

L3T4(CD4)-, Lyt-2(CD8)- and Mac-1(CD11b)-phenotypic leukocytes in murine cryptococcal meningoencephalitis.

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Abstract

An immunohistological study of L3T4(CD4)+ and LYT-2(CD8)+ lymphocytes, Mac-1(CD11b)+ monocytes and granulocytes in experimental murine cryptococcal meningoencephalitis was conducted. To assess the concomitant inflammatory reaction in an extracerebral site, livers were examined in parallel. Mice were infected i.v. with *Cryptococcus neoformans*, group A/D, and organs were examined immunohistologically for CD4-, CD8- and monocyte- and granulocyte-specific CD11b-phenotypic leukocytes over a period of 60 days. Intracerebrally, agglomerations of cryptococci formed pseudocysts that were surrounded by CD4+ and CD8+ lymphocytes at the end of the second week post-infection, followed by the invasion of monocytes and granulocytes into the lesions. After the fourth week post-infection, most of the invaded lesions were transformed into gliosis scars. Meningitis was usually marked and showed a homogenous distribution of CD4-, CD8- and CD11b-phenotypic cells, with a predominance of monocytes and CD4+ lymphocytes. Inflammatory infiltrates in the liver were found already 4 days post-infection. CD4+ lymphocytes and monocytes were distributed homogeneously in the infiltrates, with a lower number of CD8+ lymphocytes being located rather in the periphery of the infiltrates. Comparing leukocyte kinetics in brain and liver, an important observation was the delayed immigration of immune cells at the intracerebral cryptococcal lesions as compared with the liver, and the different migration patterns of T-lymphocyte subgroups and macrophages. These results suggest that there are differential leukocyte migration patterns in the liver and brain following disseminated cryptococcosis. The immunological aspects of the observed leukocyte kinetics are discussed.

Key words: *Cryptococcus*, Immunity, Immunohistology, Meningoencephalitis, Mice

Introduction

The ubiquitous yeast *Cryptococcus neoformans* is an important pathogen predominantly in patients with impaired T-cell-dependent immunity, e.g. in the acquired immunodeficiency syndrome. Infection usually occurs via the aerogenic route. If the host is unable to clear the primary pulmonary infection, the fungi disseminate systemically, and meningoencephalitis commonly evolves as the predominant clinical presentation. Meningitis is the most common presentation of cryptococcal brain infection, concurrent intracerebral cryptococcomata are found in less than 10% of cases with cryptococcal meningitis [1]. In patients with cryptococcal meningitis, underlying immunocompromising disorders are found much more frequently than in

patients with cryptococcomata [2]. While the infection of the brain and meninges dominates the outcome of generalized cryptococcosis [3], involvement of the liver usually remains clinically asymptomatic [4]. However, the inflammatory reaction in the liver seems to be representative for the clearance of the fungi from extracerebral sites in the host [5].

Immunohistological studies have provided useful information about the in-situ-conditions for the interaction of lymphocyte subsets and macrophages in infections with facultative intracellular organisms, e.g. in listeriosis, brucellosis and leprosy [6, 7, 8, 9]. Since the course of cryptococcosis in mice and humans shows many similarities, the mouse model has predominantly been used for experimental research in cryptococcosis.

The histopathology of murine cryptococcosis is well studied with conventional methods [5, 10]. Immunohistological differentiation of leucocyte subsets has been performed for the lung [11, 12, 13] after intratracheal infection with *C. neoformans* and in human pulmonary cryptococcosis [14], but not for cryptococcal meningoencephalitis after intravenous infection. Due to the crucial role of the CNS in the outcome of generalized cryptococcosis and the importance of T-cell dependent immunity in host defense against *C. neoformans*, our intention was to study the kinetics of CD4- and CD8-phenotypic T-lymphocytes and CD11b-phenotypic monocytes in murine cryptococcal meningoencephalitis. We performed a parallel examination of the livers of the infected animals to assess the immunological defense in an extracerebral site of the host.

In mice, helper/inducer-T-lymphocytes are characterized by the L3T4-antigen, equivalent to CD4 in human lymphocytes. Cytotoxic/suppressor-T-lymphocytes are characterized by the LYT-2-antigen, equivalent to the human marker CD8. Mac-1 is a mouse macrophage cell surface antigen expressed on blood monocytes, certain macrophage-populations, and in lesser amounts on granulocytes and NK cells [15]. It represents the receptor for inactivated complement C3b, equivalent to the human leukocyte antigen CD11b. In the following text, only the CD-nomenclature will be used.

Materials and methods

Mice. Male C57BL/6 mice raised in our own breeding facilities were used at the age of 9–11 weeks.

Cryptococcus neoformans. A moderately encapsulated strain of *Cryptococcus neoformans*, group A/D, was used. The strain had been isolated from an AIDS-patient with cryptococcal meningitis at the University Hospital Steglitz of the Free University of Berlin, Germany. The fungi were maintained on Sabouraud agar plates.

Preparation of Inocula and Inoculation of Mice. The isolate was grown in Trypticase soy broth, washed three times in sterile phosphate buffered saline (PBS), dispensed in vials of 1.8-ml lots and stored at -70°C . To assess the concentration of viable organisms in the suspension, an aliquot was thawed and serial 10-fold dilutions were prepared. Viable cell counts were deter-

mined by plating on Trypticase soy and Sabouraud agars and counting the number of colony-forming units (CFU) after incubation at 37°C for 6 d. For intravenous challenge, the appropriate number of vials from suspension was thawed and diluted in PBS to contain the desired number of 6×10^5 viable cryptococci in 0.2 ml of inoculum size. The concentration of viable cells in the inoculum was verified by determining the number of CFU as described above. The infection dose of 6×10^5 cryptococci per mouse was chosen because it allowed most animals to survive the infection, but was high enough to elicit a strong inflammatory reaction.

Assay for Organ CFU. At various time points post-infection (p.i.) until 72 d p.i., brains and livers were removed from mice and homogenized in saline. The number of CFU per organ was calculated by plating 10-fold serial dilutions of organ-homogenates on Sabouraud and Trypticase soy agar. The detection limit of this procedure was 10^2 cryptococci per organ. Colonies were counted after 6 d of incubation at 37°C .

Immunohistology. Immunohistology was performed starting day 2 p.i. until day 60 p.i., first in intervals of 2 days, later in intervals of 6 days. For histological examination, organ specimens were snap frozen in liquid nitrogen immediately after killing of the animals by cervical dislocation, and stored at -70°C . After fixation, frozen sections were incubated with the primary antibody. Anti-L3T4 (Paesel & Lorei, Frankfurt, FRG; rat-anti-mouse, IgG2a, dilution 1: 60), anti-LYT-2 (Becton Dickinson, Heidelberg, FRG; rat-anti-mouse, IgG2b, dilution 1: 30) and anti-Mac-1 (Culture supernatant of the hybridoma M 1/70; dilution 1: 50) were used as primary antibodies. Sections with anti-LYT-2 and anti-Mac-1 were incubated with free avidine (0.01%) and d-biotin (0.001%) to reduce nonspecific background stain followed by a biotinylated rabbit-anti-rat secondary antibody (Dianova; IgG2b, dilution 1: 300). Sections were then incubated with streptavidine-alkaline-phosphatase-complex (Dianova, dilution 1: 200) and developed with new fuchsin and naphthol-AS-BI-phosphate (Sigma). Sections with anti-L3T4 were incubated with alkaline-phosphatase-conjugated goat-anti-rat IgG secondary antibodies (Dianova; dilution 1: 200) and developed as described above. Sections were counterstained with hematoxyline.

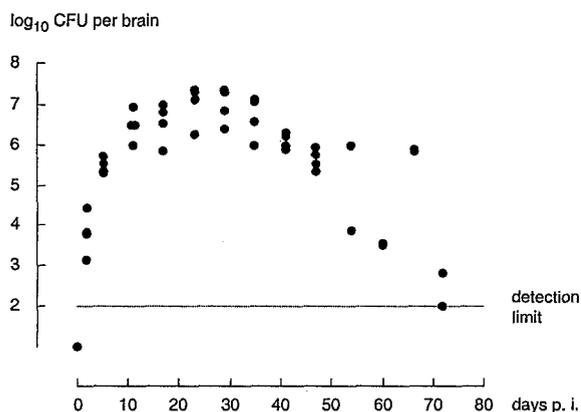


Fig. 1. Murine cryptococcosis: Quantitative cultures of brain homogenates after i.v. challenge with 6×10^5 *C. neoformans*. Brains were removed from mice and homogenized in sterile saline. The numbers of CFU per brain were calculated by plating 10-fold serial dilutions of organ-homogenates on Sabouraud and Tryptic soy agar. The detection limit of this procedure was 10^2 cryptococci per organ. Colonies were counted after 6 days of incubation at 37 °C.

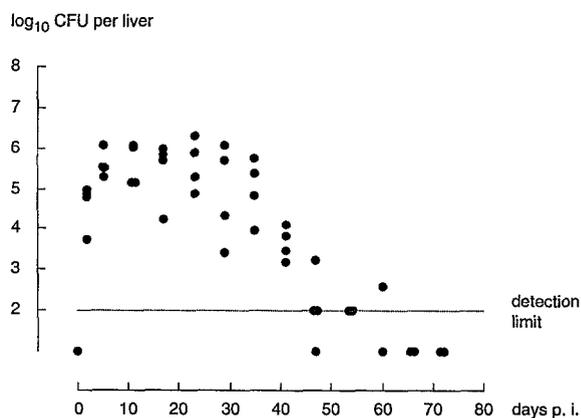


Fig. 2. Murine cryptococcosis: Quantitative cultures of liver homogenates after i.v. challenge with 6×10^5 *C. neoformans*. Livers were removed from mice and homogenized in sterile saline. The numbers of CFU per liver were calculated by plating 10-fold serial dilutions of organ-homogenates on Sabouraud and Tryptic soy agar. The detection limit of this procedure was 10^2 cryptococci per organ. Colonies were counted after 6 days of incubation at 37 °C.

For each staining procedure, murine spleens were used as positive controls for the leukocyte antigens L3T4, LYT-2 and Mac-1. Biological validity of the marked antigens was confirmed by comparing the observed distribution with the anatomically expected distribution of positive cells in the spleens. For negative control, a pure PBS-solution was used instead of the primary antibody solution.

The relative portions of leucocyte subsets in inflammatory lesions were assessed by dividing the number of immunolabeled cells in histological sections by the total number of leukocytes in an arbitrarily defined area, e.g. in all granulomas in a liver section.

Results

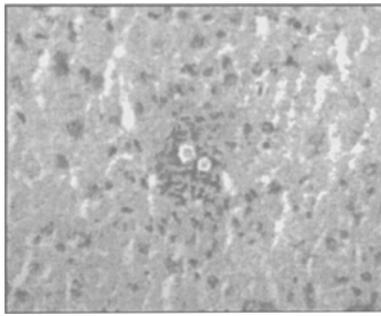
Quantitative culture of organs

The fungi rapidly grew in brains and livers. From the end of the first week until 4–5 weeks after infection, counts in the livers were 10^5 – 10^6 and in the brains 10^6 – 10^7 cryptococci per organ, respectively. Later, the cryptococcal loads were found to be reduced in the examined livers and in most of the brains (Figs. 1 and 2).

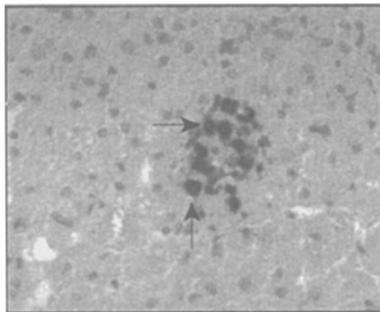
Immunohistology

Liver

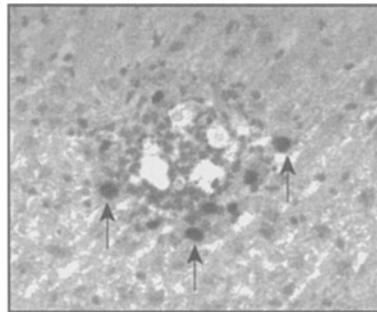
(Fig. 3) Inflammatory infiltrates were seen in increasing size and number from d 4 post-infection (p.i.) on. Encapsulated cryptococci could be identified microscopically in many infiltrates during the first week p.i., whereas they were rarely seen in the infiltrates after d 16 p.i.. Figure 3 shows four different liver infiltrates at d 6 p.i. Encapsulated cryptococci are visible on the control- and CD8-stained sections. The number and size of granulomatous infiltrates increased until the end of the 4th week p.i., but they were found to be drastically reduced in all animals examined after d 40 p.i.. Immunohistologically, the portion of CD4+ lymphocytes was about 25% of all mononuclear cells in the infiltrates during the first 4 weeks after infection and rose up to 40–50% in the following period. The portion of CD8+ lymphocytes was about 6% of all leukocytes in the infiltrates and rose to 14% by d 60 p.i.. The portion of Cd11b-phenotypic cells was increasing during the first week p.i., and later accounted for 20% of inflammatory cells in the infiltrates. Microabscesses containing granulocytes were found mainly during the first week p.i.. Cd11b-phenotypic monocytes and CD4-lymphocytes were found to be distributed quite homogeneously in the infiltrates, whereas CD8-lymphocytes were located predominantly in the periphery (Fig. 3).



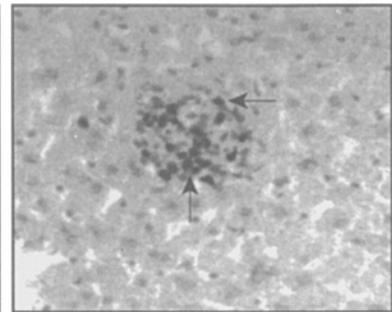
Control



L3T4 (CD4)



Lyt2 (CD8)



Mac-1 (CD11b)

Fig. 3. CD4- and CD8-phenotypic T-lymphocytes and Cd11b-positive leukocytes (monocytes) in liver inflammatory infiltrates 6 days after intravenous challenge with 6×10^5 viable *C. neoformans*. The sections show the typical portion and distribution of the marked immune cells in the infiltrates at d 6 p.i.. Cryptococci were partly washed out during the staining procedure, but some fungi can be seen in the center of the infiltrates. Representative positive cells are marked with arrows. The negative control (PBS in place of primary antibody) shows two cryptococci surrounded by inflammatory cells at d 6 p.i. $\times 200$.

Brain

(Fig. 4) Minor pseudocysts, containing densely packed agglomerations of encapsulated cryptococci, were found already on d 4 after infection. No inflammatory reaction was detected histologically in the brain and meninges on d 2 and 4. On d 10, the first inflammatory cells appearing at the intracerebral cryptococcal lesions were CD4-lymphocytes. On d 16, CD11b+ monocytes and granulocytes could be seen in and around the cryptococcal pseudocysts. CD8-lymphocytes were seen in lower numbers, but were found to be similarly distributed as CD4-lymphocytes, being located mainly in the periphery of the pseudocysts. The CD4/CD8-ratio in the periphery of the lesions was 3–4: 1. After the first 4 weeks p.i., most mice succeeded in transforming the leucocyte-invaded cryptococcal lesions into gliosis scars, concomitant with a significant reduction of visible yeasts in the intracerebral lesions.

During the 4th week post-infection, all examined animals developed a marked meningitis which was found to be practically resolved at the end of the observation period in all examined animals. During the meningitis period, CD4-lymphocytes constituted 20–30%, CD8-lymphocytes about 5%, Cd11b-phenotypic mononuclear cells 35–55%, and granulocytes constituted 5–25% of the meningeal inflammatory cells.

Discussion

Like other facultatively intracellular pathogens, *Cryptococcus neoformans* has shown to be predominantly cleared from the infected host by T-cell dependent mechanisms [16, 17]. Activation of macrophages by T-lymphocytes, e.g. via Interferon- γ [18], appears to be essential in the killing of ingested cryptococci [19, 20]. Infected hosts, in whom T-cells have been deplet-

ed, are at higher risk of developing systemic spread of the fungus [21], with cryptococcal meningoencephalitis resulting as a lethal complication [22].

Quantitative culturing of brains for cryptococci suggests that the blood-brain-barrier does not represent a significant protection against early invasion of the brain by *C. neoformans*. The delay of immigration of lymphocytes and phagocytes into the meninges and the brain parenchyma after systemic infection with *C. neoformans*, compared to the liver, was a central observation in our experiments. The observed leukocyte kinetics in brain and liver are almost identical with a comparable histological study by Bergmann from 1961 [5]. The well-documented predilection of the fungus for the brain [23] may thus be supported by a delay of cellular immunity beyond the blood-brain-barrier already in the immunocompetent host. Moreover, other than in lung [19] and liver [24], the efficacy of resident phagocytes in the brain parenchyma remains controversial [23, 25, 26, 27], thus stressing the importance of a sufficient invasion and T-cell derived activation of blood-borne phagocytes into the cryptococcal lesions.

It should be noted that comparable studies on leukocyte kinetics in experimentally induced meningoencephalitis are rare, partly because few organisms for which adequate animal models exist have a predilection for the brain comparable to *Cryptococcus neoformans*, partly because in most experimental studies on the neuropathology of infections, interest was focused on other aspects. However, experimental studies of myelomonocytic recruitment after injection of lipopolysaccharide into the brain and other organs showed a delayed entry of lymphocytes and monocytes into the brain parenchyma, thus suggesting a nonspecific 'protection' of the brain against the potentially damaging invasion of inflammatory cells [28]. However, by far not all pathogens predominantly cleared from the host by T-cell-dependent phagocyte activity have a predilection for the brain. A better understanding of the pathogenesis of cryptococcal meningoencephalitis will presumably require further research on the – probably inhibitory – interaction of cryptococcal capsular material and local components of the blood-brain-barrier, e.g. endothelial adhesion molecules [29–31]. Recent in-vitro studies [32] indicate a faster migration of activated CD4+ lymphocytes across the brain endothelium compared to CD8+ lymphocytes. Our observations indicate a possible in-vivo relevance of these findings.

In our experiments, the sequential immunohistological study of intracerebral lesions after intravenous infection with *C. neoformans* showed that the appearance of CD4+ and, in lower quantity, CD8+ lymphocytes regularly anteceded the immigration of Cd11b-phenotypic monocytes into the intracerebral lesions. Other than in the liver, we could not find a different distribution of CD4+ and CD8+ lymphocytes in cryptococcal brain lesions. Since our experiments consisted of the sequential immunohistological observation of inflammatory reactions against infection with a defined pathogen, conclusions on immunological mechanisms must be drawn with caution. However, our observations suggest that in fully immunocompetent mice, CD4+ (and CD8+) lymphocytes together with CD11b+ monocytes constitute the successful local cellular immune reaction in the brain in disseminated cryptococcosis. The kinetics of our immunohistological results correspond well with the hypothesis of Huffnagle and co-workers [12] that CD4+ T cells – in contrast to CD8+ T cells – may be more important in recruiting and activating effector cells to eliminate cryptococci that have disseminated to nonreticuloendothelial organs of the body such as the brain. Interestingly, immunohistological studies on viral meningoencephalitis showed a striking predominance of CD8+ lymphocytes in intracerebral inflammatory lesions [33, 34].

Despite intensive research, the differential role of CD4+ and CD8+ lymphocytes in cryptococcosis remains a subject requiring further evaluation. Immunohistological studies may provide important informations. In murine listeriosis, another well studied animal model of an infection with a facultatively intracellular pathogen, it could be demonstrated by depletion of CD4+ and CD8+ lymphocytes that attraction of blood-borne Cd11b-phenotypic monocytes into liver granulomatous infiltrates is a function of CD4+ lymphocytes in vivo [7]. However, depletion experiments in murine cryptococcosis [11, 12, 35] showed that both CD4+ and CD8+ lymphocytes alone are able to elicit a strong cellular inflammatory response in the lungs of mice infected with *C. neoformans* via the aerogenous route. The infiltration of CD4+ and CD8+ cells into the liver and brain of infected mice provide additional support to these findings. Similarly, depletion experiments showed that CD8+ cells are essential for the generation of delayed-type hypersensitivity (DTH) in murine cryptococcosis [36]. Moreover, induction of DTH against *C. neoformans* in mice by immunization with live or heat-killed cryptococ-

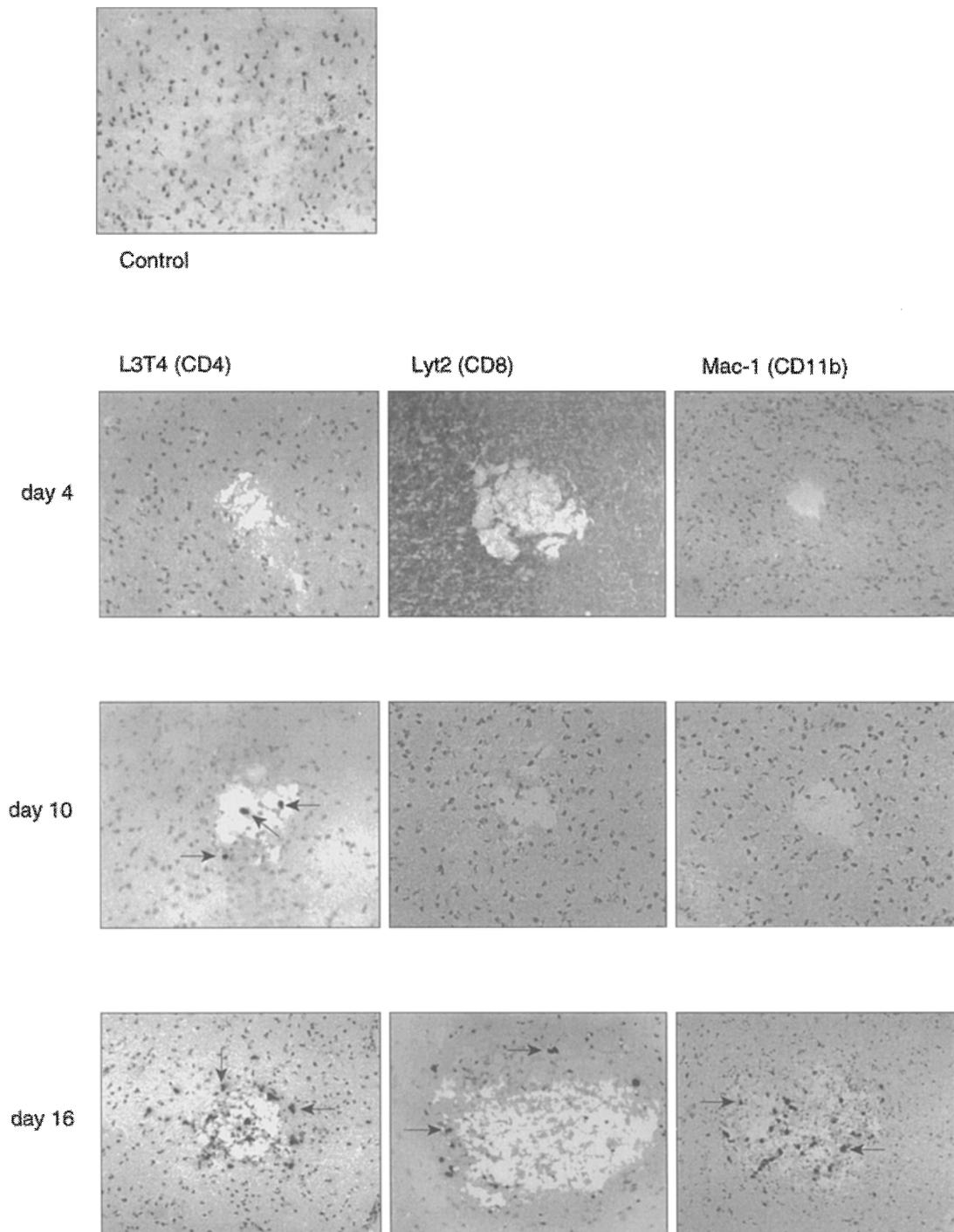


Fig. 4. Kinetics of CD4+ and CD8+ T-lymphocytes and CD11b+ leukocytes (monocytes) in the brain of mice infected with 6×10^5 *C. neoformans*. Sections are from different parts of the brain, thus resulting in different parenchymal background patterns. The Control shows astrocytes and microglial cells in the white matter, no leukocytes are stained immunohistologically. In the further sections, cryptococcal lesions of different size are visible. Cryptococci were partly washed out during the staining procedure. At d 10 post-infection, three CD4+ lymphocytes can be seen at the cryptococcal lesion, whereas CD8- and Cd11b-stained sections remain negative. At d 16, CD4-, CD8- and Cd11b-phenotypic leukocytes can be seen in typical distribution at the cryptococcal lesions. Representative positive cells are marked with arrows. $\times 200$.

ci was found to be associated with better protection against secondary infection with the fungus, although no histological differences in pathology of the internal organs between immunized and unimmunized mice could be demonstrated [10]. These works, with the exception of a recent publication by Hill and Aguirre [37], focused on the importance of local immunity in the lungs against dissemination of the fungus to extrapulmonary sites. In contrast, our intention was to describe the extrapulmonary leukocyte migration pattern in disseminated cryptococcosis by differentiating CD4+ and CD8+ lymphocyte and monocytes in the brain and liver.

Further depletion experiments of lymphocyte subsets together with immunohistological studies should provide a better understanding of the role of leukocyte subsets in the clearing of cryptococcal brain infection in this well reproducible model of a systemically induced metastatic fungal meningoencephalitis.

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