Definitive identification of a gene that confers resistance against *Toxoplasma* cyst burden and encephalitis

C. R. BROWN,*† C. A. HUNTER,‡ R. G. ESTES,* E. BECKMANN,§ J. FORMAN,¶ C. DAVID,††
J. S. REMINGTON‡ & R. MCLEOD*†‡‡ *Department of Medicine and §Department of Pathology, Michael Reese
Hospital and Medical Center and †The Committee on Immunology, The University of Chicago, Chicago, Illinois, ‡Department of
Immunology and Infectious Diseases, Research Institute, Palo Alto Medical Foundation, Palo Alto and Division of Infectious
Diseases, Department of Medicine, Stanford University School of Medicine, Stanford, California, ¶Department of Microbiology,
University of Texas Southwestern Medical Center, Texas, ††Department of Immunology, Mayo Medical Foundation, Rochester,
Minnesota, and the ‡‡Departments of Medicine, Microbiology, Immunology and Genetics, The University of Illinois at Chicago,
Chicago, Illinois, USA

SUMMARY

Control of resistance to cyst burden following per-oral infection with *Toxoplasma gondii* has been mapped previously to a region of mouse chromosome 17 of approximately 140 kb. This region is contiguous with and contains the class I gene, L^d. Resistance to development of toxoplasmic encephalitis has also been reported to be controlled by genes in this region of H-2. TNF-α, D and L genes, as well as unidentified genes, are also in this region. The work described here was performed to identify definitively the gene(s) in this 140 kb region that confers resistance to cysts and encephalitis. The study demonstrates that relative resistance to *T. gondii* organisms and cyst burden in brain, and toxoplasmic encephalitis, 30 days following per-oral *T. gondii* infection is correlated absolutely with the presence of the L^d gene in inbred, recombinant, mutant and C3H.L^d transgenic mice. Mice with the L^d gene had lower cyst burdens and less encephalitis than those without the L^d gene. Specifically, 30 days after infection mice with the L^d gene had minimal perivascular inflammation and meningeal inflammation and very few *Toxoplasma* cysts or organisms in immunoperoxidase-stained preparations of their brains. Mice without the L^d gene had a similar pattern of inflammation, but in addition they had collections of inflammatory cells in the brain parenchyma. Free tachyzoites were found within these foci of inflammation and cysts were present in these areas as well as in contiguous areas without inflammatory cells. There were CD4$^+$ and CD8$^+$ T lymphocytes in the areas of inflammation and throughout the brain parenchyma. Mice that were resistant to cysts and encephalitis had little detectable brain cytokine mRNA expression, while mice that were susceptible had elevated levels of mRNA for a wide range of cytokines, consistent with their greater amounts of inflammation. The present work definitively demonstrates that a L^d-restricted response decreases the number of organisms and cysts within the brain and thereby limits toxoplasmic encephalitis and levels of interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-6, IL-10, transforming growth factor-β (TGF-β), IL-1α, IL1β and macrophage inhibiting protein (MIP) mRNA in the brain 30 days after per-oral infection.

INTRODUCTION

The purpose of this study was to define the gene(s) that determines the numbers of cysts in the brain, severity of toxoplasmic encephalitis, and levels of cytokine mRNA produced in the brain, and to elucidate the relationship of these parameters during the early phase of primary infection

with *Toxoplasma gondii* in a murine model. The experiments were performed because definition of the gene responsible will provide a means to define the mechanisms involved in the development and containment of cysts and pathogenesis of toxoplasmic encephalitis in subsequent studies. Such information is important because this disease has emerged as a major cause of morbidity and mortality for patients with acquired immune deficiency virus (AIDS) or who have other immunocompromising illnesses or who are congenitally infected.[1-3] The present study did not address the relationship of cyst persistence, rupture or development of toxoplasmic encephalitis during chronic infection, which may be regulated differently.[4]

Resistance to acute infection with *T. gondii* varies among inbred strains of mice.[5-11] Cyst numbers and toxoplasmic encephalitis in the early phase of infection have both been demonstrated to be controlled by a gene(s) located in a 140 kb (for cysts) or larger (for encephalitis) region, which contains or is contiguous to the mouse major histocompatibility complex (MHC) D/L region.[4,6,9] In the present study, inbred recombinant and congenic strains of mice and mutant mice with deleted genes were used initially to map the resistance gene within the D/L region. The precise margins of the gene region, e.g. the extent of the deletion in the L region in BALB/c-H-2^{dm2} mice[12] or the precise location of the cross-over in B10.RKDB mice,[13] are not known. It is known that the D/L region contains more than five genes and probably also a substantial number of as yet unidentified and unmapped genes. These include D, $D2$, $D3$, $D4$ and L. In most strains $D2$, $D3$ and $D4$ are not transcribed. In BALB/c-H-2^{dm2} mice, the deletion occurs telomeric to D. Thus the chromosomal segment $D2$ to L, which contains $D2$, $D3$, $D4$ and L, is missing.[14] As L^d is the only known functional class I gene in this deletion, we inferred that the L^d gene was responsible for limiting *T. gondii* cyst burden in the brain.[6] However, this analysis did not exclude the possibility that an unknown gene(s) contained within the 140 kb deletion conferred resistance. Also, deletion of a gene and absence of an effect would not exclude synergistic or additive effects with another gene (e.g. L^d and *TNF-α*). Thus, to identify the gene responsible, and to determine whether L^d acted alone to limit the number of *T. gondii* cysts in brain and encephalitis, L^d transgenic mice were used.[15] The present study demonstrates definitively that the L^d gene is responsible for limiting *T. gondii* cyst burden and development of toxoplasmic encephalitis. This is consistent with the protective role of CD8$^+$ T cells,[6,16-18] which interact with such class I molecules on antigen-presenting or infected target cells.

Cytokine production within the brain has been implicated in resistance to toxoplasmic encephalitis[17,19] and polymorphisms in the TNF-α gene have been reported to correlate with resistance.[20] To characterize better the mechanisms responsible for limitation of toxoplasmic encephalitis, the gene(s) that regulates toxoplasmic encephalitis early in primary infection and certain of the immune functions influenced by these gene(s) were also studied. The link between cyst number, toxoplasmic encephalitis and levels of cytokine mRNA in brain during the acute phase of infection with *T. gondii* was investigated using transgenic, mutant and recombinant strains of mice to define the role of different genes in the D/L region in protection (Fig. 1), and in particular to evaluate the separate roles of the *TNF-α*, D, and L genes in cyst formation, toxoplasmic encephalitis and cytokine production.

MATERIALS AND METHODS

Mice

BALB/cJ, C57BL/10J and BALB/c-H-2^{dm2} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.RKDB mice were bred in one of our laboratories (C. David). B10.RKDB mice have the recombinations shown in Fig. 1.[13] The founder RQDB mouse had a recombination inside the D/L region with crossing-over between $D4$ and L.[21] Thus, the D to $D4$ genes are d and these mice acquired the L gene from a b haplotype mouse.[21] The B10.RKDB strain derived the D/L
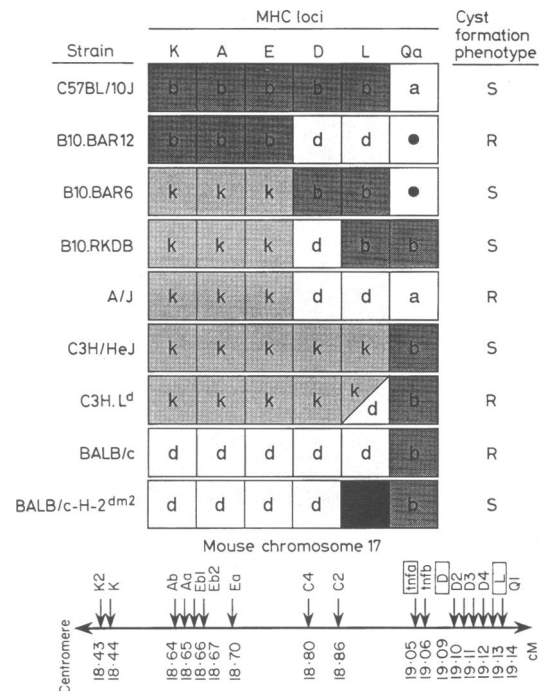
Figure 1. MHC haplotypes of mouse strains used to determine controlling locus for cyst formation following per-oral *T. gondii* infection, and schematic diagram that indicates location of these MHC loci on mouse chromosome 17. A solid square indicates deletion of the L^d gene, and ● indicates that the haplotype at this locus has not been determined.

region from the RQDB mice. The only known difference in the D/L region between BALB/c and B10.RKDB mice is the L gene. C3H/HeJ and C3H.L^d mice were produced and bred in one of our laboratories (Department of Microbiology, University of Texas Southwestern Medical Center, TX) and are described by Aldrich *et al.*[15] Female mice were used for experiments when they were between 6 and 16 weeks of age.

Per-oral infection of mice

The Me49 strain of *T. gondii* was maintained by long-term passage in female Swiss–Webster mice, as described elsewhere.[6] Mice were killed and their brains removed and homogenized in sterile phosphate-buffered saline (PBS).[6] Cyst number was quantified by placing a 50-μl aliquot under a 24 × 50-mm coverslip on a slide and counting the entire field under a ×100 magnification. The homogenate was diluted to contain 200 or 20 cysts/ml and 0·5 ml was administered by gavage using a 18-gauge, 1·5-inch intubation needle.[6]

Enumeration of brain cysts

Thirty days after infection one-half of the brains removed above were homogenized in 1 ml saline. A 10 μl aliquot of this suspension was placed under a 22 × 22-mm coverslip and the number of cysts quantified by scanning the entire slide under a ×100 magnification.

Histology and immunocytochemistry

To assess histopathology and perform cytokine analysis simultaneously, the circulatory system of the mice was perfused through the left ventricle with 30 ml PBS (pH 7·4) to

remove blood. Then brains of mice infected for 7 or 30 days were removed *en bloc* and the hemispheres divided sagitally. One half was used for cyst quantification, and the other half was either fixed immediately in 10% formalin, 70% ethanol and 5% acetic acid, or quick frozen in OCT embedding medium (Miles, Elkhart, IN). Sagital sections (5 μm thick) were stained with haematoxylin and eosin (H&E). Immunoperoxidase staining[22] was used for detection of *Toxoplasma* organisms and antigens using anti-*Toxoplasma* polyclonal rabbit antiserum. For immunocytochemistry to evaluate CD4$^+$ and CD8$^+$ T cells in inflammatory infiltrates, the brains of infected mice were excised and frozen in OCT compound (Miles). Frozen tissue blocks were sectioned using a Leica cryostat and the sections (4–6 μm), placed on fibronectin-coated microscope slides. Sections were fixed in acetone at $-20°$ for 30 seconds and washed with Hank's balanced salt solution (HBSS; Gibco BRL, Long Island, NY) plus 10 mM HEPES (Gibco BRL). The wash solution was used to rinse sections between each step and for the dilution of antibodies and formation of avidin–peroxidase complexes. Sections were incubated with 0·3 M sodium azide for 30 min before the addition of rat anti-mouse CD4 or rat anti-mouse CD8α (Pharmingen, San Diego, CA) at a dilution of 1/40 for 30 min. After washing, a secondary biotinylated mouse absorbed rabbit anti-mouse antibody (Vector, Burlingame, CA), at a dilution of 1/200, was applied to the sections for 30 min. This was followed by incubation with an avidin–peroxidase complex (ABC Elite; Vector) for 30 min. Sections were then rinsed before addition of the chromogen diaminobenzamide (Vectastain substrate kit I, Vector Laboratories, Burlington, CA).

The histopathology was evaluated in a blind manner with four sections from two different areas of the brain. Inflammation, numbers of organisms, cysts and different cell types were graded on a scale of 0 (none) to 5 (most), and relative numbers of CD4$^+$ and CD8$^+$ cells were noted; these parameters defined the presence of encephalitis.

Cytokine analysis
RNA was isolated from cryostat cut sections of brain by using a guanidium thiocyanate–phenol–chloroform single-step method.[19,23] The optical density at 260 nm was used to estimate the concentration of total mRNA. Approximately 2 μg of total RNA was used for cDNA synthesis in a total volume of 100 μl of reverse transcriptase buffer (Gibco BRL, Grand Island, NY) containing 1·25 mM deoxynucleoside triphosphates (dNTPs; Pharmacia, Alameda, CA), 20 U Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) and 2 μg random hexamers (Gibco BRL), and incubated at 42° for 60 min. The polymerase chain reaction (PCR) was performed with 2·5 μl of the original cDNA reaction mixture subjected to repeated cycles of amplification (28 for β-actin, 35 for all other cytokines) at 94° for 45 seconds, 60° for 45 seconds, and 72° for 2 min, with a Geneamp 9600 thermocycler (Perkin-Elmer, Emeryville, CA) in a 50-μl reaction volume of 1× Taq polymerase buffer containing 0·25 mM dNTPs, 5 μM of 3' and 5' primer, and 2 U of Taq polymerase (Promega, Madison, WI). Specific primers for β-actin, interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-6, IL-10, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), macrophage inflammatory protein 1 (MIP-1), and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Clontech, Palo Alto, CA)

were designed to span at least one intron. This allowed determination of whether amplified target DNA was derived from cDNA versus genomic DNA, which would not be transcribed because the intron would not be present in the cDNA. There were no signs of amplification of genomic DNA in the PCR used in this study. Negative controls consisted of the same cDNA reaction but in the absence of reverse transcriptase. Positive controls were provided by cDNA synthesized from total RNA extracted from the brains of CBA/Ca mice chronically infected with *T. gondii*. These samples were screened for their suitability to provide positive signals for the different cytokines employed in this study. PCR products were electrophoresed through 1·5% agarose gels and stained with ethidium bromide, and products were visualized with ultraviolet (UV) illumination. The amounts of PCR products were graded according to the intensity of staining from none ($-$) to the largest amount ($+ + +$). Analysis of diluted cDNA amplified with the β-actin-specific primers revealed only minor differences between samples. This methodology allowed comparison of cytokine transcript levels but did not allow direct semiquantitative comparisons between different cytokines.

Statistical analysis of data
Significance of differences in numbers of cysts was determined by the Tukey or Student's t-tests.[24]

RESULTS

Cyst numbers in brains of per-orally infected mice

Cyst numbers contained in homogenates of brain are listed in Table 1. BALB/c and C3H.L^d mice had lower numbers of cysts than did C57BL/10, B10.RKDB, BALB/c-H-2^{dm2} and C3H/HeJ mice. Increasing the inoculum size from 10 to 100 cysts had no significant effect on the numbers of cysts formed in BALB/c mice (Table 1, $P > 0·95$), but significantly increased the number of cysts formed in B10.RKDB mice (Table 1, $P < 0·01$). These data definitively identify L^d as a gene that controls cyst numbers.

Table 1. Cyst number in brains of per-orally infected mice

Strain of mouse	n^*	Inoculum	Brain cysts/10 μl tissue Mean ± SD	Range
C57BL/10J	9	10	45 ± 31	9–100
B10.RKDB	11	10	19 ± 10	6–42
BALB/c	9	10	1 ± 1	0–2
C57BL/10J	3	100	73 ± 16	63–91
B10.RKDB	11	100	52 ± 25	24–104
BALB/c	22	100	1 ± 1	0–5
BALB/c-H-2^{dm2}	6	100	29 ± 14	16–54
C3H/HeJ	15	100	36 ± 17	16–84
C3H.L^d	11	100	1 ± 1	0–2

*n, total number of mice in two or three experiments. Mice were per-orally infected with 10 or 100 Me49 cysts contained in 0·5 ml saline. Resistant mice had significantly fewer brain cysts than susceptible mice ($P < 0·01$) and had the L^d gene.

Histopathology

Mice examined at day 30 revealed two patterns of inflammation that correlated with cyst number and presence of L^d (Fig. 2). BALB/c and C3H.L^d mice, which were resistant to cyst formation, developed only very mild meningitis and perivascular inflammation (Fig. 2A–C). Immunoperoxidase staining did not demonstrate the presence of *T. gondii* in either the meninges (data not shown) or in association with vessels (Fig. 2C). Organisms were only rarely present in the brain parenchyma (data not shown), and Fig. 2D demonstrates the absence of
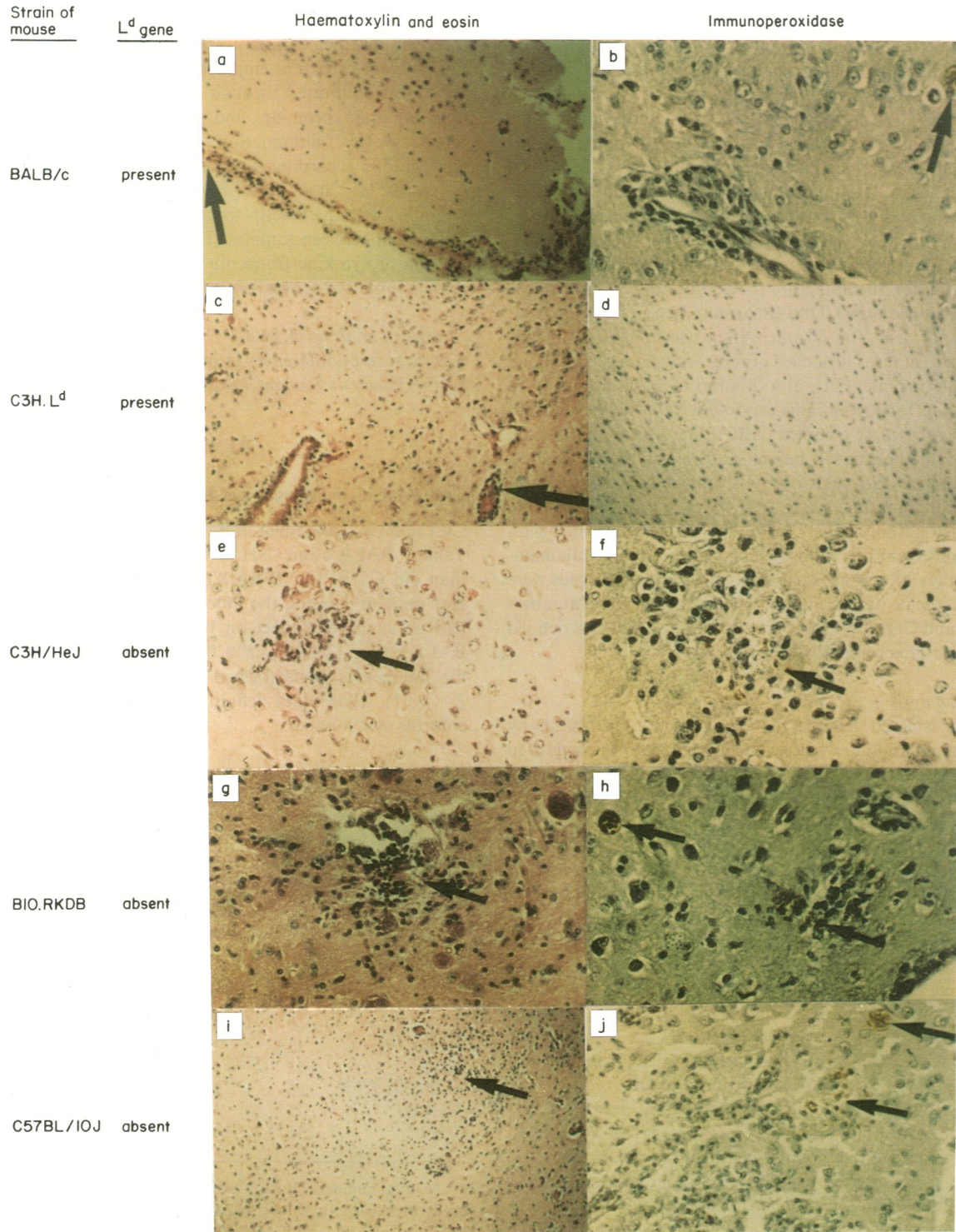

Strain of mouse	L^d gene	Haematoxylin and eosin	Immunoperoxidase
BALB/c	present	a	b
C3H.L^d	present	c	d
C3H/HeJ	absent	e	f
BIO.RKDB	absent	g	h
C57BL/IOJ	absent	i	j

Table 2. Correlation of MHC haplotype, cyst tachyzoite, inflammation and cytokine phenotype

	Haplotype			Phenotype			
Strain of mouse	TNF	D	L	Cyst	Tachyzoite	Brain inflammation	Cytokine
C57BL/10J	b	b	b	High	High	Present	Detectable
B10.BAR12	NA (b or d)	d	d	Low	NA	NA	NA
B10.BAR6	NA (b or k)	b	b	High	NA	NA	NA
B10.RKDB	NA (k or d)	d	b	High	High	Present	Detectable
BALB/c	d	d	d	Low	Low	Absent	Very low or absent
BALB/c-H-2^{dm2}	d	d	−	High	High	Present	Detectable
C3H/HeJ	k	k	k	High	High	Present	Detectable
C3H.L^d	k	k	k, d	Low	Low	Absent	Very low or absent

NA, data not available.
−, not present.

organisms in the brain parenchyma in the resistant mice. In contrast, the C57BL/10, B10.RKDB, BALB/c-H-2^{dm2} and C3H/HeJ mice, which are susceptible to cyst formation, all had meningitis and perivascular inflammation in addition to substantial parenchymal inflammation (Fig. 2E–J). Immunoperoxidase staining revealed the presence of *T. gondii* organisms within foci of inflammatory cells (Fig. 2F, H and J). Cysts were also readily seen in these preparations, both associated with and, more often, without accompanying inflammation. The immunoperoxidase staining indicated that there were organisms present outside cysts.

The presence of CD4$^+$ and CD8$^+$ T lymphocytes in brain was studied 30 days after per-oral infection in two C57BL/10 mice and three mice of each of the other strains (Fig. 3). CD4$^+$ T lymphocytes were present in the foci of parenchymal inflammation in the susceptible mice. They were present throughout the brain parenchyma and in the perivascular cellular infiltrates in both the resistant and susceptible strains of mice. In general, fewer CD4$^+$ T cells were present in the brain parenchyma and perivascular and meningeal infiltrates of the resistant mice. Interestingly, although CD8$^+$ T cells were present in the brains of mice of all the strains, C57Bl/10 and
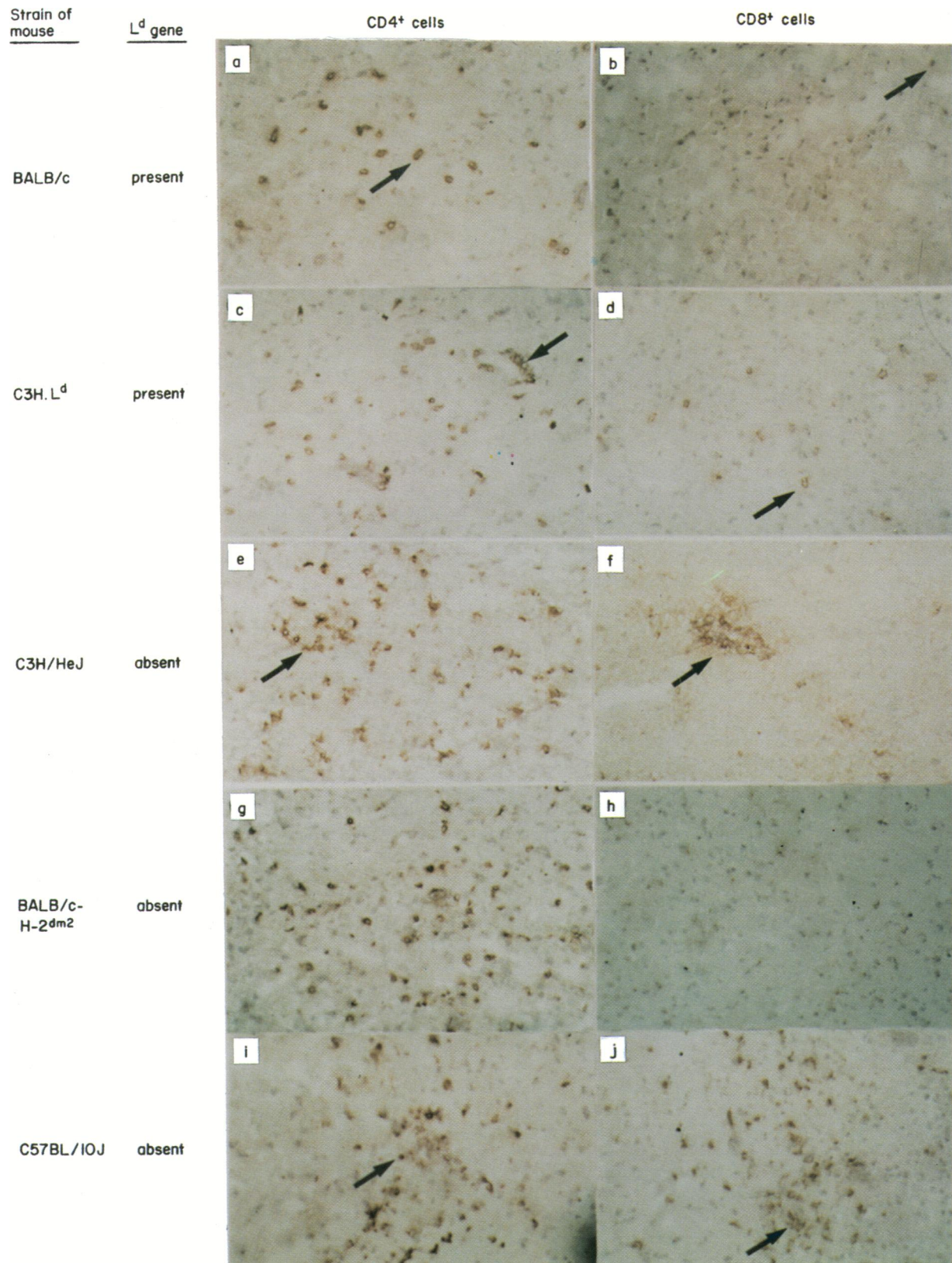
BIO.RKDB mice had substantially larger numbers of CD8$^+$ T cells in parenchymal and perivascular infiltrates (Fig. 3).
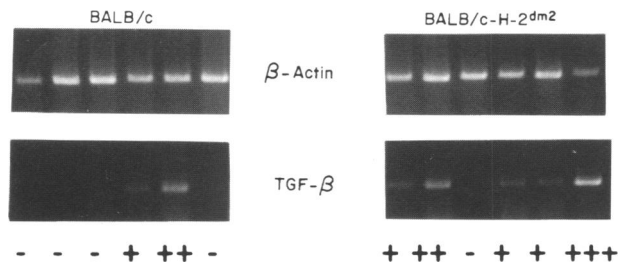
Mouse strains resistant to development of toxoplasmic encephalitis were those strains resistant to development of brain cysts (Table 2). This absolute correlation of increase in severity of toxoplasmic encephalitis in strains of mice without L^d (i.e. the representative data presented in Figs 2 and 3 for each of the recombinant, mutant and transgenic mouse strains) provides the novel and definitive evidence that L^d is the controlling gene.

Cytokine mRNA production

At 30 days after infection, message for cytokines present in the brains of infected mice was scored visually using ethidum bromide-stained gels (Fig. 4). Resistant BALB/c and C3H.L^d mice had little or no detectable cytokine mRNA expression in their brains (Tables 2 and 3). Only one of three mice had low levels of IFN-γ or TNF-α message. In contrast, the susceptible C57BL/10, B10.RKDB, BALB/c-H-2^{dm2} and C3H/HeJ mice had more IFN-γ, IL-2 and TNF-α message. Detectable levels of IL-10 message were also present in the susceptible mice, along

Figure 2. Representative examples of brains of resistant and susceptible strains of mice 30 days after per-oral infection. Resistant mice, with the L^d gene, had minimal inflammation and parasite burden, and susceptible mice, without the L^d gene, had greater inflammation and parasite burden. Brains of resistant BALB/c (a,b) and C3H.L^d (c,d) mice. Brains of susceptible C3H/HeJ (d,e), B10.RKDB (f,g) and C57BL/10J (h,i) mice. In (a) and (c), H&E-stained sections (×250) from the resistant mice demonstrated only mild meningeal and perivascular inflammation (marked by arrows). These figures are representative of mild inflammation graded as ≤2 by the pathologist (see the Materials and Methods). In (b) and (d), immunoperoxidase-stained sections representative of resistant mice did not demonstrate presence of *T. gondii* tachyzoites in either the meninges or in association with vessels. Arrow in (b) marks a *T. gondii* cyst, seen only rarely in sections from resistant mice. In (e), (g) and (i), H&E-stained sections (×250) from the susceptible mice demonstrated substantial meningeal and perivascular inflammation and, in contrast to the cyst-resistant strains, there was also substantial parenchymal inflammation (each marked by arrows). These figures with larger amounts of inflammation are representative of those graded ≥ 3 by the pathologist (see the Materials and Methods). In (f), (h) and (j), immunoperoxidase stains of representative brain sections from susceptible mice demonstrated many extracellular *T. gondii* tachyzoites or bradyzoites (marked by arrows) within foci of inflammatory cells. The morphologically distinct cysts (e.g. in the upper right-hand corner of b and g) were also readily demonstrated both with and without accompanying inflammatory cell infiltrates. Results with BALB/c-H-2^{dm2} mice were similar (data not shown). The polyclonal anti-*Toxoplasma* antisera used for immunoperoxidase staining recognizes both tachyzoites and bradyzoites.

Strain of mouse	L^d gene	CD4$^+$ cells	CD8$^+$ cells
BALB/c	present		
C3H.L^d	present		
C3H/HeJ	absent		
BALB/c-H-2^{dm2}	absent		
C57BL/IOJ	absent		

Figure 4. TGF-β and actin mRNA in brains of resistant and susceptible mice and demonstration of semiquantitative grading presented in Table 2. Ethidium bromide-stained gels, which demonstrate reverse transcriptase PCR products from brains of resistant and susceptible mice. Neither further semiquantification of cytokine messenger RNA[31] or study of additional cytokines such as IL-5 was needed because of the conclusive, all or none, results. In uninfected controls, cytokine message was absent (data not shown).

with detectable levels of IL-1α, IL-1β and TGF-β, whereas resistant mice had lower levels or no message for these cytokines.

Tables 2 and 3 demonstrate that all resistant mice had little detectable cytokine mRNA expression in their brains, and all susceptible mouse strains had substantially increased levels of many cytokines, probably reflecting the increased levels of inflammation present.

DISCUSSION

The studies described here define the role of the L^d gene in determining resistance to per-oral *T. gondii* infection. Resistance was measured as cyst burden in brain and toxoplasmic encephalitis, and was correlated with levels of cytokine mRNA in brain. This work demonstrates that resistance to brain cyst burden and toxoplasmic encephalitis early in *T. gondii* infection are both regulated by the L^d gene. The L^d gene product presumably presents a protective peptide(s) to CD8$^+$ T cells, which are then responsible for the differences in parasite burden and encephalitis observed in resistant and susceptible mice. In this manner, L^d may limit the number of organisms that reach the brain, and thus there are fewer cysts, and encephalitis manifested by brain parenchymal infiltrates is not present.

Although we have demonstrated previously[6] that BALB/c-H-2^{dm2} mice, which have a deletion of approximately 140 kb, including the entire gene for L^d, and no other known functional class I genes,[14] were susceptible to cyst formation, it was

possible that another gene that co-segregated with L^d was the gene which was critical for resistance to cysts, either alone or in conjunction with L^d. Formal proof of whether L^d was or was not the gene which confers resistance to cysts required the use of L^d-transgenic mice. The studies described here demonstrate definitively, through the use of such C3H.L^d transgenic mice, that L^d is the gene that alone limits *Toxoplasma* cysts numbers in brain. Supporting data are presented in Table 1 and Figs 2 and 3.

The severity of toxoplasmic encephalitis is also regulated by the L^d gene, as indicated by the present experiments. This is clearly demonstrated by the representative histological data from each of the strains of mice. For example, lack of the L^d gene in B10.RKDB and BALB/c-H-2^{dm2} mice induces susceptibility, while the introduction of the L^d transgene in C3H mice renders them resistant. This work extends the findings of Suzuki *et al.*[4] who reported that a gene within the H-2D/L region conferred resistance to development of toxoplasmic encephalitis. All these results correlate well with our hypothesis[6] that the mode of action of L^d in protection against cysts and encephalitis is by limiting parasite numbers through interaction with CD8$^+$ T lymphocytes. Specifically, if L^d mediates its effect through a reduction of parasitaemia, there would be fewer *Toxoplasma* organisms reaching the brain and, therefore, fewer organisms to form cysts or to cause an inflammatory response within the brain parenchyma. However, another report[20] correlated polymorphisms in the TNF-α gene (also located near the H-2D/L region), along with increased levels of TNF-α, with resistance to development of toxoplasmic encephalitis. This suggested the presence of a cytokine associated with protective immunity in the brain, perhaps working in conjunction with L^d, that could protect mice from toxoplasmic encephalitis. The influence of L^d or D^d could not be ruled out in this previous study[20] because of the complete co-segregation of these genes in the mouse strains used.

Several other lines of evidence have also suggested that TNF-α could be important in protection. This includes the work of Blackwell *et al.*[25] and Chang *et al.*,[26] which demonstrated a protective role for TNF-α in murine toxoplasmosis, as well as recent obervations that there is a positive association between the *TNF2* allele, a variant which gives higher levels of transcription of TNF-α than the *TNF1* allele, and susceptibiltiy to infection with human cerebral malaria.[27] However, the experiments described here indicate that the TNF-α gene is not the resistance gene protective against toxoplasmic encephalitis early after primary per-oral infection of mice. This is apparent from each of the comparative

Figure 3. Representative examples of CD4$^+$ and CD8$^+$ T cells in brains of resistant BALB/c and C3H. L^d (a–d) and susceptible C3H/HeJ, BalB/c-H-2^{dm2} and C57 BL 10/J (e–i) mice that were per-orally infected 30 days earlier. CD4$^+$ and CD8$^+$ T cells were demonstrated by immunoperoxidase stain. (a) BALB/c mouse, CD4$^+$ cells. Note that there was only a small number of CD4$^+$ cells (arrow). These cells were also present around a blood vessel and in the meninges (data not shown). (b) BALB/c mouse, CD8$^+$ cells. Note that there were also occasional CD8$^+$ cells (arrow). (c) C3H.L^d mouse, CD4$^+$ cells. Arrow demonstrates a small number of CD4$^+$ cells around a blood vessel and in the adjacent parenchyma. (d) C3H.L^d mouse, CD8$^+$ cells. Arrow indicates CD8$^+$ cell. (e) C3H/HeJ mouse, CD4$^+$ cells. Arrow demonstrates substantial numbers of CD4$^+$ cells in the brain parenchyma. (f) C3H/HeJ mouse, CD8$^+$ cells. Arrows demonstrate CD8$^+$ cells in the wall of a blood vessel in the brain parenchyma. (g) BALB/c-H-2^{dm2} mouse, CD4$^+$ cells. Large numbers of CD4$^+$ T cells were present. (h) BALB/c-H-2^{dm2} mouse, CD8$^+$ cells. Very small numbers of CD8$^+$ T cells were present. (i) C57BL/10 mouse, CD4$^+$ cells. Large numbers of CD4$^+$ cells were present in clusters in the brain parenchyma (arrow). (j) C57BL/10 mouse, CD8$^+$ cells. Large numbers of CD8$^+$ lymphocytes were present in the perivascular areas (data not shown) and in clusters in the brain parenchyma (arrow). CD4$^+$ and CD8$^+$ lymphocytes were not present in brain parenchyma of control mice that had not been infected with *T. gondii* (data not shown).

Table 3. Cytokine expression in btrains of per-orally infected mice

Strain	Phenotype	IFN-γ	TNF-α	IL-2	IL-4	IL-6	IL-10	TGF-β	IL-1α	IL-1β	GM-CSF	MIP
BALB/c	R	+	ND	−	−	−	−	ND	−	−	−	−
		−	ND	−	−	−	−	ND	−	−	−	−
		−	ND	−	−	−	−	ND	−	−	−	−
		−	−	−	−	−	−	−	−	+	−	−
		−	+	−	−	−	−	−	−	+	−	−
		−	−	−	−	−	−	−	−	+	−	−
		−	−	−	−	−	−	−	−	+	−	−
		−	+	−	−	−	−	−	−	+	−	−
		−	−	−	−	−	−	+	−	+ +	−	−
		−	+	−	−	−	−	+ +	+	+ +	−	+
		−	+	−	−	−	−	−	+	+	−	−
C3H.L^d	R	−	−	−	−	−	−	+	+	+ +	−	−
		−	−	−	−	−	−	−	−	+ +	−	−
		−	−	−	−	−	−	+	+	+ +	−	−
		−	+ +	−	−	−	−	−	−	+	−	−
		−	−	−	−	−	−	+	−	+	−	−
		−	−	−	−	−	−	−	−	+ +	−	−
C3H/HeJ	S	+ +	+ + +	+	−	−	+	+ + +	+ +	+ + +	−	+
		+	+ + +	+	−	−	+	+ +	+ +	+ + +	−	+
		−	+ + +	+	−	−	+	−	+ +	−	−	−
		−	+ + +	+	−	−	+	+	+	+ + +	−	+
		+	+ + +	+	−	−	+	+ +	+ +	+ + +	−	+
		+ +	+ + +	+	−	−	+	+ + +	+ +	+ + +	−	+ +
B10.RKDB	S	+ + +	ND	+ +	−	+ +	+ +	ND	+ + +	+ +	−	−
		+	ND	+ +	−	+	+ +	ND	−	+	−	−
		+ +	ND	+ +	−	+ +	+	ND	+ +	+	−	−
BALB/c-H-2^{dm2}	S	+	+ + +	+	−	−	+	+	+ +	+ + +	−	+
		+ +	+ + +	+	−	−	+	+ +	+ +	+ + +	−	+
		+	+ + +	+	−	−	+	−	+ +	+ + +	−	+
		+	+ + +	+	−	−	+	+	+ +	+ + +	−	+
		+ +	+ + +	+	−	−	+	+	+ +	+ + +	−	+
		+	+ + +	+	−	−	+	+ + +	+ +	+ +	−	+
C57BL/10J	S	+	ND	+ +	−	+	+	ND	−	+	−	−
		+ +	ND	+ +	−	+ +	+	ND	−	+	−	−
		+ + +	ND	+ +	−	+	+	ND	+ +	+	−	−
		−	+ +	+	−	+	+	+	+	+	−	+
		−	+ +	+	−	+	+	+	+	+	−	+

Magnitude of message was quantified from none (−) to largest amount (+ + +).
ND, not done.

experiments, e.g. C3H/HeJ versus C3H.L^d, C57BL/10 versus B10.RKDB, BALB/c versus BALB/c-H-2^{dm2}. The experiments with the BALB/c-H-2^{dm2} mutant mice, which have a resistant phenotype (H-2d) in the TNF-α locus, demonstrated that these mice are susceptible to cyst formation and toxoplasmic encephalitis (Figs 1–3 and Table 3). This could indicate that L^d or another molecule(s) encoded in the D/L region, which is deleted along with L^d in BALB/c-H-2^{dm2} mice, is needed in addition to TNF-α . The experiment with the L^d transgenic and L^d-lacking B10.RKDB mice indicates, however, that it is L^d that is critical and sufficient alone for protection and limitation of toxoplasmic encephalitis. Thus TNF-α polymorphisms[20] do not account for resistance to cyst formation and susceptibility

to toxoplasmic encephalitis following early per-oral infection of mice with the Me49 strain of *T. gondii*. As there appear to be differences in magnitudes of cyst numbers between B10 and other mouse strains, other genes besides L^d could also influence parasite burden.

Since other cytokines also are important in resistance to toxoplasmic encephalitis,[17,19,28] studies were performed to attempt to correlate resistance with the presence of these other cytokines. Cytokine mRNA expression in the brain was minimal in resistant mice. This was consistent with the limited inflammation present. The susceptible mice had substantial amounts of many cytokine transcripts in their brains, and this may simply have reflected the increased amount of inflammation

present. There was no specific single cytokine produced in the resistant mouse brains that could account for a protective immune response. There was no greater TNF-α or IFN-γ production in brains of resistant mice. These observations do not contradict the previous demonstration that endogenous IFN-γ and TNF-α both have a protective activity against toxoplasmic encephalitis.[17,28] Rather, they suggest that the infection is contained before encephalitis occurs, and in susceptible mice the substantial cytokine mRNA reflects the greater inflammation. It is also possible that production of these cytokines in the brain or other sites at earlier times could be the critical effector function regulated by the L^d gene. Alternatively, the presence of IL-10 message that we observed in the susceptible strains of mice raises another possible way cytokines might be involved in the different degrees of toxoplasmic encephalitis in resistant and susceptible mice. Specifically, excess cytokines that inhibit certain cell-mediated immune responses (e.g. IL-10 or TGF-β) produced in susceptible mice could abrogate a protective cytokine response. For example, IL-10 could down-modulate the protective effect of IFN-γ or TNF-α, which provides enhanced macrophage inhibition or killing of *T. gondii*.[29,30] If this were the case, perhaps, L^d-restricted cells could down-modulate this IL-10 production.

Interestingly, when resistance was measured as survival after per-oral infection in the strains of mice indicated in Fig. 1 and Table 1, increased survival did not correlate with the presence of the L^d gene ($P > 0.05$, data not shown). Thus, consistent with our earlier work,[10] other genes appear to influence survival critically. Also, these mice, which died 8 days following per-oral infection, did not have encephalitis (data not shown). This is consistent with an earlier report by Suzuki *et al.*,[31] which demonstrated that, following parenteral infection, mice that died during the acute infection did not have encephalitis.

In summary, at 30 days after primary *T. gondii* infection acquired per-orally, this study demonstrates conclusively the key role of the L^d gene in determining outcome, measured as parasite burden in brain and encephalitis. Previously, a 140-kb (or greater) region of mouse chromosome 17 was noted to be associated with regulation of numbers of cysts present in brain 30 days after per-oral infection and development of toxoplasmic encephalitis. Our data, using multiple mouse strains including L^d transgenic mice, prove that neither the TNF-α gene nor any other already defined or as yet undiscovered genes, besides the L^d gene, in this partially characterized 140-kb region of mouse chromosome 17, is essential for regulation of the critical, protective response(s) that limits parasite burden and toxoplasmic encephalitis. Reduction in brain cyst number and encephalitis 30 days after per-oral infection is mediated by the L^d gene product.

ACKNOWLEDGMENTS

REFERENCES

1. LUFT B.J., HAFNER R., KORZUN A. *et al.* (1993) Toxoplasmic encephalitis in patients with the acquired immunodeficiency syndrome. *New Engl J Med* **329**, 995.
2. McLEOD R. & REMINGTON, J.S. (1995) Toxoplasmosis. In: *Nelson's Textbook of Pediatrics*, 15th edn (eds R.L. Behrman, R. Kliegman & A.M. Arvin). WB Saunders, Philadelphia (in press).
3. MC AULEY J., BOYER K., PATEL D. *et al.* (1994) Early and longitudinal evaluations of treated infants and children and untreated historical patients with congenital toxoplasmosis: the Chicago collaborative treatment trial. *Clin Infect Dis* **18**, 38.
4. SUZUKI Y., JOHN K., ORELLANA M.A., CONLEY F.K. & REMINGTON J.S. (1991) A gene(s) within the H-2D region determines the development of toxoplasmic encephalitis in mice. *Immunology* **74**, 732.
5. ARAUJO F.G., WILLIAMS D.M., GRUMET F.C. & REMINGTON J.S. (1976) Strain-dependent differences in murine susceptibility to *Toxoplasma*. *Infect Immun* **13**, 1528.
6. BROWN C.R. & McLEOD R. (1990) Class I MHC genes and CD8$^+$ T cells determine cyst number in *Toxoplasma gondii* infection. *J Immunol* **145**, 3438.
7. JONES T.C. & ERB P. (1985) H-2 complex-linked resistance in murine toxoplasmosis. *J Infect Dis* **151**, 739.
8. McLEOD R., ESTES R.G., MACK D.G. & COHEN H. (1984) Immune response of mice to ingested *Toxoplasma gondii*. *J Infect Dis* **149**, 234.
9. McLEOD R., EISENHAUER P, MACK D.G., FILICE G. & SPITALNY G. (1989) Immune responses associated with early survival after per-oral infection with *Toxoplasma gondii*. *J Immunol* **142**, 3247.
10. McLEOD R., SKAMENE E., BROWN C.R., EISENHAUER P. & MACK D. (1989) Genetic regulation of early survival and cyst number after per-oral *Toxoplasma gondii* infection of AXB/BXA recombinant inbred and B10 congenic mice. *J Immunol* **143**, 3031.
11. WILLIAMS D.M., GRUMET F.C., & REMINGTON J.S. (1978) Genetic control of murine resistance to *Toxoplasma gondii*. *Infect Immun* **9**, 416.
12. MELVOLD R.W. & KOHN H.I. (1976) Eight new histocompatibility mutations associated with the H-2 complex. *Immunogenetics* **3**, 185.
13. PEDRINACI S., HANSON J. & DAVID C. (1994) Hierarchy in the assembly and transport of HLA-B27 and HLA-Cw3 molecules in transgenic mice. *Immunogenetics* **39**, 130.
14. RUBOCKI R.J., HANSEN T.H. & LEE D.R. (1986) Molecular studies of murine mutant BALB/c-H-2^{dm2} define a deletion of several class I genes including the entire H-2L^d gene. *Proc Natl Acad Sci USA* **83**, 9606.
15. ALDRICH C.J., HAMMER R.E., JONES-YOUNGBLOOD S. *et al.* (1991) Negative and positive selection of antigen-specific cytotoxic T lymphocytes affected by the $\alpha 3$ domain of MHC I molecules. *Nature* **352**, 718.
16. PARKER S.J., ROBERTS C.W. & ALEXANDER J. (1991) CD8$^+$ T cells are the major lymphocyte population involved in the protective immune response to *Toxoplasma gondii* in mice. *Clin Exp Immunol* **84**, 207.
17. GAZZINELLI R.T., ELTOUM I., WYNN T.A. & SHER A. (1993) Acute cerebral toxoplasmosis is induced by *in vivo* neutralization of TNF-α and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. *J Immunol* **151**, 3672.
18. KAHN I.A., ELY K.H. & KASPER L.H. (1994) Antigen-specific CD8$^+$ T cell clone protects against acute *Toxoplasma gondii* infection in mice. *J Immunol* **152**, 1856.
19. HUNTER C.A., ROBERTS C.W. & ALEXANDER J. (1992) Kinetics of cytokine mRNA production in the brains of mice with progressive toxoplasmic encephalitis. *Eur J Immunol* **22**, 2317.
20. FREUND Y.R., SGARLATO G., JACOB C.O., SUZUKI Y. & REMINGTON J.S. (1992) Polymorphisms in the tumor necrosis factor α (TNF-α)

gene correlate with murine resistance to development of toxoplas-mic encephalitis with levels of TNF-α in infected brain tissue. *J Exp Med* **175**, 683.

21. DURAN L.W., ZELLER J.K., LUNDY J.K. *et al.* (1987) Genetic analysis of the H-2D region using a new intra-D-region recombinant mouse strain. *J Immunol* **139**, 2818.

22. CONLEY F.K. & JENKINS K.A. (1981) Immunohistological study of the anatomic relationship of *Toxoplasma* antigens to the inflammatory response in the brains of chronically infected mice with *Toxoplasma gondii*. *Infect Immun* **31**, 1184.

23. CHOMCZYNSKI P. & SACCHI N. (1987) Single step method of RNA isolation by guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156.

24. ZAR J.H. (1984) *Biostatistical Analysis*, 2nd edn. Prentice-Hall Inc., New Jersey.

25. BLACKWELL J.M., ROBERTS C.W. & ALEXANDER J. (1993) Influence of genes within the MHC on mortality and brain cyst development in mice infected with *Toxoplasma gondii:* Kinetics of immune regulation in BALB H-2 congenic mice. *Parasite Immunol* **15**, 317.

26. CHANG H.R., GRAU G.E. & PECHERE J.C. (1990) Role of TNF and IL-1 in infection with *Toxoplasma gondii*. *Immunology* **69**, 33.

27. McGUIRE W., HILL V.S.A., ALLSOPP E.M.C., GREENWOOD B.M. & KWJATKOWSKI D. (1994) Variation in the TNF-α promoter region associated with susceptibility to cerebral malaria. *Nature* **371**, 508.

28. SUZUKI Y., CONLEY F.K. & REMINGTON J.S. (1989) Importance of endogenous IFN-γ for prevention of toxoplasmic encephalitis in mice. *J Immunol* **143**, 2045.

29. GAZZINELLI R.T., HAKIM F.T., HIENY S., SCHEARER G.M., & SHER A. (1991) Synergistic role of CD4$^+$ and CD8$^+$ T lymphocytes in IFN-γ production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J Immunol* **146**, 286.

30. SIBLEY L.D., ADAMS L.B., FUKUTOMI Y. & KRAHENBUHL J.L. (1991) Tumor necrosis factor-α triggers antitoxoplasmal activity of IFN-α primed macrophages. *J Immunol* **147**, 2340.

31. SUZUKI Y., ORELLANA M.A., WONG S.Y., CONLEY F.K. & REMINGTON J.S. (1993) Susceptibility to chronic infection with *Toxoplasma gondii* does not correlate with susceptibility to acute infection. *Infect Immun* **61**, 2284.