schleicher & Schuell, Keene, NH) and crosslinked using a UV Stratalinker (240; Stratagene, San Diego, CA). Filters were prehybridized at 42°C in 5x SSPE, 50% formamide, 5x Denhardt's, 0.65% SDS, and 100 μg/ml salmon sperm DNA, and hybridized to a 300-bp PvuII fragment encoding a portion of the fourth exon of the TNF-α gene. This fragment was labeled with ^32P-dCTP by hexamer-primer (18) to a spedfic activity of 8-10 x 10^6 cpm/μg, and filters were hybridized with 1-2.5 x 10^7 cpm per filter for 24 h. Filters were washed in 2x SSC/0.1% SDS at room temperature and in 0.1x SSC/0.1% SDS at 60°C for 30 min and 2 h, respectively. Filters were exposed to XAR Kodak film for 48 h with two Cronex lightening-plus intensifying screens.

Northern Blot Analysis. 2-mo-old female mice of the BALB/c, CBA/Ca, A/J, and C57BL/10 strains were infected intraperitoneally with 10 cysts of the ME49 strain of T. gondii in sterile, endotoxin-tested PBS (Sigma Chemical Co., St. Louis, MO). Control mice were injected with endotoxin-tested PBS. 6 wk after infection, mice were killed using CO2, and brains were processed according to the method of Chomczynski and Sacchi (19) to extract RNA. 100 μg of total RNA per lane was loaded onto a 1.5% agarose gel and subjected to electrophoresis in 1x MOPS buffer with 3% formaldehyde (17). RNA was transferred to Nytran membranes, crosslinked as described above, and hybridized to the PvuII fragment of the murine TNF-α probe as described. Filters were washed according to the manufacturer's recommendations and exposed to x-ray film as described.

Oligonucleotides. Oligonucleotides were synthesized on an DNA synthesizer (391; Applied Biosystems, Inc., Foster, City, CA) and then desalted by passing the samples through a Sephadex G-25 column. Oligonucleotides were end-labeled using 10 pmol of primer in a mixture containing 50 mM Tris-Cl, pH 7.6, 10 mM MgCl2, 5 mM dithiothreitol, 0.1 mM spermidine-Cl, 0.1 mM EDTA, pH 8.0, 10 pmol 3,000 Ci/mmol γ-[32P]ATP, and 2 U T4 polynucleotide kinase.

Amplification of Microsatellites. The oligonucleotides flanking the mouse TNF-α promoter (AC)n repeat that were used as PCR primers were: primer 1, GGAATTCGCTTGAATGAGGTTTC; primer 2, TCGAATCTGGGCGCAAATCCGAGGG. The oligonucleotides flanking the mouse HSP70 (TA)n repeat that were used as primers in PCR were: primer 1, GATAATGGCTTTGACTGTAAAT; primer 2, TCGAATCTGGGCGCAAATCCGAGGG.

Results and Discussion

The presence of the d haplotype at the D locus of the H-2 complex has been shown by Suzuki et al. (10) to correlate with resistance to development of TE and with absence of inflammation in brain tissue. A/J, BALB/c, and congenic B10.A(3R) and (18R) mice, which express the d haplotype at the H-2 D locus, showed little inflammation in brain tissue after infection, whereas CBA/J, C57BL/6, C57BL/10, and B10.A(4R) mice, which do not express the d haplotype at D, had remarkable meningeal and perivascular infiltration and foci of acute inflammation.

Genomic DNA from the TE-resistant BALB/c, A/J, B10.A(3R), and B10.A(18R) mice and from susceptible CBA/Ca, C57BL/10, and B10.A(4R) mice was digested with BamHI or with XbaI, and Southern blots were probed with a PvuII fragment from the fourth exon of the TNF-α gene (Fig. 1, A and B). When DNA was digested with BamHI, a 9-kb fragment was observed in TE-resistant BALB/c, A/J, B10.A(18R), and B10.A (3R) mice, whereas a 10.5-kb fragment was observed in susceptible CBA/Ca, C57BL/10, and B10.A(4R) mice. When DNA was digested with XbaI, a 6.3-kb fragment was generated in TE-susceptible CBA/Ca, C57BL/10, and B10.A(4R) mice, and a 5.9-kb fragment was generated in resistant BALB/c, A/J, B10.A (18R), and B10.A(3R) mice. The PvuII fragment, which was used as a probe, contained a HincII restriction site. For this reason, double digestion with BamHI and HincII was used to determine whether the difference in BamHI digestion could be localized 5' or 3' of the fourth exon (Fig. 1, C). A 4.3- or a 4.2-kb band was observed in all mouse strains tested. We deduced from the location of the 5' BamHI site in the sequence of Shakhov et al. (20) that these fragments covered the region 3' of the HincII site in the fourth exon. The size of the fragment covering the 5' portions of the TNF-α gene was 2.7 kb in susceptible strains and 1.2 kb in resistant strains. The 1.2-kb band would place the 5' BamHI site of resistant mouse strains in the first intron of the TNF-α gene. When the first intron was analyzed using the sequence from C57BL/6 (20), a site that differed by one nucleotide from that of a
BamHI restriction site (GGACCC rather than GGATCC) was found at 1.2 kb from the HincII site. This suggested that a point mutation may have occurred to generate this difference between susceptible and resistant mice. Shirai et al. (21) have sequenced a murine TNF-α gene from a mouse strain not designated by the authors. Analysis of this sequence revealed a BamHI site in the first intron at a location that corresponded to the site noted in the C57BL/6 sequence. Comparison of the TNF-α sequence from Shirai et al. (21) with that of the C57BL/6 revealed five other differences between the strains in the first intron. It has been suggested that introns may be involved in regulation of gene expression (22, 23), and the role of intron 1 in regulation of TNF-α expression will be investigated.

The location of the polymorphic XbaI site was also determined using double digestion of DNA with BamHI and XbaI. A 6.1-kb fragment was generated in TE-susceptible strains and a 4.2-kb fragment in resistant mouse strains. The 1.9-kb difference in size between these fragments appears to be due to a shift in the BamHI site of resistant mice 1.5 kb closer to the 3' XbaI site and an additional 400 bp difference, which is the result of a shift in the XbaI site of resistant mice 400 bp closer to the 5' end of the TNF-α gene. The region of differences in XbaI restriction sites is ~3.3 kb from the 3' end of the fourth exon of the TNF-α gene.

DNA polymorphisms with only two alleles, as in the TNF-α RFLPs, have a minimum polymorphic information content (PIC) value of 0.375 (24). On the other hand, DNA polymorphisms based on length variation in simple-sequence tandem repeats, called microsatellites, have an average PIC value of 0.61, about twice the average for all two allele RFLPs (25). For this reason, we have utilized as a polymorphic marker an (AC)n repeat within the promoter region of TNF-α. This (AC)n repeat is highly polymorphic (26, 27) and shows length variation that correlates with resistance to TE (Fig. 2 A, lanes 1–4). Thus, the described correlation of resistance

Figure 1. RFLP patterns in the TNF-α gene in mouse strains that differ in resistance to TE. (A) DNA digested with BamHI. (B) DNA digested with XbaI. (C) DNA digested with BamHI and HincII. (D) DNA digested with BamHI and XbaI. Gels in B, C, and D were subjected to electrophoresis for >24 h to optimize separation of bands and allow increased accuracy of size determination.
to TE with the d haplotype in the D region is also a correlation with an RFLP in the first intron and with the (AC)n microsatellite pattern in the promoter of the TNF-α gene. In a further attempt to map murine resistance to TE, we have utilized an additional polymorphic microsatellite marker. The 3′ untranslated region of a heat shock protein gene (HSP70), located centromeric to the TNF-α gene (27), contains a (TA)n microsatellite. This microsatellite is highly

Table 1. Mapping the Murine Susceptibility to Toxoplasmic Encephalitis to the Central Portion of the H-2 Complex

| Strain       | H-2 haplotype | Inflammation in brain* | No. of cysts/coronal section of brain† | H-2 haplotype in D region |
|--------------|---------------|------------------------|----------------------------------------|---------------------------|
|              | K  | A  | E  | S  | D  |                     | S  | HSP70  | TNF  | D  |
| A/J          | k  | k  | k  | d  | d  | −                        | low | d      | d    | d  | d  |
| BALB/c       | d  | d  | d  | d  | d  | −                        | low | d      | d    | d  | d  |
| CBA/J        | k  | k  | k  | k  | k  | +                        | high| k      | d    | k  | k  |
| C57BL6       | b  | b  | b  | b  | b  | +                        | high| b      | b    | b  | b  |
| B10.A(3R)    | b  | b  | k  | d  | d  | −                        | low | d      | aq   | d  | d  |
| B10.A(4R)    | k  | k  | b  | b  | b  | +                        | high| b      | b    | b  | b  |
| B10.A(18R)   | b  | b  | b  | b  | d  | −                        | low | b      | b    | d  | d  |

* Mice were injected intraperitoneally with 10 cysts of the ME49 strain of T. gondii. 10 wk after infection, mice were killed and histological studies were carried out. Three to six mice were used in each experimental group. Positive inflammation: meningeal and perivascular infiltration of mononuclear cells and acute foci of inflammation in the tissue.
† Low = <1.0; high = 10–20 cysts.
‡ The HSP70 and TNF-α haplotype designations are based on size variation in tandem repeats within the 5′ or 3′ untranslated region of HSP70 and the 5′ regulatory region of TNF-α. The mapping of one member of the HSP70 family of genes is from Jacob and Hwang (27).
Northern blot analysis of total RNA from brain tissue of mice infected intraperitoneally with 10 cysts of the ME49 strain of *T. gondii*. RNA was extracted 6 wk after infection. 100 µg RNA was loaded in each lane. Blot was probed with a 300-bp PvuII fragment from exon 4 of the TNF-α gene.

polymorphic and shows different size variants in the relevant strains of mice (Fig. 2 B). Using the three B10.A congenic mouse strains, we can clearly map resistance to TE telomeric to HSP70 (Table 1).

To determine whether the observed RFLPs in the TNF-α gene correlated with expression of TNF-α transcripts during TE, mice from two susceptible and two resistant strains were infected with the ME49 strains and RNA was isolated from brain tissue at 6 wk after infection. In Fig. 3, Northern blot analysis of total RNA revealed that mRNA for TNF-α was detectable only in susceptible CBA/Ca and C57BL/10 mice that had been infected with ME49. No RNA was detectable in brains of infected, TE-resistant BALB/c or A/J mice, nor in brains from uninfected control mice, which were injected with sterile PBS. The fact that no detectable TNF-α mRNA was expressed in uninfected brain tissue suggested that the gene was regulated in part through an increase in transcription. We have preliminary evidence, using immunohistochemical staining of fixed brain tissue, that TNF-α protein is expressed in brains of infected CBA/Ca mice but not detected in infected BALB/c mice, nor in uninfected mice of either strain (data not shown).

Strain-specific differences in regulation of TNF-α expression in brain tissue have been observed in inbred rats (28), and high levels of TNF-α protein expression in CBA/Ca, but not BALB/c mice have been linked to susceptibility to cerebral malaria (6). Although it is possible that expression of TNF-α in brains of susceptible mice is just one aspect of the general inflammatory response that occurs during TE, the fact that TNF-α has been demonstrated to play roles both in protection and pathology in other neurological diseases suggests that this cytokine warrants further study to determine how its expression in brain tissue from TE-susceptible mice influences the course of disease.

This work was supported by U.S. Public Health Service grants AI-04717, AI-30320, and BRSG SO7 RR-005513 from the National Institutes of Health, the MacArthur Foundation grant in Molecular Parasitology, and the Naito Foundation, Japan.

Address correspondence to Yvonne R. Freund, Department of Immunology and Infectious Diseases, Research Institute, Palo Alto Medical Foundation, 860 Bryant St., Palo Alto, CA 94301.

Received for publication 11 October 1991.

References

1. Sharief, M.K., M. Phil, and R. Hentges. 1991. Association between tumor necrosis factor-α and disease progression in patients with multiple sclerosis. *N. Engl. J. Med.* 325:467.
2. Hofman, F.M., D.R. Hinton, K. Johnson, and J.E. Merrill. 1989. Tumor necrosis factor identified in multiple sclerosis brain. *J. Exp. Med.* 170:607.
3. Schijns, V.E., R. Van der Neut, B.L. Haagmans, D.R. Bar, H. Schellekens, and M.C. Hornzinek. 1991. Tumour necrosis factor-alpha, interferon-gamma and interferon-beta exert antiviral activity in nervous tissue cells. *J. Gen. Virol.* 72:809.
4. Selmaî, K.W., and C.S. Raine. 1988. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann. Neurol.* 23:339.
5. Robbins, D.S., Y. Shirazi, B. Drysdale, A. Lieberman, H.S. Shin, and M.L. Shin. 1987. Production of cytotoxic factor for oligodendrocytes by stimulated astrocytes. *J. Immunol.* 139:2593.
6. Grau, G.E., L.F. Fajardo, P.F. Piguet, B. Allè, P.H. Lambert, and P. Vassalli. 1987. Tumor necrosis factor/cachectin as an essential mediator in murine cerebral malaria. *Science (Wash. DC).* 237:1210.
7. DeFittos, E.H., J.R. Catterall, and J.S. Remington. 1986. Activity of recombinant tumor necrosis factor on *Toxoplasma gondii* and *Trypanosoma cruzi*. *J. Immunol.* 137:1342.
8. Black, C.M., D.M. Israelski, Y. Suzuki, and J.S. Remington. 1989. Effect of recombinant tumor necrosis factor on acute infection in mice with *Toxoplasma gondii* or *Trypanosoma cruzi*. *Immunology.* 68:570.
9. Chang, H.R., G.E. Grau, and J.C. Pechère. 1990. Role of TNF
and IL-1 in infections with *Toxoplasma gondii*. Immunology. 69:33.

10. Suzuki, Y., K. Joh, M.A. Orellana, F.K. Conley, and J.S. Remington. 1991. Gene(s) within the H-2D region determine development of toxoplasmic encephalitis in mice. *Immunology.* In press.

11. Brown, C.R., and R. McLeod. 1990. Class I MHC genes and CD8+ T cells determine cyst number in *Toxoplasma gondii* infection. *J. Immunol.* 145:3438.

12. Frenkel, J.K., and A. Escaljado. 1987. Cyst rupture as a pathogenic mechanism of toxoplasmic encephalitis. *Am. J. Trop. Med. Hyg.* 36:517.

13. Suzuki, Y., F.K. Conley, and J.S. Remington. 1989. Importance of endogenous IFN-γ for prevention of toxoplasmic encephalitis in mice. *J. Immunol.* 143:2045.

14. Muller, U., C.V. Jongeneel, S.A. Nedospasov, K.F. Lindahl, and M. Steinmetz. 1987. Tumour necrosis factor and lymphotixin genes map close to the H-2D in the mouse major histocompatibility complex. *Nature (Lond.)* 325:265.

15. Jacob, C.O., and H.O. McDevitt. 1988. Tumour necrosis factor-α in murine autoimmune ‘Lupus’ nephritis. *Nature (Lond.)* 331:56.

16. Gardner, S.M., B.A. Mock, J. Hilgers, K.E. Huppi, and W.D. Roeder. 1987. Mouse lymphotixin and tumor necrosis factor: structural analysis of the cloned genes, physical linkage and chromosomal position. *J. Immunol.* 139:476.

17. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

18. Feinberg, A.P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 137:266.

19. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.

20. Shakhov, A.N., M.A. Collart, P. Vassalli, S.A. Nedospasov, and C.V. Jongeneel. 1990. κB-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor α gene in primary macrophages. *J. Exp. Med.* 171:35.

21. Shirai, T., N. Shimizu, S. Shijoji, S. Horiguchi, and H. Ito. 1988. Cloning and expression in *Escherichia coli* of the gene for mouse tumor necrosis factor. *DNA (NY).* 7:193.

22. Palmet, R.D., E.P. Sandgren, M.R. Avarbock, D.D. Allen, and R.L. Brinster. 1991. Heterologous introns can enhance expression of transgenes in mice. *Proc. Natl. Acad. Sci. USA.* 88:478.

23. Messner, G., U. Spengler, M.C. Jung, G. Honold, K. Blomer, G.R. Pape, G. Riethmuller, and E.H. Weis. 1991. Polymorphic structure of the tumor necrosis factor (TNF) locus: an NcoI polymorphism in the first intron of the human TNF-β gene correlates with a variant amino acid in position 26 and a reduced level of TNF-β production. *J. Exp. Med.* 173:209.

24. Botstein, D. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32:314.

25. Weber, J.L. 1990. Human DNA polymorphisms based on length variation in simple sequence tandem repeats. In *Genomic Analysis*. K.E. Davis and S.M. Tilghman, editors. Cold Spring Harbor Press, Cold Spring Harbor, NY. 159-181.

26. Jongeneel, C.V., H. Acha-Orbea, and T. Blankenstein. 1990. A polymorphic microsatellite in the tumor necrosis factor α promoter identifies an allele unique to the NZW mouse strain. *J. Exp. Med.* 171:2141.

27. Jacob, C.O., and F. Hwang. 1992. Definition of microsatellite size variants for TNF-α and HSP70 in autoimmune and non-autoimmune mouse strains. *Immunogenetics.* In press.

28. Chung, I.Y., J.G. Norris, and E.N. Benviste. 1991. Differential tumor necrosis factor-α expression by astrocytes from experimental allergic encephalomyelitis-susceptible and -resistant rat strains. *J. Exp. Med.* 173:801.

29. Lowe, D.G., and L.A. Moran. 1986. Molecular cloning and analysis of DNA complementary to three mouse Mr = 68,000 heat shock protein mRNAs. *J. Biol. Chem.* 261:2102.