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# Cerebral Malaria in Mice

## *Interleukin-2 Treatment Induces Accumulation of γδ T Cells in the Brain and Alters Resistant Mice to Susceptible-Like Phenotype*

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**In this study, we report that infection with *Plasmodium yoelii* 17XL, a lethal strain of rodent malaria, does not result in death in the DBA/2 strain of mice. In contrast to BALB/c mice, DBA/2 mice developed significantly less parasitemia and never manifested symptoms of cerebral malaria (CM) on infection with this parasite. Moreover, the histological changes evident in the brain of susceptible BALB/c were absent in DBA/2 mice. Interestingly, the resistant DBA/2 mice when treated with recombinant interleukin (IL)-2, were found to develop CM symptoms and the infection became fatal by 6 to 8 days after infection. This condition was associated with an augmented interferon-γ and nitric oxide production. Unexpectedly, IL-10 levels were also elevated in IL-2-treated DBA/2 mice during late stage of infection (at day 6 of infection) whereas the inverse relationship between IL-10 and interferon-γ or nitric oxide was maintained in the early stage of infection (at day 3 after infection). The level of tumor necrosis factor-α production was moderately increased in the late phase of infection in these mice. Histology of brain from IL-2-treated mice demonstrated the presence of parasitized erythrocytes and infiltration of lymphocytes in cerebral vessels, and also displayed some signs of endothelial degeneration. Confocal microscopical studies demonstrated preferential accumulation of γδ T cells in the cerebral vessels of IL-2-treated and -infected mice but not in mice treated with IL-2 alone. The cells recruited in the brain were activated because they demonstrated expression of CD25 (IL-2R) and CD54 (intercellular adhesion molecule 1) molecules. Administration of anti-γδ mAb prevented development of CM in IL-2-treated mice until day 18 after infection whereas mice treated with control antibody showed CM symptoms by day 6 after infection. The information concerning creating pathological sequelae and death in an otherwise resistant mouse strain provides an interesting focus for the burden of pathological attributes on death in an infectious disease. *(Am J Pathol 2001, 158:163–172)***

The pathogenesis of cerebral malaria (CM) has been a subject of considerable interest, and it remains a major cause of death associated with severe *Plasmodium falciparum* infection in many tropical countries.[1] The early events that lead to human CM are difficult to study experimentally because of ethical constraints and the limitations of postmortem materials. Because of these difficulties, investigators turned to murine models to investigate mechanisms of CM pathogenesis. Considerable effort has been devoted to study the cerebral pathogenesis in the murine models involving *Plasmodium berghei* ANKA and *Plasmodium yoelii* 17XL, however, there are many aspects of this pathogenesis that are still poorly understood.[2] Previous studies in the *P. berghei* ANKA murine model have indicated that CM might be a lymphocyte-mediated disease, in which CD4+, and CD8+ T cells have been postulated to play a role.[3–5] It was recently demonstrated that on treatment with anti-γδ T cell antibody, the susceptible mice failed to develop CM after *P. berghei* ANKA infection indicating some pathological role of γδ T cells.[6] However, the extent of T cell activation that is required for the development of CM and whether actual T cell infiltration occurs at the site of brain has not been known. Morphological and biochemical studies have so far not recognized a focal or global determinant(s) for CM pathogenesis or a clear relationship between brain lesion and death.

*P. yoelii* 17XL regularly induces a CM syndrome in susceptible mice that parallels human disease in several

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respects including cytoadherence of parasitized red blood cells (PRBCs) in the brain and pathological symptoms.[7,8] Whether CM represents an inflammatory pathogenesis was not clarified by studying *P. yoelii* infection in susceptible mice. It remains debatable whether human CM presents an inflammatory pathogenesis.[9,10] Development of CM in humans infected with *P. falciparum* has often been accompanied by increased production of interferon (IFN)-$\gamma$ and tumor necrosis factor (TNF)-$\alpha$.[11,12] Interleukin (IL)-2 is a potent T cell activator and has the ability to induce production of these pro-inflammatory cytokines.[13,14] Considering that IL-2 is central in the regulation of cell-mediated immune responses, we have hypothesized whether the systemic administration of IL-2 can induce pathological manifestations of CM in a strain of mouse that is otherwise resistant to this syndrome. If it does so, this will be invaluable for analysis of the immune response, particularly of T cell responses and the associated pathology. In this report, we demonstrate that enhanced immunoreactivity induced by IL-2 treatment alters CM-resistant mice to susceptible-like phenotype as revealed by development of symptoms and histological changes characteristic of CM. This condition in resistant DBA/2 mice was associated with increased production of IFN-$\gamma$ and nitric oxide (NO). Confocal microscopic studies showed preferential recruitment of $\gamma\delta$ T-bearing cells in cerebral vessels of IL-2-treated mice manifesting CM symptoms. To our knowledge, this is the first direct evidence of accumulation of $\gamma\delta$ T cells in the brain of a host that developed CM. Treatment with anti-$\gamma\delta$ T cell mAb arrested the development of CM in IL-2-treated DBA/2 mice. These results suggest that $\gamma\delta$ T cells play an important role in the pathways of CM pathogenesis.

## Materials and Methods

### Parasites and Infection

Female DBA/2 (H-2d) mice, 5 to 6 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and from Iffa Credo (L'Arbresle, France). All animals were housed in the accredited Animal Research Facility and maintained under the guidelines established by the institution for their use. There are two strains of *P. yoelii* available, one is lethal and the other is nonlethal. Throughout our present study, we used the lethal (17XL) strain (PYL). The parasite was stored in liquid nitrogen and then used to infect source mice. PRBCs obtained from source mice were suspended in RPMI 1640 and were injected intraperitoneally ($5 \times 10^5$ PRBCs/mouse) in all experimental mice. Parasitemia was monitored by making blood smears and counting Giemsa-stained fields. The survival of mice was assessed until day 30 after infection.

### Monoclonal Antibody PRBCs

Antibodies directed against CD3 (mAb 145-2C11), $\gamma\delta$ TCR-expressing T cells [mAb GL3, fluorescein isothiocyanate (FITC)-conjugated], $\alpha\beta$ TCR-bearing T cells (mAb H57-597, FITC-conjugated), CD25 (mAb 7D4, FITC-conjugated), and CD54 [intercellular adhesion molecule 1 (ICAM-1)] (mAb 3E2, PE-conjugated) were used in this study. All these monoclonal antibodies were obtained from PharMingen (San Diego, CA). Hybridoma secreting anti-$\gamma\delta$ TCR mAb (GL3) was obtained from Dr. Pablo Pereira, Institut Pasteur, Paris, France.

### Interleukin 2 (IL-2) Treatment

Recombinant IL-2 was a kind gift from Dr. Tom Ciardelli, Department of Toxicology, Dartmouth Medical School. DBA/2 mice were treated with rIL-2 as essentially reported previously.[15] Mice were administered rIL-2, 1 $\mu$g in the presence of 1% of normal mouse serum in 200 $\mu$l of physiological saline per injection. Administration was via intraperitoneal inoculation three times daily for total of 8 days. It was shown previously that IL-2 was detectable in the serum samples from the mice treated three times a day with the above dose. Two days after the initiation of therapy, mice were infected with *P. yoelii* 17XL ($5 \times 10^5$ PRBCs/mouse). Age-matched control mice were given similar doses of rIL-2 injections in 1% normal mouse serum but they did not receive any infection.

### Histopathological Analysis

The brain of each mouse was removed immediately after anesthesia and placed in 10% buffered neutral formaldehyde (Polyscience Inc., Warrington, PA). Paraffin-embedded brain tissues were sectioned and stained with hematoxylin and eosin. These are random sections of the cerebrum. The slides were examined and then photographed.

### T Cell Preparation and Culture

Mice were killed on days 3 and 6 after infection, spleens were removed and gently dissociated into single-cell suspensions. Red blood cells were removed by using lysing buffer (Sigma Chemical Co., St. Louis, MO). Cell suspensions were passed through nylon wool columns to enrich for T cells. These cells were ~90% T cells. Bulk cultures were set up in 24-well plates with $2 \times 10^6$ cells/well, and stimulated with cross-linked anti-CD3 mAb as described previously.[15] For anti-CD3 mAb (145–2C11 mAb; PharMingen,) driven activation, culture-plate wells were precoated with goat anti-hamster IgG (14 $\mu$g of anti-hamster IgG) (Jackson Immunology Research Laboratories, West Grove, PA) overnight at 4°C. After washing, the wells were incubated with 5 $\mu$g/ml of anti-CD3 mAb at 37°C for several hours.

### Enzyme-Linked Immunosorbent Assay Quantitation of Cytokine in Splenic T-Cell Culture Supernatants

Supernatants from cultures of cross-linked anti-CD3 mAb stimulated splenocytes from the various groups of mice

were collected at 24 and 48 hours after the beginning of culture. The bulk cultures were set up in 24-well plates (Nunc, Rochester, NY) with $2 \times 10^6$ cells/well, in a final volume of 1 ml of RPMI 1640 medium supplemented with fetal calf serum, HEPES, L-glutamine, 2 mercaptoethanol, and gentamicin. In preliminary experiments, a 48-hour incubation time was found to be optimum for cytokine and nitrite productions. Cytokine enzyme-linked immunosorbent assays were conducted using paired capture and biotinylated detection antibodies from R&D Systems (Minneapolis, MN) following the manufacturer's recommendations. The cytokine level was calculated by reference to standard units provided by the manufacturer. The results were presented as pg/ml.

### *Assay for Nitrite Production*

Nitrite production in 48-hour culture supernatants was measured using Griess reagent.[16] Briefly, 0.05 ml of supernatant was mixed with 0.05 ml of Griess reagent (0.5% sulfanilamide and 0.05% *N*-1-naphthylenedimine hydrochloride in 2.5% $H_3PO_4$) then incubated for 10 minutes at room temperature and the absorbency was read at 570 nm by using an automated plate reader. The nitrite concentration was calculated from a $NaNO_2$ (Sigma Chemical Co.) standard curve. The results were presented as μmol/L.

### *Immunofluorescence for T Cell Visualization in Brains by Confocal Microscopy*

The blocks of brain tissue were trimmed, and 30- to 70-μm sections were cut using a vibratome (V1000, TPI; Energy Beam Sciences, Agawam, MA). Sections were maintained in ice-cold phosphate-buffered saline (PBS) throughout processing. To phenotype the immune cells present in the brain, immunofluorescent staining of tissue sections was conducted according to the method described previously.[17] Briefly, immediately after cutting, for direct staining, 2 μg/100 μl each of FITC-, PE-labeled Abs in PBS/1% bovine serum albumin/0.1% azide (PBA)-containing mouse Ig (6 mg/ml to block nonspecific binding) were added to sections in 96-well plates and incubated overnight at 4°C in the dark with continuous gentle agitation. Unbound Ab was removed from the sections by aspiration followed by four 20-minute washes in PBA. Washed sections were then fixed overnight in the same buffer containing 1% paraformaldehyde. Stained sections were wet-mounted in anti-fade (Molecular Probes, Eugene, OR), sealed with nail varnish, and stored at 4°C in the dark for up to 10 days before confocal imaging. Unstained and fluorescein isotype controls were used to control for autofluorescence and nonspecific Ab binding, respectively.

### In Vivo *Administration of Anti-γδ TCR mAb*

Protein G-purified anti-γδ TCR mAb was injected intraperitoneally at a dose of 500 μg as described previously.[6] Groups ($n = 7$) of IL-2-treated DBA/2 mice were used to assess the effect of depletion of γδ T cells on development of CM. One group was administered with anti-γδ TCR mAb on days −1 and +2 after infection, and another group received injections on days +3 and +6 after infection. Control groups were administered with purified hamster IgG (Jackson Immunology Research Laboratories) diluted with PBS in the manner as in experimental groups. The efficacy of γδ T-cell depletion was determined by flow cytometry of spleen lymphocytes. The percentage of γδ T cells was <0.02% after anti-γδ mAb treatment.

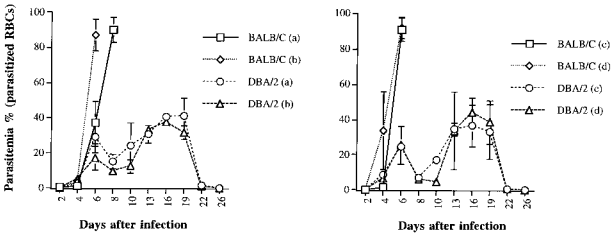


**Figure 1.** Levels of parasitemia in BALB/c and DBA/2 mice ($n = 5$/group) after infection with *P. yoelii* (17XL strain). Mice were infected intraperitoneally with different doses of PRBCs: $0.2 \times 10^5$ (**a**), $1.0 \times 10^5$ (**b**), $5.0 \times 10^5$ (**c**), $1.0 \times 10^7$ (**d**). This experiment was repeated three times.

### *Statistical Significance*

Probability significance was determined by two-tailed Student's *t*-test, assuming equal variances. Statistical significance was set at $P$ of <0.05 for all comparisons except murine survival after immunization. Fisher's exact test was used for murine survival experiments.

## *Results*

### *DBA/2 Mice after Infection with* P. yoelii *17XL (a Lethal Strain) Did Not Develop CM Pathology or Succumb to Infection*

In a previous study, we showed that BALB/c mice infected with *P. yoelii* 17XL developed symptoms of CM and died by days 6 to 8 after infection.[18] In this study, BALB/c and DBA/2 mice were given different doses of blood-stage infection with *P. yoelii* 17XL and we then evaluated parasitemia and survival in these two strains of mice. Whatever inoculum was given the level of parasitemia was almost the same in both BALB/c and DBA/2 mice at day 4 after infection but it increased rapidly and became significantly higher in BALB/c mice than in DBA/2 mice on day 6 of infection ($P < 0.005$). When infection was given with a lower dose ($0.2 \times 10^5$ PRBCs), the peak of parasitemia was slightly delayed in BALB/c mice compared to that after infection with a higher dose ($1 \times 10^5$ PRBCs) (Figure 1). The results presented in Figure 2 clearly demonstrate that whatever dose of inoculum ranging from $0.2 \times 10^5$ to $1 \times 10^7$ was given, all of DBA/2 mice survived the infection whereas all of the BALB/c mice died by day 6 to 8 after infection. Both DBA/2 and BALB/c mice were infected with $5 \times 10^5$ PRBCs, and then brain

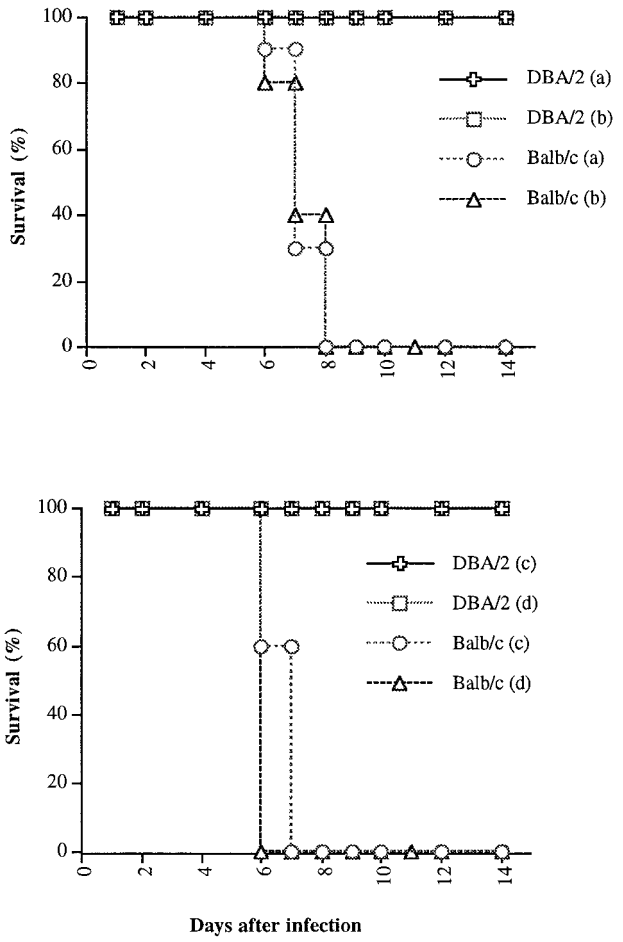


**Figure 2.** Survival of BALB/c and DBA/2 mice ($n$ = 10/group) given infection with *P. yoelii* (17XL strain). Both strains of mice received the same dose of PRBCs: $0.2 \times 10^5$ (**a**), $1.0 \times 10^5$ (**b**), $5.0 \times 10^5$ (**c**), $1.0 \times 10^7$ (**d**). DBA/2 infected mice survived until day 30, end of observation period (not shown).

sections were obtained from them on day 6 of infection. The results of brain histology showed the presence of parasitized erythrocytes adjacent to endothelial wall, infiltration of lymphocytes, and signs of endothelial cell activation in susceptible BALB/c (Figure 3A) but not in DBA/2 mice (Figure 3B).

## *IL-2 Treatment Alters CM-Resistant Mice to Susceptible-Like Phenotype*

To determine whether CM pathology is related to an enhanced T cell activation, resistant DBA/2 mice were administered with rIL-2. After the treatment, all of the DBA/2 mice that were infected with *P. yoelii* 17XL (TI) developed symptoms of CM by day 6 after infection. The symptoms include: appearance of ruffled hair, convulsions, seizure, ataxia, and coma, whereas none of the nontreated but infected mice (IO) showed any of these CM signs. All TI animals succumbed to infection by 6 to 8 days after infection whereas none of the IO mice died by the same time period (Figure 4). The regimen of IL-2 treatment was not toxic for DBA/2 mice because all of the IL-2-treated but noninfected mice (TO) were in good health and survived until the end of observation period (Figure 4). There was no significant difference in parasitemia between treated and untreated mice at day 3 after infection. The IL-2-treated moribund mice at day 6 after infection showed slightly higher parasitemia than nontreated infected mice (Figure 5).

## *IL-2 Treatment Induced Augmented Production of Pro-inflammatory Cytokine in Moribund Mice with CM*

The extent of T cell activation after administration of rIL-2 was assessed by measuring cytokine production by T-cell enriched populations obtained from spleens. Splenic cells were enriched for T lymphocytes by passage on nylon-wool columns and then were stimulated with cross-linked anti-CD3 mAb. This TCR-mediated activation has allowed potential generation of cytokines by mouse T cells. A significantly lower IFN-$\gamma$ production was found at day 3 after infection in TI mice than in TO, IO, or in normal mice ($P < 0.005$) (Figure 6A). The diminished production of IFN-$\gamma$ at an early stage of infection (on day 3 of infection) in TI animals was accompanied by an enhanced production of IL-10, an immunoregulatory cytokine (Figure 6A). The level of IFN-$\gamma$ was significantly augmented in TI mice at day 6 after infection compared to that in the early stage of infection ($P < 0.005$) (Figure 6B). There was no significant difference in the level of IFN-$\gamma$ between IO and TO mice on day 6 of infection (Figure 6B). Our findings showed significantly higher IL-10 production in the early stage of infection in TI mice than in IO or TO mice ($P < 0.005$). No such difference in IL-10 production was seen between these groups in the late stage of infection ($P > 05$). The level of TNF-$\alpha$ production was lower in TI mice (532 $\pm$ 57 pg/ml) than in IO animals (820 $\pm$ 170 pg/ml) during the early stage of infection whereas this became higher in TI mice (772 $\pm$ 57 pg/ml) than in IO animals (508 $\pm$ 94 pg/ml) in the late stage of infection. There was an increase in the production of TNF-$\alpha$ in TI mice in the late stage of infection compared to that in the early stage. Although these differences in TNF-$\alpha$ levels are significant ($P < 0.05$), they are not extremely as significant as seen in the case of IFN-$\gamma$ production. These results are summarized in Figure 6.

## *Effect of IL-2 Treatment on Nitrite Production*

The production of NO by T-cell enriched splenic cell populations after stimulation with cross-linked anti-CD3 mAb was assayed. Nitrite production in TI mice was significantly reduced on day 3 of infection compared to that in TO animals ($P < 0.05$). The reduced production of nitrite in TI was associated with higher production of IL-10 at this stage. When the IL-2 treatment continued and the infection progressed to the late stage (day 6 of infection), the level of NO production augmented in TI mice and became significantly higher than in TO mice or in normal mice ($P < 0.005$). These results are presented in Figure 7.

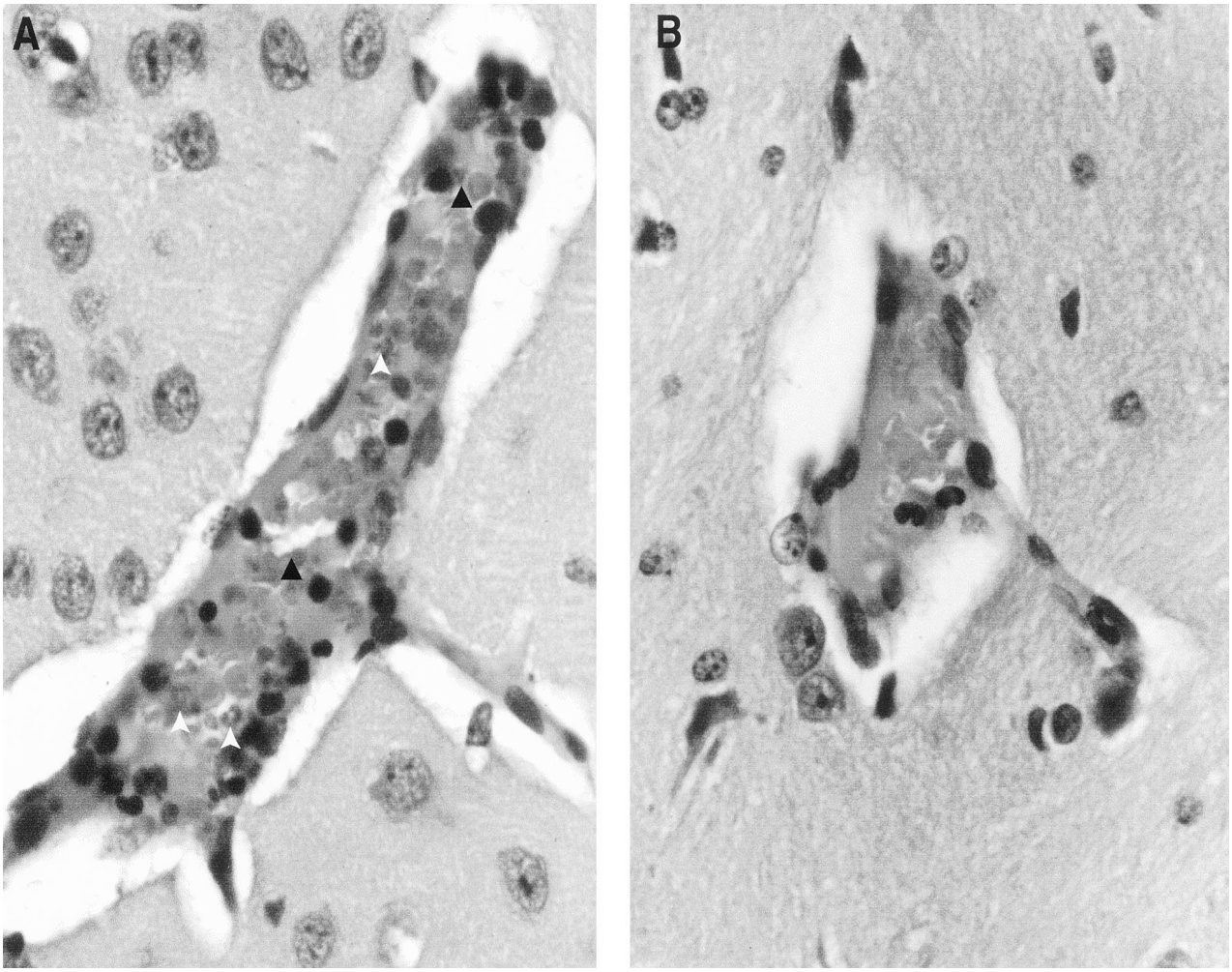


**Figure 3.** Histology of the brain (original magnification, 20 × 1.6) shows accumulation of parasitized erythrocytes (**open arrow**) and infiltration of lymphocytes (**dark arrow**) within cerebral vessels of BALB/c (**A**) but not in DBA/2 (**B**) mice after infection with *P. yoelii* (17XL strain).

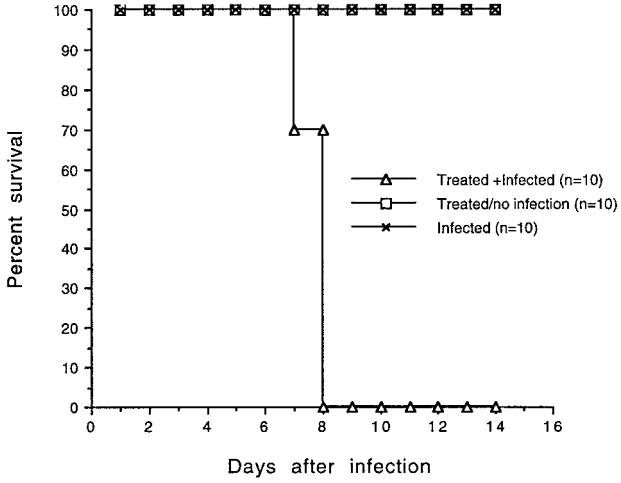


**Figure 4.** Survival of *P. yoelii* 17XL-infected and IL-2-treated mice. DBA/2 mice treated with IL-2 and infected with *P. yoelii* (17XL strain; $n = 10$); mice treated with IL-2 but not given infection ($n = 10$); or mice received infection alone ($n = 10$) (**C**). DBA/2 mice treated/no infection or mice given infection only survived until day 35, the end of observation period (not shown).

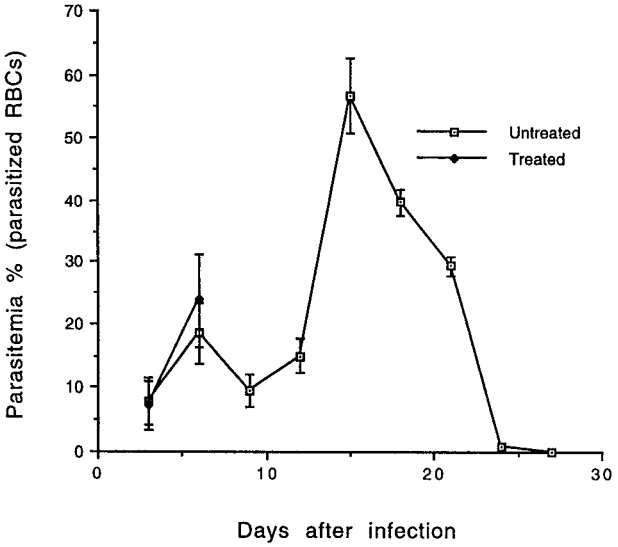


**Figure 5.** Effect of IL-2 treatment on parasitemia during the course of infection with *P. yoelii* (17XL strain) in DBA/2 mice ($n = 5$/group). The parasitemia in IL-2-treated and -infected moribund DBA/2 mice was slightly increased compared to mice given infection alone at day 6 after infection. Evaluation of parasitemia could not be continued in treated and infected mice beyond day 6 of infection as they died by this time period, whereas parasitemia was shown in surviving mice given infection alone until day 30 of infection. This experiment was repeated three times.

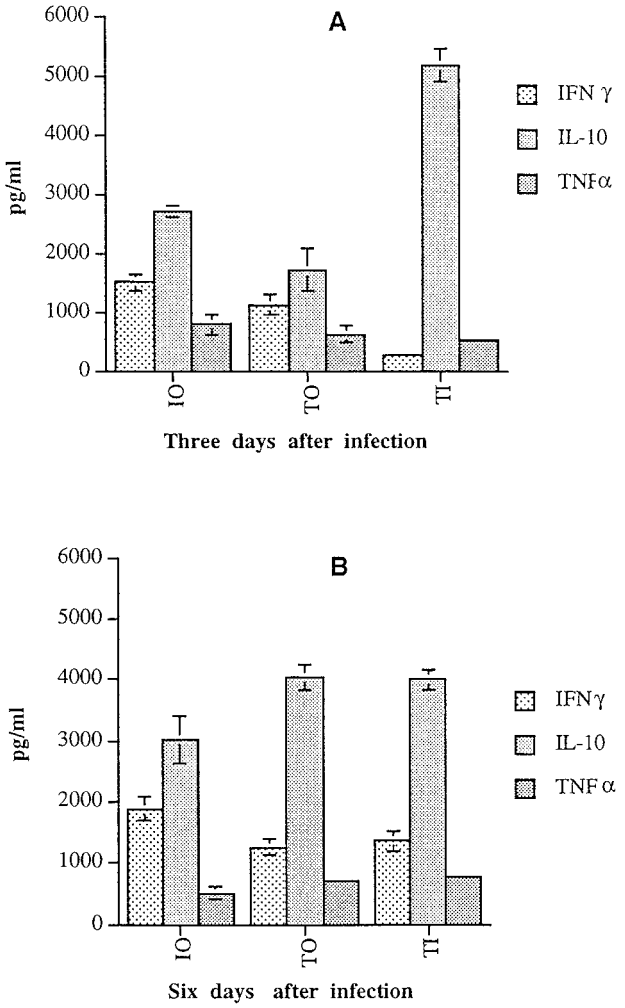


**Figure 6.** Cytokine production in DBA/2 mice treated with IL-2 and infected with *P. yoelii* (17XL strain). Mice ($n = 3$) were either given IL-2 treatment only or treated with IL-2 and given infection with *P. yoelii*. Spleen cells were harvested from mice infected for 3 or 6 days and enriched for T cells by passage on nylon-wool column and bulk cultures were set up with $2 \times 10^6$ cells/well. Cells were stimulated for 48 hours with cross-linked anti-CD3 mAb and the cytokine production in culture supernatants was assessed. The production of IFN-γ, IL-10, and TNF-α by normal mouse cells stimulated with cross-linked anti-CD3 mAb was 966 ± 321 pg/ml, 1,500 ± 102 pg/ml, and 456 ± 58 pg/ml, respectively. The production of IFN-γ, TNF-α, or IL-10 in the absence of stimulation (ie, medium alone) in culture supernatants of cells from treated or untreated mice with or without infection was <200 pg/ml.

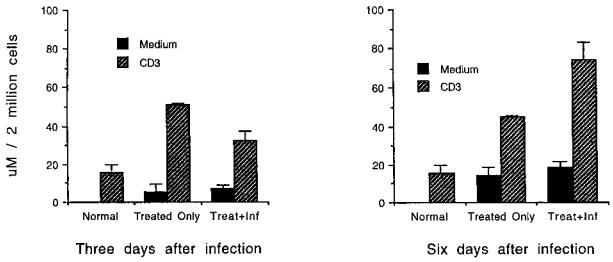


**Figure 7.** Effect of IL-2 treatment on nitrite production in DBA/2 mice after infection with blood stages of *P. yoelii* (17XL strain). Mice ($n = 3$) either received IL-2 treatment only or were treated with IL-2 and given infection with *P. yoelii*. Spleen cells were harvested at days 3 and 6 after infection. They were then enriched for T cells by nylon-wool purification and were stimulated with cross-linked anti-CD3 mAb. Nitrite levels were measured in 48-hour culture supernatants. Cells were unstimulated (**solid bars**) or stimulated with anti-CD3 mAb (**hatched bars**).

## *Histological Changes in the Brain of IL-2-Treated Mice*

Histological analysis revealed the changes in the brain in IL-2-treated DBA/2 mice at day 6 after infection that closely resembled those described in susceptible BALB/c mice. These include the accumulation of PRBCs adjacent to endothelial wall, signs of activation of endothelial cells by enlargement of their nuclei, weakness in the integrity of blood-brain barrier, and signs of hemorrhage. In addition, we observed infiltration of lymphocytes within cerebral vessels of IL-2-treated and infected mice (Figure 8D). In contrast, no such histological alterations were seen in the brains of normal mice (Figure 8A), in mice that were given IL-2 only (Figure 8B), or in mice that received infection but no IL-2 treatment (Figure 8C).

## *IL-2 Treatment Evokes Accumulation of γδ T Cells within Cerebral Vessels of DBA/2 Mice Infected with* P. yoelli *17XL*

Confocal microscopy was performed with brain specimens obtained from TI mice to characterize the nature of the cellular infiltrates. Infiltration of lymphocytes was not observed in the brain of TI mice at day 3 after infection in histological analysis (data not shown). For this reason, brain samples were obtained only from the moribund TI mice at day 6 after infection. Thin sections from brains were prepared and these sections were incubated with fluorescent conjugated antibodies to αβ or γδ TCRs. When analyzed by confocal microscopy, we could observe recruitment of a few T cells belonging to αβ lineage in the brains of TI mice (Figure 9D). Interestingly, the T cells that were predominantly accumulated in the brains of mice with CM were seen to be γδ-bearing T cells (Figure 9E). Further, we were able to detect the expression of CD25 (IL-2R)-positive cells in the brains of TI but not in TO mice (data not shown). This finding suggested that T cells recruited in the brains were in a state of activation. Another important activation marker, CD54 (ICAM-1) molecules were detected on the surface of recruited cells (Figure 9F).

## *Effects of* in Vivo *Administration of Anti-γδ mAb on CM Development in DBA/2 Mice*

Having observed the preferential recruitment of γδ T cells within cerebral vessels of TI mice, we wanted to investigate the *in vivo* role of γδ T cells in CM pathology and subsequent mortality. Because of the nonavailability of γδ T-cell-deficient mice with DBA/2 background, we studied the effect of depletion of γδ T cells by antibody treatment on the outcome of infection in IL-2-treated mice. None of the anti-γδ mAb-treated TI mice developed CM symptoms at day 6 after infection whereas all of the control mice demonstrated signs of CM on day 6 of infection. Five of the seven anti-γδ mAb-treated TI mice ultimately

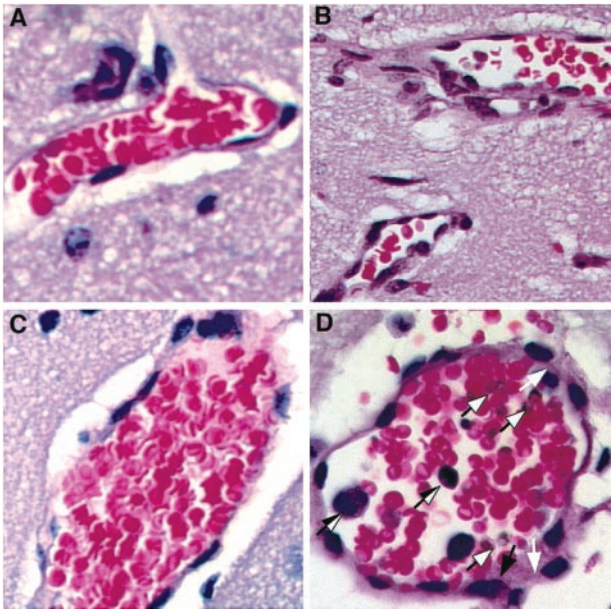


**Figure 8.** Histology of the brain from DBA/2 mice treated with IL-2 and given infection with *P. yoelii* (strain 17XL). Sections of brain vessel (original magnification, 40 × 1.6) from normal mouse (**A**); from mouse that received no treatment but was infected for 6 days (**B**); from mouse receiving treatment only (**C**); from treated and infected mouse at day 6 after infection (**D**) showing presence of several parasitized erythrocytes in the vessel (**dark-bordered arrow**), infiltration of lymphocytes (**one-side dark arrow**), endothelial cell activation with enlarged nuclei (**dark arrow**) and areas of vessel disruption (**clear arrow**).

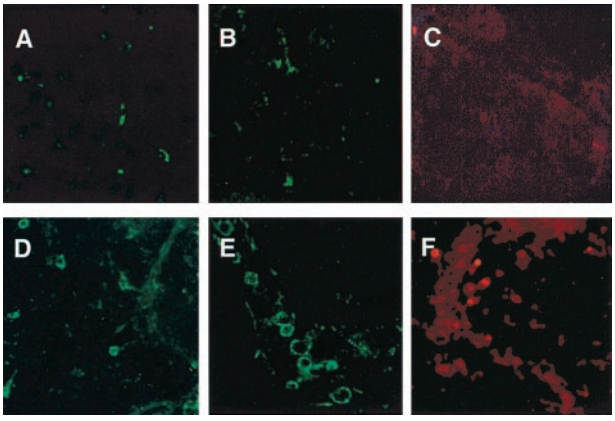


**Figure 9.** Confocal microscopy performed on vibratome sections of the brain from mice that received IL-2 treatment and were infected with *P. yoelii* (strain 17XL) for 6 days. Sections of brain from a mouse given IL-2 treatment but no infection, and stained with anti-αβ TCR antibody (**A**), with anti-γδ TCR antibody (**B**), or with anti-CD54 (ICAM-1) antibody (**C**); no marked staining was seen in any of the cases. Sections of brain from a mouse given IL-2 treatment and infection, and incubated with anti-αβ TCR antibody (**D**), with anti-γδ TCR antibody (**E**), or with anti-CD54 antibody (**F**); a number of cells with positive staining was seen in each cases, notably with anti-γδ TCR antibody. Laser power, PMT gains, and confocal thresholds were set using FITC-IgG isotype controls and kept constant throughout the experiment.

developed CM symptoms and died by days 18 to 21 after infection. The remaining two mice stayed healthy until the last day of observation. In a separate experiment, TI mice that received anti-γδ T cell antibody on days −1 and +2 after infection developed CM symptoms and died by days 6 to 8 after infection. Only one out of seven mice survived the infection in this group. All of the control TI mice included in both of these experiments became moribund with signs of CM by day 6 after infection and succumbed to infection by day 8 after infection. The results of these experiments are presented in Table 1.

## *Discussion*

BALB/c mice, when infected with *P. yoelii* 17XL (a lethal strain), readily develop CM symptoms by days 6 to 8 after infection and succumb to infection.[18] In this study, we show that DBA/2 mice inoculated with the same strain of parasite survived infection and never developed signs of CM. CM syndrome in DBA/2 mice could not be induced even by increasing the inoculum by 500 times. The level of parasitemia in this mouse strain was never as high as in BALB/c mice even with a large inoculum. Further, histological examination of brain revealed a lack of parasitized erythrocytes within vessels and no observed infiltration of lymphocytes within cerebral vessels in DBA/2 mice, in comparison to susceptible BALB/c mice that showed parasitized erythrocytes and perivascular lymphocytic infiltrates. These findings clearly illustrate that DBA/2 strain of mice is resistant to the pathological hallmarks of CM.

It is still unclear whether human CM presents an immune-mediated disease.[9,10] In the current study, we have considered the possibility that large doses of IL-2 administration may enhance cell-mediated immune responses in resistant DBA/2 mice, and this in turn may stimulate pathways for the development of CM pathology from infection with *P. yoelii* 17XL. Indeed, treatment with IL-2 led to the development of CM symptoms by day 6 after infection in DBA/2 mice (TI), which subsequently died on days 6 to 8 of infection. The regimen of IL-2 treatment was not toxic for DBA/2 mice because mice treated in the same way and not given infection (TO) showed no signs of disease until the end of observation period. These findings clearly demonstrated that treat-

**Table 1.** Effects of Anti-γδ mAb Administration on Development of Cerebral Malaria in IL-2-Treated and Infected DBA/2 Mice ($n$ = 7/Group)

| Experiment | Treatment | Cerebral malaria* |
|---|---|---|
| Experiment 1† | TcR γδ mAb (on days +3 and +6 after infection) | 0/7 |
| | Isotype match control | 7/7 |
| Experiment 2‡ | TcR γδ mAb (on days −1 and +2 after infection) | 6/7 |
| | Isotype match control | 7/7 |

*Cerebral malaria symptoms were examined at day 6 after infection.
†Mice were injected with anti-γδ on days +3 and +6 after infection.
‡Mice were given injections with anti-γδ mAb on days −1 and +2 after infection.

ment with IL-2, a potent activator of T cells, induced the CM-resistant mice to demonstrate a susceptible phenotype and compromised their ability to survive a *P. yoelii* 17XL infection. Our results suggest that development of CM, at least in IL-2-treated DBA/2 mice, is independent of level of parasitemia because parasitemia was increased only slightly in treated, moribund mice compared to untreated, infected mice (IO). It seems that overall parasitemia remains unpredictable as a marker of fatal infection in malaria. In an earlier study we showed that after IL-2 treatment ~70% of BALB/c mice infected with a nonlethal strain of *P. yoelii* (17XNL strain) died, although parasitemia was markedly decreased in those mice.[15] Recently Landau and her colleagues,[19] by controlling parasitemia through subcurative drug treatment, have concluded that pathological sequelae are related not only to acute infections with high parasite levels, but also are consequent to chronic infection with low-grade parasitemias.

T-cell enriched populations from the spleen, which is an active lymphoid organ in mice and an important site for PRBC clearance,[20] were stimulated with cross-linked anti-CD3 mAb. Measurement of cytokine production by this TCR-mediated activation has allowed us to evaluate the extent of T cell activation after administration of rIL-2. Our results demonstrated that the production of IFN-$\gamma$ was increased in moribund TI mice with CM symptoms during the late stage of infection compared to that in the early stage of infection. Further, early IFN-$\gamma$ response in TI mice was markedly diminished compared to that in IO mice that had survived the infection. These results correlate with previous reports by others and by us that show a weak IFN-$\gamma$ response in the initial stage followed by an increased expression of IFN-$\gamma$ in the late stage of infection that led to fatal infections.[2,18] It has been shown that stimulation of strong IFN-$\gamma$ response in the initial stage of infection with *P. yoelii* seems to defend the host against the development of CM in susceptible mice.[2,18,21] These findings emphasize that the timing and level of IFN-$\gamma$ production is critically important in determining whether the effects would be beneficial or harmful. It has not yet been clearly established whether NO plays a role in the development of CM. Some authors have suggested that NO might be implicated in CM pathogenesis whereas others have failed to show a relationship between them.[22–24] Data presented in this study clearly show heightened NO production was associated with CM development in IL-2-treated mice. Patients undergoing IL-2 therapy have been shown to express increased NO synthesis from L-arginine.[25] IL-2 treatment of DBA/2 mice may influence a similar NO synthesis pathway, which may affect neurotransmission in the brain.[26] The effects of IL-2 treatment on production of IL-10 by primed T cells are not clear. However, it seems from our results that the well-recognized inverse relationship between IL-10 and IFN-$\gamma$,[27,28] or between IL-10 and NO[29] is maintained when the animals received IL-2 treatment for a short duration (3 days of infection and 2 days before infection).

The balance of these factors became unstable when the immune cells were exposed to IL-2 stimulation for a longer time (6 days of infection and 2 days before infection). It is of note that simultaneous elevated serum levels of IL-10 and IFN-$\gamma$ have previously been reported in patients with acute *P. falciparum* malaria.[30] It is possible that the inhibitory effect induced by IL-10 on IFN-$\gamma$ or on NO may be dependent on their respective levels and/or other regulatory mechanisms that are involved after extended IL-2-mediated stimulation.[31]

We have demonstrated in this study that there was an augmentation in TNF-$\alpha$ production in TI mice in the late stage compared to that in early stage of infection, which indicated that TNF-$\alpha$ response remained stimulated in moribund TI mice that developed CM. The differences in TNF-$\alpha$ production are significant but less marked than to changes in IFN-$\gamma$ levels observed among different groups. It may be noted that in the current study we measured cytokine production by T-cell enriched populations after TCR-mediated stimulation and thus primarily excluded production of TNF-$\alpha$ by cells from the monocyte/macrophage lineage. The moderately elevated TNF-$\alpha$ response that we observed in TI mice may, along with other factors, participate in the development of CM. TNF-$\alpha$ may not be the sole factor responsible for CM. Recently Shear et al[32] described that TNF-$\alpha$ levels were not significantly different between infections with lethal and nonlethal strains of *P. yoelii*. Treatment of patients with a mAb against TNF-$\alpha$ did not diminish the incidence of CM.[33] Also, therapy with pentoxyfylline, an inhibitor of TNF-$\alpha$, did not improve the condition of patients in a controlled study of *P. falciparum* infection.[34] However, another study reported reduction of both TNF-$\alpha$ and duration of coma in CM in children after treatment.[35] Production of cytokine in TO mice is likely to involve a cytokine cascade initiated by the administration of IL-2 and is not dependent on antigen stimulation because TO mice were not exposed to infection and thus not primed with *P. yoelii* blood-stage antigens.

In this study, histological analysis of brains from TI mice demonstrated several anomalies, which include activation of endothelial cells by enlargement of their nuclei, infiltration of erythrocytes through the blood-brain barrier, and subsequent edema and hemorrhage. This condition was associated with accumulation of PRBCs and infiltration of lymphocytes in the perivascular space of cerebral venules. These changes clearly correlated with the development of clinical CM in TI mice. Some of the infiltrating lymphocytes were seen to advance toward the endothelial wall. Examination of multiple brain sections from TI mice did not show the presence of lymphocytes in the parenchyma. Sequestration of recruited T lymphocytes to activated microvascular endothelial cells may well occur via receptor-ligand interaction. Weakness in the blood-brain barrier has been reported in *P. falciparum* CM patients but transmigration of leukocytes in brain parenchyma was not clearly demonstrated.[36] No infiltration of lymphocytes and parasitized erythrocytes or endothelial damage was seen in the brains of mice that were infected but received no IL-2 treatment, or in mice that were given IL-2 only. The histological changes seen in the brain of IL-2-treated and infected DBA/2 mice closely resemble those described in susceptible mice (see Figure 3 in this study and Yoelii and Hargreaves[7] and Kaul et al[8]).

A critical issue concerning pathology might be the preferential recruitment of some activated T cells in certain microvascular beds, particularly in the brain. The results of our confocal microscopical studies showed infiltration of only a few αβ T cells in cerebral vessels. The T cells accumulated in the brain vessels of mice with CM were predominantly γδ-bearing T cells. Recruitment of γδ T cells was not observed in the brain of mice given IL-2 only. As expected, IL-2 treatment evoked a cascade of cytokine response that was not sufficient to induce recruitment of γδ T cells in cerebral vessels of TO mice in the absence of infection. It has been postulated that only memory T cells can enter and be retained in the central nervous system, and only if the relevant antigens are present.[37] These results clearly demonstrated that recruitment of γδ T cells in the brain of TI mice was infection-induced, and retention of γδ T cells may be dependent on the local presence of antigens from accumulated PRBCs. Some of the activation markers such as CD25 (IL-2R) and CD54 (ICAM-1) molecules were detected on the surface of recruited cells.[38,39] This indicated that T cells recruited in cerebral vessels of TI mice were in a state of activation. ICAM-1 can be expressed on endothelial cells, but the cells that showed positive staining with anti-ICAM-1 antibody looked morphologically more like T cells than endothelial cells. The ICAM-1 molecules expressed on activated T cells can induce aggregation, adherence, and sequestration of T lymphocytes in the cerebral venule.[40] Moreover, accumulated γδ T cells that are in a state of activation can induce local production of IFN-γ and TNF-α,[41,42] and also perhaps generate NO.[43] These mediators will induce/up-regulate the expression of receptors at the brain endothelium where accumulated PRBCs and T lymphocytes could sequester, and this will lead to endothelial degeneration.

The evidence of an important role of γδ T cells in the induction of CM pathology in TI mice was bolstered by reversal of CM in these mice by treatment with anti-γδ antibody. Approximately 70% of TI mice treated with anti-γδ mAb succumbed to infection by days 18 to 20 after infection. This may be explained either by the pathological activities of other cell types in the absence of γδ T cells or by the restoration of γδ T cell expansion later in the infection, because anti-γδ treatment terminated at day 6 after infection in our experiments. Of note, treatment was inefficient when first given 1 day before infection and stopped at day 2 after infection. This observation suggests a biphasic mechanism of action of γδ T cells (early protective/late pathological) in this murine malaria model. It has recently been shown that mice depleted of γδ T cells by antibody failed to develop CM after infection with *P. berghei* ANKA, however, mice deficient in γδ TCR genes developed CM pathology.[6] The biphasic activity of γδ T cells may explain the failure to inhibit CM pathology in γδ-deficient KO mice. It may be noted that several authors have reported a protective role of γδ T cells in malaria infections.[44–46] An early infection-induced response of natural killer cells and γδ T cells, as a part of innate immunity, may develop followed by, eventually, an adaptive immune response. But abundant response of γδ T cells late in malarial infection could be detrimental for hosts, as has recently been described in influenza.[47]

The results of the present study suggest a potential mechanism of CM pathogenesis and support the hypothesis that CM is an immune-mediated disease. Our findings show that the genetic resistance in mice to CM syndrome may be altered by provoking enhanced immunoreactivity in the hosts. Our results provide strong evidence that preferential accumulation of γδ T cells in the brain is crucial for induction of the pathogenesis of CM.

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