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Characterization of brain-infiltrating mononuclear cells during infection with mouse hepatitis virus strain JHM

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Summary

The eradication of infectious virus from the central nervous system (CNS) following infection with the neurotropic JHM strain of mouse hepatitis virus (JHMV) is thought to be immune-mediated. Furthermore, a significant decrease of infectious virus coincides with the appearance of prominent inflammatory infiltrates in the brain and spinal cord. In the present study, mononuclear cells infiltrating the brain during JHMV infection were isolated and characterized. While all subsets of immune cells were present, there appeared to be a temporal relationship between the peak incidence of $CD8^+$ T cells (40% of total isolated cells) and reduction of virus at day 7 post-infection. Cells with the natural killer (NK) phenotype (at least 30%) were also present throughout infection. These data suggest that $CD8^+$ T cells and NK cells are prominent among cells which infiltrate the brain during JHM virus infection and may have important roles in reduction of virus within the CNS.

Introduction

Intracerebral inoculation with the JHM strain of mouse hepatitis virus (JHMV) results in an acute encephalomyelitis with the development of both acute and chronic demyelination in mice (Lampert et al., 1973; Weiner, 1973). While recovery from acute encephalitis following infection with JHMV is rare in susceptible C57BL/6 or BALB/c mice, infected mice can nevertheless be protected from fatal infections by the timely passive transfer of JHMV-specific antibodies (Buchmeier et al., 1984; Wege et al., 1984; Nakanaga et al., 1986; Fleming et al., 1989) or by adoptively-transferred immune T cells (Stohlman et al., 1986; Sussman et al., 1987). Furthermore, the reduction of virus in the central nervous system (CNS) appears to be mediated by cells of the host immune system as mice immunosuppressed by X-irradiation, or by in vivo depletion of T cells, are unable to clear virus from their CNS (Sussman et al., 1987; Williamson and Stohlman, 1990).

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Following infection with JHMV, viral titers in the CNS increase rapidly, reaching a peak around day 5 post-infection (p.i.) and declining thereafter. Between days 7 and 9, a significant reduction in virus titer is observed and by day 11 when infection is fatal for the majority of the animals, only low numbers or no detectable virus are found (Sussman et al., 1987; Williamson and Stohlman, 1990). The rapid decline in viral titer after day 5 p.i. coincides with the observation of prominent perivascular cuffs in the parenchyma (Erlich et al., 1989). This temporal relationship between the amount of virus present and the appearance of infiltrating inflammatory cells in the CNS, suggests that infiltrating cells may play a direct role in reduction of infectious virus. The infiltrate consists of both polymorphonuclear and mononuclear cells which penetrate the parenchyma of both gray and white matter (Erlich et al., 1989). However, the phenotypes of the mononuclear cells have not been identified.

The purpose of the present study was to isolate and characterize the cells infiltrating the brain at various times following infection with JHMV. The kinetics of cellular infiltration observed corresponded closely with that observed histologically (Erlich et al., 1989) with cells first appearing around day 5 p.i. and increasing thereafter. The predominance of $CD8^+$ cells at day 7 p.i. and the presence of cells with the NK phenotype throughout the course of infection suggested that both cell types may have roles in reducing virus in the CNS.

Materials and methods

Mice

Six-week-old BALB/c mice were purchased from the Jackson Laboratories, Bar Harbor, ME, U.S.A. Mice were maintained on standard laboratory chow and water ad libitum. Representative mice were bled and ascertained to be seronegative for JHMV prior to infection.

Virus

The DS small plaque variant of the neurotropic JHM strain of mouse hepatitis virus was used for intracerebral (i.c.) infection (Stohlman et al., 1982). Virus was propagated in DBT cells, a murine astrocytoma. Mice were inoculated i.c. with approximately 5×10^3 plaque-forming units (PFU) of JHMV DS in a volume of 0.03 ml.

Isolation of brain mononuclear cells

At various times after infection, 5–20 mice were exsanguinated by cardiac puncture, sacrificed, and their brains removed. Mononuclear cells were isolated according to a procedure described by Clatch et al. (1990) with slight modifications. Brain tissue was dissociated by pressing through 200 μm Nitex gauze (Tetko, Lancaster, NY, U.S.A.) and resuspended in phosphate-buffered saline (PBS). The dissociated brain suspension (two brains per 15 ml tube) was allowed to settle at room temperature for 15 min to remove debris. Supernatants were collected and centrifuged for 7 min at $300 \times g$. The pellets, containing cells and myelin debris, were resuspended in 3 ml of 68% isotonic Percoll (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) diluted in PBS. Discontinuous gradients were prepared by layering 5 ml of 30% Percoll over the cell suspension. The gradients were centrifuged for 25 min at $400 \times g$ at room temperature. Viable mononuclear cells were collected from the 30%/68% Percoll interface, washed twice and counted. Cells were also isolated from brains of mice which received an i.c. injection of sterile PBS 7 days prior to sacrifice.

Spleen cells

Spleens were obtained from uninfected mice. Cell suspensions obtained by pressing spleens through 200 μm Nitex gauze (Tetko) were washed once and erythrocytes removed by lysis with Gey's solution. The cells were washed 2 times in PBS and 1×10^6 cells/sample were stained for use as positive controls in flow cytometry. Spleen cells from immunized mice were obtained in the same manner but were not treated with Gey's solution when used in the natural killer cell (NK) assay.

Staining of cells for flow cytometry

Approximately 2×10^5 brain mononuclear cells or 1×10^6 spleen cells were centrifuged in 1.5 ml Eppendorf tubes and resuspended in monoclonal antibodies reactive against the following cell surface markers: T200 (clone M1/9.3 HL, Boeh-

ringer Mannheim Biochemicals, used at 1:10); L3T4 (CD4, hybridoma supernatant from clone GK1.5, ATCC TIB 207); Lyt-2 (CD8, hybridoma supernatant from clone 2.43, ATCC TIB 210); Mac-1 (hybridoma supernatant from clone M1/70, ATCC TIB 128); Thy-1.2 (hybridoma supernatant from clone H0-13.4, ATCC TIB 99); $ASGM_1$ (polyclonal rabbit anti-asialo GM_1, Wako Chemicals, Richmond, VA, U.S.A.). Cells were incubated with 20 μl of the primary antibodies for 15–20 min at 4°C, washed once with PBS containing 0.1% gelatin, followed by staining with the appropriate fluorescein-isothiocyanate (FITC)-conjugated secondary antibody. These included FITC-$F(ab')_2$ goat anti-rat Ig, FITC-$F(ab')_2$ goat anti-mouse Ig or FITC-goat anti-rabbit Ig (Cappel, Malvern, PA, U.S.A.). Cells were washed 2 times following 15 min incubation with the appropriate secondary antibody and resuspended in PBS with 0.1% formaldehyde and analyzed on a FACS (FACStar, Becton Dickinson, Mountain View, CA, U.S.A.).

In some experiments, double-immunofluorescence staining was used to examine the co-expression of T-200 or Mac-1 on $ASGM_1^+$ cells. Brain mononuclear cells were incubated with anti-T200 and anti-$ASGM_1$, or anti-Mac-1 and anti-$ASGM_1$ for 20 min on ice. Following washing, FITC-goat anti-rabbit Ig was added to the cells for 15 min. Cells were washed again and resuspended in 20 μl of phycoerythrin (PE)-conjugated rabbit anti-rat Ig (Southern Biotechnical Associates, Birmingham, AL, U.S.A.; used at 1:20) and reincubated for 15 min. Thereafter, cells were treated as described for single staining and analyzed accordingly on the FACS.

Assay for NK activity

NK-sensitive YAC-1 target cells were labelled with 100 μCi sodium ^{51}Cr (DuPont NEN Research Products, Boston, MA, U.S.A.) and incubated with spleen or brain-derived mononuclear cells at various effector: target (E:T) ratios for 4 h at 37°C in microtiter plates as described previously (Stohlman et al., 1983). Spontaneous release was determined by incubating medium with targets for 4 h. Maximum ^{51}Cr release was determined by adding 0.25% Triton X-100 to target cells. Percentage of lysis was calculated as: 100 × (cpm test sample–cpm spontaneous release)/(cpm maximum release–cpm spontaneous release). NK-insensitive P815 target cells prepared in an identical manner were also used in some experiments.

Results

Isolation of inflammatory cells from brains

BALB/c mice infected with JHMV show clinical signs of disease by days 4 and 5 p.i. Inflammatory cells were isolated from brains of mice on days 5, 7, 9 and 11 following infection. The total number of mononuclear cells obtained are shown in Fig. 1. A small but significant number of cells (2×10^5/brain) were isolated on day 5 p.i. This number increased, reaching a peak on day 9 p.i. By day 11 p.i., at which time surviving mice were moribund, the number of cells recovered had declined to approximately 6×10^5/brain. Less than 5×10^4 cells/brain could be isolated from uninfected mice using this protocol. Since trauma following an i.c. injection may also result in an inflammatory cell infiltration of the brain, we also examined the cells isolated from brains of mice which had received sham i.c. injections. Sham-injected mice were sacrificed at day 7 post-injection.

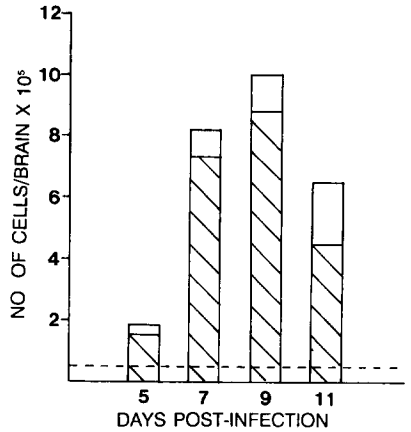

Fig. 1. Total number of mononuclear cells isolated from brains of BALB/c mice on different days following infection with JHMV variant DS. Mononuclear cells were recovered from the 30%/68% interface of a discontinuous Percoll gradient. Hatched area represents cells of bone marrow origin ($T200^+$). The dashed line represents the number of cells isolated from uninfected mice. Data shown are from one of two similar experiments.

The number of cells isolated from these mice (9×10^4/brain), while exceeding that found in untreated mice, was significantly less (9 times) than the number isolated from virus-infected mice at 7 days p.i.

Phenotypes of inflammatory cells

The surface phenotypes of mononuclear cells recovered from brains of JHMV-infected mice were determined by flow cytometry. Mononuclear cell preparations from brains were found to contain a more heterogeneous population than spleen as revealed by forward light scatter. The brain-derived mononuclear cells were therefore analyzed using an acquisition gate based on splenic lymphocytes. Under these conditions, spleen cells were always > 95% positive for the common leukocyte antigen, T200 (data not shown) and brain mononuclear cells were usually > 80% positive (Fig. 1).

The data in Table 1 show that T cells (Thy-1$^+$, CD8$^+$ and CD4$^+$), B cells (sIg$^+$), macrophages (Mac-1$^+$) and NK cells (ASGM$_1^+$) are present within the population of mononuclear cells isolated from the brains of infected mice. The percentage of Thy-1$^+$ cells increased with increasing numbers of total infiltrating cells, reached a peak on day 9 and decreased by day 11. However, different patterns of infiltration were observed for the CD4$^+$ and CD8$^+$ T cell subsets. Both T cell subsets were detectable on day 5 p.i. albeit in small numbers. CD4$^+$ cells made up only 6% of the total cells recovered and only 3% were CD8$^+$. The percentage of CD4$^+$ cells rose gradually during the course of infection and continued to increase on day 11. The percentage of CD8$^+$ cells, however, increased dramatically, reaching a peak on day 7 (40% of the total cells) after which a gradual decline was observed. Throughout the course of infection, the majority of the cells infiltrating the brain were ASGM$_1^+$ (greater than 60%). Double-immunofluorescent staining (Fig. 2*a*) revealed that the majority of the ASGM$_1^+$ cells were of bone-marrow origin (T200$^+$, ASGM$_1^+$). Furthermore, Fig. 2*b* shows that some of the ASGM$_1^+$ cells also express Mac-1 suggesting the presence of activated macrophages. NK cells (Kasai et al., 1980; Young et al., 1980), cytotoxic cells (CD8$^+$) (Stuttles et al., 1986) and activated macrophages (Wiltrout et al., 1985) have been shown to express surface ASGM$_1$. Since the percentage of ASGM$_1^+$ cells always exceeded the sums of the percentages of CD8$^+$ and Mac-1$^+$ cells, these results suggest that at least 30% or more of the cells infiltrating the brain during JHMV infection may be NK cells. A large percentage (35%) of the cells present at day 5 appear to be macrophages (Mac-1$^+$); however, their presence apparently declined with progression of disease. The percentage of B cells (as identified by surface immunoglobulin staining) remained fairly constant throughout the disease (10–15%). The phenotypes of cells present in brains of sham-injected mice are also listed in Table 1. Since the majority of the cells were Mac-1$^+$ or ASGM$_1^+$, these data suggest that T lymphocyte infiltration is not a prominent feature following a stab wound lesion in the brain.

Data from FACS analysis were used to estimate the numbers of each cell subpopulation present in brain infiltrates in virus-infected mice (Fig. 3). Since greater than 75% of the cells were T200$^+$,

TABLE 1

PHENOTYPES OF BRAIN MONONUCLEAR CELLS FROM JHMV-INFECTED MICE

Time post-infection/days	Percentage of positive cells [a]					
	Thy-1	CD4	CD8	Mac-1	ASGM$_1$	sIg
5	39	6	3	35	69	10
7 [b]	69	9	40	12	89	15
9	78	18	25	5	61	11
11	50	20	15	11	60	16

[a] Data shown from one of two similar experiments.

[b] The phenotypes found in sham-injected mice at day 7 were as follows: Thy-1$^+$ (8%); CD4$^+$ (3%); CD8$^+$ (2%); Mac-1$^+$ (20%); ASGM$_1^+$ (47%); sIg (3%). Cells analyzed were > 70% T200$^+$.

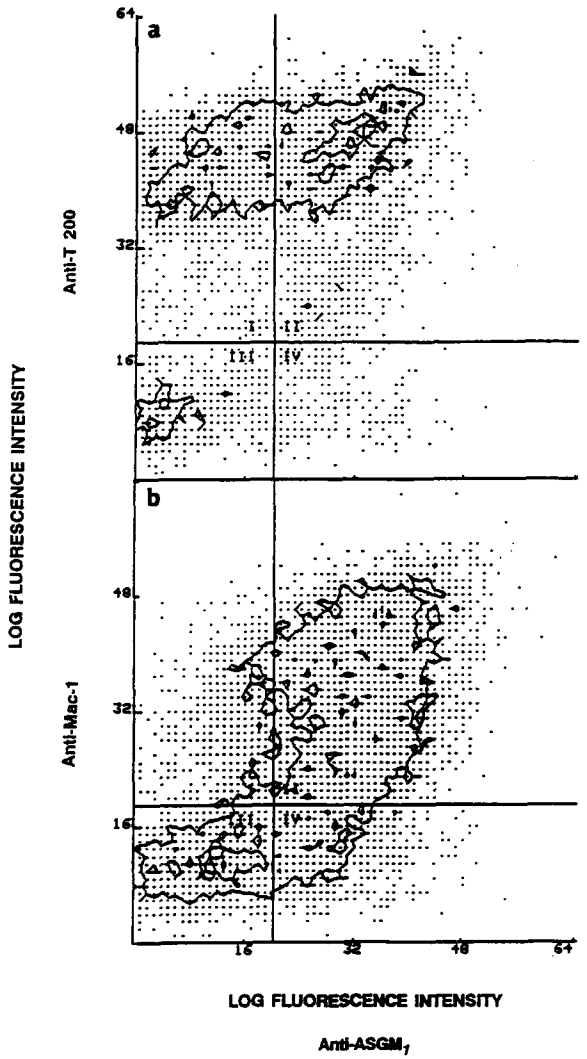

Fig. 2. Double-immunofluorescence staining of brain-derived mononuclear cells showing co-expression of $ASGM_1$ and T200 (*a*), and $ASGM_1$ and Mac-1 (*b*).

the number of bone marrow-derived cells closely matches the total number of cells recovered from the brain. On day 5 approximately 1.5×10^5 bone marrow-derived cells/brain were obtained. By day 9, this number had increased 6-fold to 8.7×10^5 cells/brain, with a substantial number still remaining on day 11 (4.5×10^5 cells/brain). A similar distribution pattern was observed with Thy-1^+, $CD4^+$ or B cells, i.e. these numbers peaked at day 9 and declined by day 11 p.i. Interestingly, $CD8^+$ cells and $ASGM_1^+$ cells were at a maximum on day 7, with substantial numbers remaining at day 9 and declining rapidly on day 11.

NK activity in brain-infiltrating cells

The presence of large numbers of $ASGM_1^+$ cells throughout the course of infection suggested that a significant number of NK cells may infiltrate the brain during JHMV infection. Brain-derived mononuclear cells were assayed for cytolytic activity to confirm the presence of functional NK cells in the brain infiltrates.

Twenty BALB/c mice were inoculated intracerebrally with JHMV and brain infiltrating mononuclear cells were isolated at 7 days p.i. since the maximum number of $ASGM_1^+$ cells was present at this time. Furthermore, the higher numbers of cells present at day 7 p.i. enabled the recovery

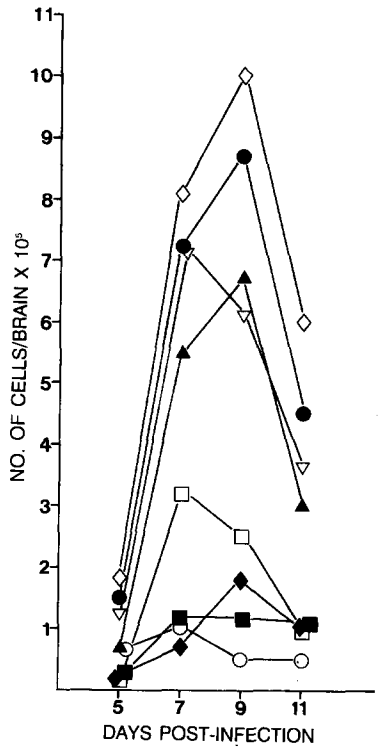

Fig. 3. Absolute numbers of subpopulations of mononuclear cells found within the cells isolated from the brains of JHMV-infected BALB/c mice. These numbers were derived from data obtained for the total numbers (◇) of cells recovered and percentages of each subpopulation present as revealed by FACS analysis. The following cell subpopulations were present: $T200^+$ (●); $ASGM_1^+$ (▽); Thy-1.2^+ (▲), $CD8^+$ (□); $CD4^+$ (◆); Mac-1^+ (○); sIg^+ B cells (■).

TABLE 2

CYTOLYTIC ACTIVITY OF BMNC AND IMMUNE SPLEEN CELLS FOR NK-SENSITIVE, UNINFECTED YAC-1 TARGET CELLS

Source of effectors	E:T ratio	Specific cytotoxicity (%)		
		Expt. 1	Expt. 2	Expt. 3
BMNC [a]	100:1	32.6±1.3	51.7±14.7	32.1±4.1
	50:1	19.9±0.4	36.9± 8.4	12.5±3.6
	25:1	11.6±0.4	20.7± 2.2	10.2±1.6
Immune spleen [b]	100:1	14.4±0.1	10.9± 5.6	28.6±2.3
	50:1	9.3±0.6	6.2± 9.0	15.5±0.5
	25:1	4.8±0.8	4.9± 1.6	11.6±1.6

[a] NK activity assayed on day 7 p.i.

[b] NK activity assayed on day 3 following i.p. injection of 1×10^6 PFU of JHMV.

of a sufficient number of cells to assess their functional activity. Spleen cells from mice immunized intraperitoneally with JHMV 3 days previously were used as positive controls. Results from these experiments show that the brain-infiltrating mononuclear cells were cytotoxic for uninfected YAC-1 target cells (Table 2). The cytolytic activity observed with brain-derived mononuclear cells from infected mice was comparable to, or higher than that observed with spleen cells from intraperitoneally immunized mice. Mononuclear cells from the brains of infected mice were unable to lyse uninfected non-NK sensitive P815 target cells (Fig. 4). These results show that cells displaying both phenotype and cytotoxicity typical of NK cells are present among the bone marrow-derived cells which infiltrate the brain following JHMV infection.

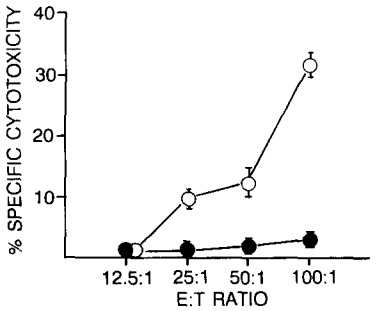

Fig. 4. Cytotoxicity of brain-infiltrating mononuclear cells from day 7 JHMV-infected BALB/c mice on uninfected YAC-1 (○) or uninfected P815 (●) target cells. Data shown are mean values of triplicates with standard deviations of less than 10%.

Discussion

Following infection with JHMV, the viral titer in the brain increases rapidly, peaking at day 5 p.i. and decreasing thereafter to almost undetectable levels by day 11. The exact mechanism(s) by which viral replication is kept in control is unknown. That the immune response plays a role is shown by the observations that mice which are immunosuppressed prior to infection, either by irradiation or by specifically depleting host T cells, are unable to clear virus from the CNS. Moreover, the appearance of an inflammatory infiltrate at a time when a significant decrease in replicating virus occurs further suggests that the inflammatory cells may have a role in reducing virus. Hence, the identification of the subpopulations of the cells infiltrating the brain based on their phenotypic and potential functional characteristics may provide information regarding their role(s) in viral clearance in the local CNS environment.

Results from these studies show that a full complement of the cells of the immune response (T cells, B cells, macrophages and NK cells) is represented in the CNS infiltrates during JHMV infection. However, a detailed study of the kinetics of infiltration and relative numbers of subpopulations shows that $CD8^+$ cells are prominent on day 7 post-infection. This corresponds to a time when virus is rapidly cleared from the brain suggesting that $CD8^+$ T cell-mediated, class I-restricted cytotoxicity for virus-infected cells may be an important mechanism for controlling viral replication within the CNS (Sussman et al., 1987; Williamson and Stohlman, 1990). The importance of the cytotoxic T lymphocyte (CTL) response in the CNS is further emphasized by recent observations that both astrocytes (Skias et al., 1987) and oligodendrocytes (Ruijs et al., 1990) are susceptible to cytolysis by class I-restricted lymphocytes.

The existence of $CD8^+$ CTL specific for mouse hepatitis virus (MHV) was indirectly demonstrated only recently by Yamaguchi et al. (1988). Although cytotoxic activity has not been demonstrated directly from spleens of immunized mice

or following in vitro stimulation in secondary cultures, clearly the observation that JHMV-specific cytolytic clones can be established suggests that virus-specific CTL are induced following intraperitoneal injection (Yamaguchi et al., 1988).

In 1987, Natuk and Welsh observed that following infection with lymphocytic choriomeningitis virus (LCMV), large granular lymphocytes (LGL) accumulated at the injection site, with maximum numbers appearing at day 7. While similar numbers of LGL were observed initially in MHV-infected mice, they did not continue to increase or reach the numbers observed following LCMV infection. Since CTL activity within the LGL population following LCMV infection peaked at day 7 p.i., Natuk and Welsh (1987) suggested that the failure to detect an MHV-specific CTL response may be due to the lack of accumulation of MHV-specific CTL/LGL in the injection site. Our finding that a large percentage of cells infiltrating the CNS on day 7 p.i. are $CD8^+$ suggests that the CNS may be an area of accumulation of virus-specific CTL following intracerebral infection. At this time the CD4 : CD8 ratio of cells recovered from the CNS of infected mice is 0.2–0.6 : 1, which is in contrast to that observed in their cervical lymph nodes (1.4–2 : 1, data not shown). This suggests that there may be a differential accumulation of $CD8^+$ cells in the brain. It is presently not known what proportion of these may be JHMV-specific cytotoxic cells. CTL infiltration of the CNS has also been observed in mice infected with other neurotropic viruses such as West Nile virus (Liu et al., 1989) and LCMV (Oldstone et al., 1986). That the differential accumulation of $CD8^+$ cells in the brain resulted from virus infection rather than a non-specific breaching of the blood–brain barrier was further supported by the observation that few $CD8^+$ cells were present among the mononuclear cells isolated from sham-injected mice. The fact that Mac-1^+ or $ASGM_1^+$ cells and not lymphocytes (Thy-1^+, $CD4^+$, $CD8^+$ or sIg^+) were prominent in brains of sham-injected mice correlates well with the observation that cells which accumulate around stab wounds of the brain are macrophage/microglia-like cells (Kaur et al., 1987).

Another interesting finding regarding the phenotypes of cells in the brain inflammatory infiltrate is the presence of large numbers of $ASGM_1^+$ cells. Since $ASGM_1$ is found in white matter (Kusunoki et al., 1985), it is possible that some neural cells may express $ASGM_1$ on their surface. However, the $ASGM_1^+$ population within the cells we have isolated is clearly of bone marrow origin since $ASGM_1^+$ cells constitute 70–100% of the $T200^+$ cells throughout JHMV infection. This was further confirmed by double-immunofluorescent staining which showed that the majority of $ASGM_1^+$ cells were indeed also $T200^+$. In addition to the finding that cells with the NK phenotype are present among the infiltrates, cytolytic activity against the NK-sensitive YAC-1 target cell was observed. This suggests that a significant number of cells infiltrating the CNS following JHMV infection are indeed NK cells.

Following intraperitoneal MHV infection, NK activity and NK/LGL are present at the site of viral replication within 12 h p.i., and peak at 3 days p.i. (Natuk and Welsh, 1987). The present study shows that within 5 days of intracerebral infection, 65% of the infiltrating cells are $ASGM_1^+$. At this time 35% of the cells are macrophages (Mac-1^+) and only 3% are $CD8^+$. Since activated macrophages and some CTL may also be $ASGM_1^+$, at least 27% of the mononuclear cells infiltrating the brain at day 5 may be $ASGM_1^+$ NK cells. In fact, a significant number of $ASGM_1^+$ cells (exceeding the sums of $CD8^+$ and Mac-1^+ cells) and, therefore presumably NK cells are present through JHMV infection. A large percentage of cells bearing the allotypic NK cell marker NK1.1 (Burton et al., 1982; Koo and Peppard, 1984) have also been detected in infected C57BL/6 mice at day 12 p.i. (unpublished observation), further substantiating the finding that cells with the NK phenotype accumulate in the brain during JHMV infection. The presence of cells with NK phenotype and function in brain infiltrates may explain the early transient clearance of virus (prior to day 7 p.i.) in mice which have been depleted of $CD4^+$ or $CD8^+$ cells (Williamson and Stohlman, 1990).

The present study shows that inflammatory infiltrates in the brain following JHMV infection contain T cells, B cells, macrophages and NK cells. Functional NK activity was present in the isolated brain mononuclear cell preparations, suggesting that NK cells may have a role in the

eradication of virus from the CNS before CTL activity occurs. Since a significant proportion of $CD8^+$ cells is present at day 7, when viral titers decrease significantly, $CD8^+$ cells may have a major role in eventual clearance of virus through a class I-restricted process. Further studies on the specificity and frequencies of virus-specific $CD8^+$ cells in the CNS of JHMV-infected mice may provide important information regarding their roles in virus clearance and in the immunopathogenesis of demyelination.

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