

IMMUNOCYTOCHEMICAL IDENTIFICATION AND QUANTITATION OF THE MONONUCLEAR CELLS IN THE CEREBROSPINAL FLUID, MENINGES, AND BRAIN DURING ACUTE VIRAL MENINGOENCEPHALITIS

BY THOMAS R. MOENCH* AND DIANE E. GRIFFIN

*From the Departments of Medicine and Neurology, The Johns Hopkins University School of
Medicine, Baltimore, Maryland 21205*

The inflammatory response to a variety of acute viral meningoencephalitides is characterized morphologically by an infiltration of mononuclear cells into the brain, meninges, and spinal fluid. The development of the inflammatory response is known to be immunologically specific (1-4), but the identities of the participating mononuclear cells are incompletely characterized (5-12). In studies of inflammatory processes of the central nervous system (CNS)¹, the more easily accessible cells in the cerebrospinal fluid (CSF) are often assumed to reflect the cells participating in the pathologic process in the brain. Recently developed monoclonal antibody reagents directed against surface markers of mouse immune cell subsets now provide the opportunity to identify these inflammatory cells immunocytochemically (13-19). We have used antibody against markers for macrophages, B cells, T cells, and their subsets to determine the types of mononuclear cells present. CSF, meningeal exudate, and brain parenchyma perivascular cuffs were studied at various points after infecting mice with Sindbis virus, an alpha-virus that causes a well-characterized, acute, nonfatal, inflammatory meningoencephalitis (1, 20). We found that the time course of the inflammatory response and the types of cells present differed significantly between the three CNS compartments.

Materials and Methods

Animal Manipulations. 5-wk (34-39 d)-old BALB/c AnNrIBR (Charles River Breeding Laboratories, Wilmington, MA) mice were injected intracerebrally with 500 plaque-forming units of Sindbis virus, strain AR339, in 0.03 ml of Hank's balanced salt solution (HBSS). On days 3, 5, 7, 10, and 14 after injection, mice were anesthetized with methoxyflurane and exsanguinated. The dura overlying the posterior fossa was exposed

This work was supported by a grant from the National Multiple Sclerosis Society and by grants NS 18596 and NS 07000 from the National Institutes of Health.

* To whom correspondence should be addressed at The Johns Hopkins University School of Medicine, Meyer 6-181, Baltimore, MD 21205.

¹ *Abbreviations used in this paper:* CNS, central nervous system; CSF, cerebrospinal fluid; HBSS, Hank's balanced salt solution; MHC, major histocompatibility complex; NK, natural killer; PBS, phosphate-buffered saline.

surgically, and CSF was aspirated into siliconized glass capillary pipettes (21). Samples from four animals for each time point were measured, pooled, and diluted in HBSS without calcium and magnesium. Meningeal exudate was collected by unroofing the skullcap with scissors, removing the brain into 2 ml of HBSS without calcium or magnesium, and dislodging adherent cells from the meninges with spray from a pipette (12). The brain and spleen were frozen in liquid nitrogen.

Quantitation of the Inflammatory Response. Cells from the CSF and meningeal exudate were counted in a hemocytometer. The numbers are expressed as cells per cubic millimeter in undiluted CSF. Meningeal exudate cells from single mice were counted per cubic millimeter of the initial 2-ml suspension. Parenchymal inflammation was scored on a scale of 0-4 based on the extent and magnitude of perivascular cuffing and parenchymal infiltration (22).

Preparation of Cells and Tissues. Cells were sedimented onto glass slides by cytocentrifugation (Shandon Southern Instruments Inc., Sewickley, PA) and fixed in 95% ethanol. Frozen brain and spleen from each animal were mounted together, 8- μ m cryosections cut, and the sections melted onto glass slides and immediately postfixed in 95% ethanol. The spleen was sectioned in the same block to serve as a simultaneous positive control for each immune cell marker.

Immunoperoxidase Staining. Slides were immunoperoxidase stained by the avidin-biotin complex method of Hsu (23). Endogenous peroxidase was blocked by a 30-min incubation in 0.1% hydrogen peroxide in methanol. After rehydration in phosphate-buffered saline (PBS) (25 mM, pH 7.4), the slides were incubated 20 min in 2% normal rabbit serum in PBS, then for 30 min with primary antibodies at dilutions specified in Table I and with normal rat serum diluted 1:500 as a control. After two 5-min rinses in PBS, slides were incubated 30 min with 5 μ g/ml biotinylated anti-rat IgG (Vector Laboratories, Inc., Burlingame, CA) that previously had been adsorbed on a mouse IgG affinity column. Slides were washed, incubated 30 min with a complex of avidin DH and biotinylated horseradish peroxidase (freshly prepared as directed by the supplier, Vector Laboratories), washed again, then incubated 10 min in a freshly prepared solution of diaminobenzidine (Polysciences, Inc., Warrington, PA) 0.5 mg/ml and hydrogen peroxide 0.01% in PBS. After rinsing, the staining was darkened by a 5 min soak in 0.5% CuSO₄ in 0.15 M NaCl, rinsed again, then counterstained lightly with hematoxylin.

Cells were counted under a 100 \times oil immersion objective and the percentage of mononuclear cells that were stained brown were recorded for each antibody. Few, if any, polymorphonuclear cells were seen in CSF or brain. Some unavoidable contamination

TABLE I
Monoclonal Antibodies Used in Immunoperoxidase Staining for Identification of Immune Cell Subsets

Name	Dilution or concentration	Specificity	Source	References
Thy-1.2	1:100*	Pan T cell, neural tissue	Becton, Dickinson & Co. Mountain View, CA	15, 16
Lyt-1	1:100*	Helper/inducer T cells	Becton Dickinson	13, 14, 16
Lyt-2	1:50*	Suppressor/cytotoxic T cells	Becton Dickinson	13, 14, 16
F4/80	1:50 [†]	Macrophages/monocytes	Gift of Dr. Jonathan Austyn, The Rockefeller University	17, 19
RA32C2	20 μ g/ml	B cells, pre B cells	Gift of Dr. Robert Coffman, Stanford University	18

* Diluted from stock as supplied by manufacturer.

[†] Diluted from tissue culture supernatant.

with blood introduced up to 18% polymorphonuclear cells in the meningeal exudate preparations. These cells were disregarded in the analysis. Each CSF data point represents the mean of assays on three pools, each consisting of CSF from four mice. Each meningeal exudate data point represents the mean of assays on three individual mice. For brain parenchyma, only perivascular cuff cells were counted and the total number of nuclei in the cuff was used as the denominator. 4–10 cuffs were counted for each mouse and each cuff data point represents the mean for three to five mice. The normal serum control consistently gave minimal background staining.

Antibody Thy-1.2 was not used on brain parenchyma sections because of its reaction with neural tissue (24). Staining of day 3 perivascular cuffs by antibody F4/80 was not quantitated because of apparent cross-reactivity with endothelial cells, which was judged to introduce significant uncertainty into the counts of these rudimentary early cuffs.

Statistical Analysis. Student's *t* test was used to compare the means of the samples.

Results

Quantitation of the Inflammatory Response. The magnitude of the mononuclear cell infiltrate in the three compartments was examined at various times after infection (Fig. 1). Infiltration with mononuclear inflammatory cells was clearly present in CSF, meninges, and brain by day 3, but the development of maximal intensity and subsequent diminution in each location followed different time courses. The pleocytosis in the CSF peaked between days 3 and 5, while the inflammatory response in the brain parenchyma reached maximal intensity 7–10 d after infection. The inflammatory cells in the meningeal exudate increased and decreased gradually. Although inflammatory cells increased significantly (see below), the total number of meningeal cells increased only twofold because of the significant numbers of noninflammatory cells present in the baseline preparation from uninfected animals.

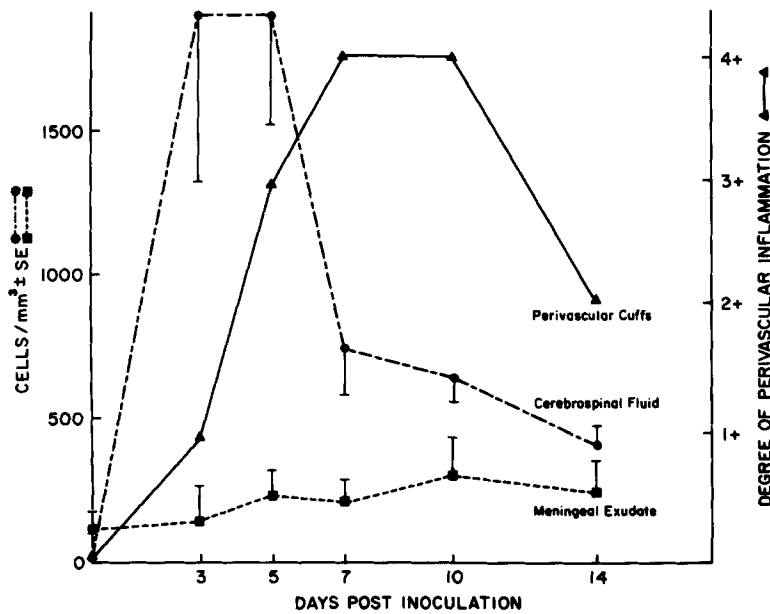


FIGURE 1. Magnitude of the inflammatory response in CSF, meninges, and brain parenchyma at various times after infection with Sindbis virus.

Evaluation of the Selectivity of Antiserum to Lyt-1 and Lyt-2. The suppressor/cytotoxic T cell subset carries low levels of Lyt-1 antigen in addition to large amounts of Lyt-2 antigen on the cell surface (16). Precursor T cells have high levels of both Lyt-1 and Lyt-2 antigens (14). Therefore, we wished to determine whether our assay was doubly identifying either of these subpopulations of T cells to a significant degree. Samples of CSF, meningeal exudate, and brain were stained with each antibody separately and with a mixture of antibodies against Lyt-1 and Lyt-2 (Table II). No significant differences were found between the percentage of stained cells with the mixed reagent and the sum of the percentages stained with anti-Lyt-1 and anti-Lyt-2 separately. This indicates that our assay is not so sensitive as to include significant numbers of suppressor/cytotoxic T cells in the Lyt-1-staining group, and that precursor T cells are probably not substantially represented among the T cells present in these preparations.

Inflammatory Cells in the CSF. Essentially all (99%) of the cells infiltrating the CSF that could be stained were stained with T cell markers (Lyt-1, Lyt-2, or Thy 1.2) (Fig. 2). The percentage of cells staining with Thy-1.2 was always approximately equal to the sum of the percentages of cells staining with Lyt-1 and Lyt-2, again indicating that double identification of cells was not a significant problem in this compartment. The percentage of Lyt-1-staining cells increased from day 3 to day 10 ($P < 0.02$) and from day 3 to day 14 ($P < 0.01$). There were no significant differences in the percentages of Lyt-2-staining cells at different time points. 1% or fewer of CSF cells at any time after infection stained with RA32C2 (B cell marker) and none stained with F4/80 (macrophage marker). However, at all time points, 14–26% of the cells present in the CSF remained unidentified.

Inflammatory Cells in the Meninges. In contrast to the CSF, which contained very few cells ($<20/\text{mm}^3$) in uninfected mice, meningeal cell preparations, even from uninfected mice, were quite cellular. However, since very few of the cells obtained from normal mice could be stained, it is considered most likely that these cells represent meningeal lining cells (Fig. 3). The percentage of cells staining with antibody to Thy-1.2, Lyt-1, and Lyt-2 rose significantly between day 0 and day 3 ($P < 0.01$). There was a trend of further increase for Thy-1.2 and Lyt-1 from day 3 to day 7. The increase in percentages of cells staining with F4/80 and RA32C2 in infected animals relative to uninfected did not reach statistical significance. There was, however, a trend of increasing over time for the percentage of RA32C2-staining cells.

TABLE II
Percentages of Cells in the CSF, Meninges, and Perivascular Cuffs Staining with Antibody to Lyt-1, Lyt-2, or a Mixture of Antibodies to Lyt-1 and Lyt-2

Inflammatory compartment*	Antibody used for staining			
	Anti-Lyt-1	Anti-Lyt-2	Sum	Mixed anti-Lyt-1 + Anti-Lyt-2
	%			
CSF	59.6 ± 3.2	21.5 ± 3	81.1	76 ± 2.3
Meningeal exudate	14 ± 3.4	8 ± 1	22	23 ± 3.9
Perivascular cuffs	33.5 ± 4	7 ± 0.3	40.5	38 ± 1.8

* Sampled 10 d after infection with Sindbis virus.

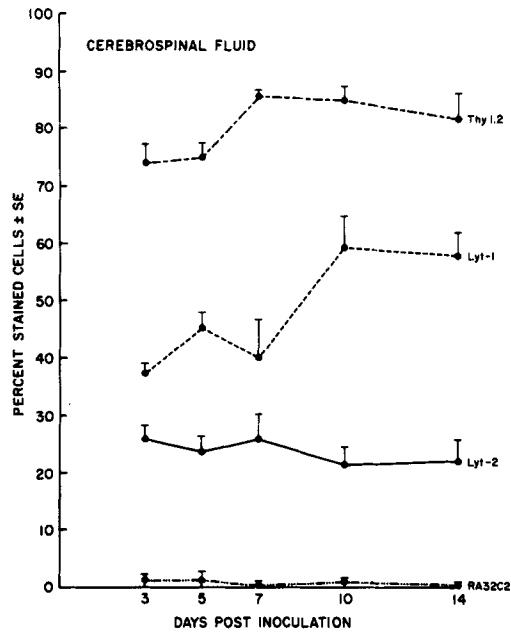


FIGURE 2. Percentages of CSF cells staining by the immunoperoxidase method with antibodies to immune cell markers at various times after infection with Sindbis virus. No cells stained with antibody F4/80 at any day postinfection.

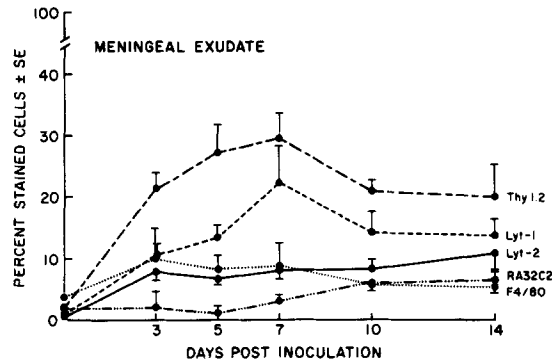


FIGURE 3. Percentages of meningeal exudate cells staining by the immunoperoxidase method with antibodies to immune cell markers at various times after infection with Sindbis virus.

Inflammatory Cells in the Brain Parenchyma. Cells infiltrating the brain parenchyma during most of the inflammatory response are located primarily in the perivascular region where they form cuffs of cells around the vessel. During particularly intense reactions, and late in the response, inflammatory cells are found in parenchymal foci, not clearly associated with vessels. Since quantitation of these latter cells was considered an uncertain process at best, only the perivascular cells were evaluated (Fig. 4). Cells staining with T cell markers were again the most numerous population. The sum of Lyt-1- and Lyt-2-staining cells represented up to 42% of total perivascular cuff cells. The percentage of Lyt-1-staining cells increased significantly with time ($P < 0.05$, day 3 vs. day 10 or 14),

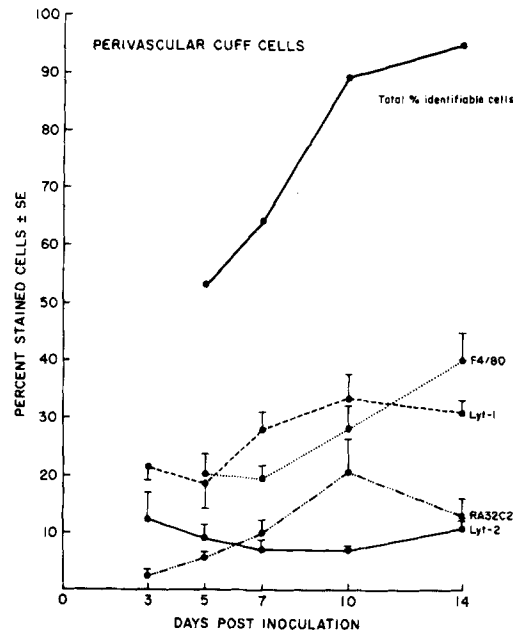


FIGURE 4. Percentage of perivascular cuff cells staining by the immunoperoxidase method with antibodies to immune cell markers at various times after infection with Sindbis virus. Uppermost curve is the sum of the lower curves and represents the total percentage of cells that could be identified with the four immune cell markers.

but there was no significant change in the percentage of Lyt-2-staining cells. In contrast to CSF, perivascular cuffs contained a significant percentage of cells staining with B cell (RA32C2) and macrophage (F4/80) markers. The percentages of these cells changed markedly over time. F4/80-staining cells increased from 20% on day 7 to 40% of total cuff cells on day 14 ($P < 0.02$). RA32C2-staining cells were initially rare (2.5% of cuff cells on day 3) but increased until they constituted 21% of the cuff cells on day 10 ($P < 0.02$). With these increases in B cells and macrophages came an increase in the total percentage of identifiable cells. Early (day 5) in the inflammatory process, ~50% of the infiltrating cells could be identified with the battery of antibodies used, while late (day 14), almost all of the cells (96%) were identifiable as either B cells, T cells, or macrophages. This suggests that another, as yet unidentified, population of mononuclear cells is also participating significantly in the initial stages of inflammation.

Relative Numbers of Cells Staining with Antibody to Lyt-1 and Lyt-2. The ratios of Lyt-1 to Lyt-2 cells were determined for each time point in each of the three compartments (Fig. 5). Lyt-1-staining cells predominated over Lyt-2-staining cells at all time points. At no time was the Lyt-1/Lyt-2 ratio less than 1.3. The ratio was lowest at the earliest stages of the inflammatory response, after which the proportion of the Lyt-1 helper/inducer phenotype increased in each compartment, with the most dramatic increase occurring (ratio 1.7 increasing to 4.9) in the brain parenchyma, compared with an increase from 1.4 to 2.8 in the CSF.

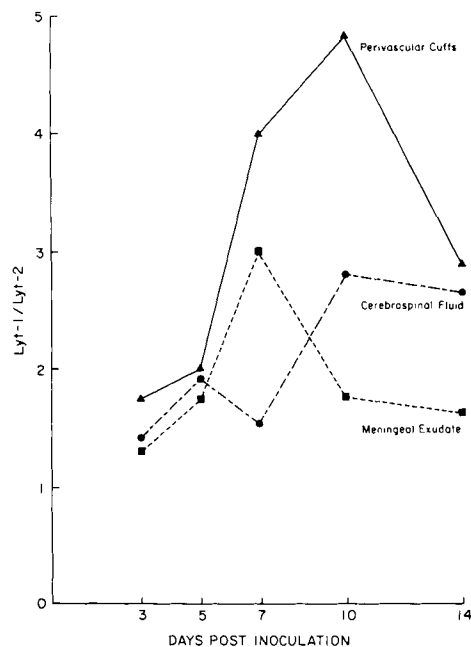


FIGURE 5. Ratio of Lyt-1- to Lyt-2-staining cells. Ratios represent the ratio of the means of the percent stained cells for each antibody at each time point and compartment.

Discussion

We have performed a quantitative study of the types of mononuclear cells entering various compartments of the central nervous system (CSF, meninges, and brain parenchyma) during the development of the inflammatory response to Sindbis virus-induced meningoencephalitis. Cells in the three locations differed both in the composition of mononuclear cells present and in the time course of the appearance and disappearance of these cells. Cells in the CSF were almost entirely T cells and peaked early, while the cells in the meninges and the parenchymal perivascular cuffs included B cells and macrophages in addition to T cells, and peaked later.

T lymphocytes are generally considered to provide the immunologic specificity for the full development of the inflammatory response to CNS viral infections (2, 4, 7, 22). In this study, T cells were the earliest cells to appear in all compartments and their arrival was temporally associated with the onset of the cellular immune response to this infection (25, 26). On day 3, most of the identifiable cells in CSF and brain were T cells, and the proportion of Lyt-1 to Lyt-2 cells was similar. These early CSF T cells may have come from the brain parenchyma into the CSF since it has been shown experimentally, in normal animals, that lymphocytes inoculated into the brain move into the CSF as well as into the cervical lymph nodes (27). Thus, there appears to be a normal recirculation of lymphocytes from the blood through the CNS to the lymphatics, which is increased dramatically during antigenic stimulation in the CNS.

T cells remained the predominant cell in the CSF during the entire period of the pleocytosis. In most other studies in which CSF cells have been examined in

inflammatory diseases of the CNS, T cells have been the predominant cell type. 60% of CSF cells in lymphocytic choriomeningitis virus-induced CNS disease (6) and 77% of CSF cells and 80% of meningeal cells in vaccinia virus-induced CNS disease (12, 28) of mice were T cells. By E-rosette assay, T cells comprised 80–90% of the CSF cells in patients with mumps virus meningitis (10). Our studies demonstrate that a similar (74–86%) proportion of CSF cells in Sindbis virus meningoencephalitis were T cells, although in a previous study with encephalitis caused by another alphavirus, Semliki Forest, only 20% of CSF cells were lysed by anti-theta serum and complement (7). Even in normal CSF, 70% of the small number of cells present are T lymphocytes (29–32).

T cells are also the major identifiable cells early in the response in the brain parenchyma, but within a few days they are joined by significant numbers of macrophages and B lymphocytes. Using other techniques, macrophages have been demonstrated in parenchymal infiltrates of mice with encephalitis caused by Japanese encephalitis virus (9), lymphocytic choriomeningitis virus (8), and Sindbis virus (5). The predominant T cells in the early lesions are cells of the Lyt-1 helper/inducer (delayed-type hypersensitivity [33]) phenotype. Upon antigenic stimulation, these lymphocytes can produce a variety of lymphokines, one of which is a chemotactic factor for monocytes (34, 35). The delayed appearance of macrophages in the perivascular cuffs would suggest that peripheral blood monocytes are recruited to the local area of virus replication secondarily by the chemotactic factors produced by the earlier appearing virus-sensitized T lymphocytes.

B cells represent a third cell population present in the perivascular cuffs. B cells comprise only a very small proportion of the mononuclear cells early in the viral inflammatory process, but, like macrophages, increase in later phases. B cells have been shown to appear late in the inflammatory response to poliomyelitis in humans (36), influenza pneumonia in mice (37), and the autoimmune demyelinating disease, experimental allergic encephalomyelitis (38, 39). It is likely that B cells, like monocytes, also enter local inflammatory reactions in response to lymphokines produced by T cells of the Lyt-1 phenotype already present at the site of antigenic stimulation in the CNS (34). This entry correlates with the appearance of locally produced antibody in the CSF during the recovery phase of infection (21).

Since T cells, including a predominance of cells of the Lyt-1 phenotype, are also present in the CSF, it is not clear why the T cell component of the CSF inflammatory response is not followed by lymphokine production and subsequent entry of monocytes and B cells into this compartment, as it is in the brain parenchyma. This phenomenon may be a manifestation of the well-documented major histocompatibility complex (MHC) region-restricted interaction of T lymphocytes with virus-infected cells or tissues (40–44). The MHC restriction implies that antigen-specific T cells, in order to lyse infected cells (43), or be stimulated to lymphokine production (41, 42), must recognize not only a specific viral antigen, but viral antigen on infected cells. Cell-associated virus for T cell recognition would be found in the brain parenchyma, but primarily cell-free virus would be found in the CSF. Monocytes and B lymphocytes would therefore be recruited into the brain parenchyma but not into the CSF.

Despite using markers for T cells, B cells, and macrophages, the major subclasses of immune cells, not all of the mononuclear cells present have been identified. In the meningeal exudate, many of the unidentified cells appear to be meningeal lining cells. In the perivascular cuffs, some of the unlabeled cells are probably endothelial cells, but this would only account for a small percentage of unlabeled cells. In CSF some meningeal lining cells may also be present. Nevertheless, it seems probable that in each compartment some of the cells participating in the inflammatory response were not stained with the markers used. A number of possible explanations exist. First, some of the cells may have subthreshold quantities of the phenotypic surface marker used. There are variations in F4/80 antigen density on macrophages (19) and of RA32C2 on B cells (18) at different stages of activation and differentiation. Second, access of antibody to the cell surface may be hindered by close apposition of cells in tissue sections compared with free individual cells. A third possibility is that some of these unlabeled cells represent an additional class (or classes) of immune cells participating in the inflammatory response. The percentage of unlabeled cells is highest in the early days of infection, raising the possibility that these cells are natural killer (NK) cells. The NK cell is known to be induced by interferon (45) and to function as a cytotoxic cell in viral infections before antigen-specific immune cells can be generated (46). Furthermore, non-T cell-mediated cytotoxic activity, compatible with NK cell activity, has recently been demonstrated in CSF cells early in the course of vaccinia meningitis in young mice (47). We speculate that NK cells may enter the local sites of viral replication early and may comprise a significant proportion of the inflammatory cells present in the early stages of infection, particularly in the brain parenchyma.

The significant differences in composition of the CSF and parenchymal compartments bear emphasis. No macrophages and very few B cells were found in the CSF, whereas these cells were prominent components of the parenchymal infiltrate, especially at later time points. In addition, among the T cells present, the representation of the Lyt-1- and Lyt-2-staining phenotypes is different in CSF and brain parenchyma. Because it is readily accessible, CSF is the compartment most commonly sampled in human or experimental disease. The present study demonstrates that this sample may not reflect, either functionally or phenotypically, the composition of inflammatory cells present at the actual site of CNS pathology.

Summary

The mononuclear cells of the central nervous system (CNS) inflammatory response were characterized in cerebrospinal fluid (CSF), meningeal exudate, and brain parenchyma of mice 3–14 d after infection with Sindbis virus. The inflammatory infiltrate in CSF peaked and resolved before that of the parenchyma or meningeal exudate. Immunoperoxidase staining with monoclonal antibodies identified CSF inflammatory cells to be almost exclusively T cells, while inflammatory cells in the brain parenchymal perivascular cuffs and the meninges were a mixture of T cells, B cells, and macrophages. The percentage of B cells and macrophages increased at the later time points. Approximately 20% of CSF and 50% of the cells present early in the perivascular cuffs were not

identified, suggesting that another subset of inflammatory cells may be present. We concluded that significant differences exist in the time course and cellular composition of the inflammatory responses in different compartments of the CNS during an acute viral infection.

We thank Queruben Mendoza for excellent technical assistance, Linda Kelly for preparation of the manuscript, Dr. Robert Coffman for the gift of antibody RA32C2, and Dr. Jonathan M. Austyn for the gift of antibody F4/80.

Received for publication 19 September 1983.

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