FUNCTIONAL HETEROGENEITY OF LYMPHOCYTIC CHORIOENCEPHALITIS VIRUS-SPECIFIC T LYMPHOCYTES

I. Identification of Effector and Memory Subsets*

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Infections produced in adult mice by the neurotropic Armstrong strain of lymphocytic choriomeningitis (LCM) virus can result in either of two contrasting outcomes, each of which depends on the route of virus administration. Inoculated intracerebrally (i.c.), any dose of infectious virus replicates predominantly in neural membranes, with little or no extension to parenchymal cells of the central nervous system (CNS) and with minimal involvement of extraneural tissues (1, 2). Although the infection is noncytopathic, an acute uniformly fatal CNS disease develops as a direct result of virus-induced immune-mediated damage to infected cells of the leptomeninges, choroid plexus, and ependyma (1, 3). Convincing evidence for the immunopathological nature of LCM comes from our previous studies in which immunosuppression with cyclophosphamide (CY) after cerebral infection was shown to prevent the development of CNS disease and to subsequently lead to the development of a chronic asymptomatic virus-carrier state (1, 4). In contrast, mice given LCM virus by an extraneural route develop a self-limiting subclinical infection confined to extraneural tissues and which eventually confers a solid protective immunity to a second challenge of virus given i.c. (5). However, under appropriate conditions, splenic lymphocytes from such immunized mice, when adoptively transferred to syngeneic CY-induced virus-carrier mice, regularly mediate acute lethal CNS disease which, histologically and clinically, is typical of classical LCM (1, 3). As first shown by Cole et al. (6), both CNS disease and virus clearance mediated by exogenous immune lymphoid cells requires the participation of thymus-derived (T) lymphocytes. Depletion of this population with anti-θ serum and complement (C) completely abrogates the ability of the transferred cells to elicit LCM but not their capacity to produce virus-specific antibody. These results and the fact that column-purified immune donor T lymphocytes can effectively mediate virus clearance in carrier recipients without accompanying antibody production (M. Volkert, personal communication) argue strongly against a significant role for antibody in producing acute disease.

The expression of LCM virus-specific cellular immune reactivity can also be demonstrated in vitro. Splenic lymphocytes, obtained from peripherally infected adult mice,
have the ability to specifically lyse cultures of virus-infected mouse fibroblasts (7). Such lytic activity, as measured by $^{31}$Cr release, has been shown to be present at high levels mainly during the 2nd wk postinfection, and also to be ablated by treatment with anti-$\theta$ serum and C (7, 8).

From previous studies (9, 10), we had noted that there was a poor correlation between the ability of immune donor splenic lymphocytes to produce LCM in CY-induced virus-carrier mice and their lytic capacity in vitro. In fact, mediation of disease in the majority of any group of carrier recipients was possible only when spleens were obtained from donors which had been immunized about 3 wk previously, well beyond the time at which they contained maximal cytolytic activity. These initial observations led us to postulate the existence of two populations of T lymphocytes differing in their functional capabilities and generated at different times during virus-specific immune induction (9). We now present the results of studies which both confirm and extend these observations and which discriminate between memory and effector T lymphocytes on the basis of their respective roles in either protecting against or mediating fatal CNS disease. The generation of a cell population containing the potential to become cytolytically active or “killer” T lymphocytes may be the final common pathway to both outcomes; however each outcome is determined by the relative kinetics of virus replication and immune induction.

Materials and Methods

**Mice.** Male BALB/c mice, aged 6-10 wk, (Flow Laboratories, Inc., Dublin, Va., or Carworth Farms, Nutley, N. J.) or C3H × C57BL F, mice (provided by Dr. Norman Morrison, The Johns Hopkins University), were employed in all adoptive transfer experiments. C3H × C57BL F, mice were either thymectomized at 4 wk of age, lethally irradiated and reconstituted with syngeneic bone marrow (AT × BM), or unmanipulated. AT × BM mice were used between 3 and 4 wk after marrow reconstitution. Adult Swiss Webster (ICR) mice (Microbiological Associates, Walkersville, Md.) were used for virus titrations.

**Virus.** The Armstrong E-350 strain of LCM virus (ATCCVR no. 134) was used throughout. Stock virus was prepared by i.e. inoculation of adult ICR mice, and brains were harvested 6 days later (4). A clarified 20% homogenate in phosphate-buffered saline (PBS) was stored at -70°C. This preparation had an adult mouse i.e. titer of 10$^6$ (LD$_{50}$)/0.03 ml. In some experiments, control animals were inoculated with dilutions of normal brain homogenate prepared in an identical fashion.

**Virus Titrations.** Brains from saline-perfused CO$_2$-anesthetized mice were removed, homogenized, and prepared as clarified 20% suspensions in PBS. Decimal dilutions were inoculated i.c. into adult ICR mice. Virus titers were calculated by the Kärber method (11) and expressed as the LD$_{50}$/0.03 g.

**Cells for Adoptive Immunization.** Immune donors received one or more intraperitoneal (i.p.) doses of 1,000 LD$_{50}$ of virus. Immune or normal donor spleen cell suspensions were prepared by tearing through a stainless steel mesh into a sterile Petri dish containing Eagle's minimum essential medium (MEM) with penicillin (100 $\mu$g/ml) and streptomycin (0.1 mg/ml). After removing rapidly settling aggregates, the cells were washed once in MEM, and adjusted to a concentration of 10 or 100 × 10$^6$ nucleated cells/ml. About 95% of these cells were viable as determined by trypan blue exclusion. All lymphoid cell transfers consisted of 1.0-ml vol administered by the i.p. route.

**CY-Induced LCM Virus Carriers.** Detailed procedures have been previously described (4). In brief, 5 days after the i.e. inoculation of 6- to 8-wk old BALB/c mice with 10$^9$ LD$_{50}$ of LCM virus, a single i.p. dose of CY (150 mg/kg of body weight) was administered. This procedure leads to the development of a permanent virus-carrier state in about 90% of infected immunosuppressed mice. Virus carriers were adoptively immunized between 10 and 15 days after virus inoculation.

**Cytotoxicity Assay.** The method is essentially that described in a previous report (7). 3 days after
inoculating 60% confluent monolayers of BALB 3T3 cells with LCM virus at a multiplicity of infection of about 10 mouse LD<sub>50</sub>/cell, the infected cultures and companion uninfected cultures were trypsinized, labeled with <sup>51</sup>Cr (sodium chromate, New England Nuclear, Boston, Mass.), and washed. Aliquots were seeded into four-chambered tissue culture slides (Lab-Tek Products, Div. Miles Laboratories Inc. Westmont, Ill.) and after adhering, the proportion of cells containing virus-specific antigens was determined by immunofluorescent staining. Only infected preparations containing 90% or more fluorescing cells were used in cytotoxicity assays. Target cells were co-cultivated with splenic lymphocytes from either normal or immune mice as mixtures standardized to contain 1 x 10<sup>5</sup> viable lymphocytes and 1 x 10<sup>5</sup> target cells. After incubation for 18 h at 36°C in air containing 5% CO<sub>2</sub>, the mixtures were centrifuged and the cell-free supernates counted for <sup>51</sup>Cr.

The percentage of specific cell lysis was calculated by subtracting from the percentage of <sup>51</sup>Cr released in the presence of immune splenic lymphocytes, that percentage of label released in the presence of normal lymphocytes. (Percentage cytolysis = counts in cell-free supernates x 100 - total counts.)

DNA Synthesis in Spleens. As an indicator of lymphoid cell proliferation in donor mice after LCM virus immunization, the rate of DNA synthesis in their spleens was measured. From preliminary experiments it was determined that essentially identical DNA-synthetic response curves were generated using either tritiated thymidine or [5-<sup>3</sup>H]-2'-deoxyuridine (H<sub>3</sub>DUR, Amersham/Searle Corp., Arlington Heights, Ill.). Therefore, the latter isotope was employed routinely because of the ease with which it could be measured. At various intervals, groups of 3-4 immunized mice, and control animals inoculated i.p. with an appropriate dilution of normal mouse brain homogenate, were injected i.p. with 2 μCi of an aqueous solution of <sup>3</sup>H]-DUR diluted in 0.5 ml of MEM. Spleens were removed 24 h later at which time all retained <sup>3</sup>H]-DUR is associated with cellular DNA (12). Just before isotope administration, the activity of several 0.5-ml aliquots was measured in a Packard Auto-Gamma spectrometer (Packard Instrument Co., Downers Grove, Ill.) to determine the total number of radioactive counts being given to each mouse. Since the number of lymphocytes in the spleen varies after LCM virus infection (13) the results were expressed as the percent of input <sup>3</sup>H]-DUR retained in replicate aliquots of 100 x 10<sup>8</sup> nucleated cells obtained from twice-washed cell suspensions prepared from the pooled spleens of a given donor group. The presence of infectious virus in these suspensions was also determined by inoculating 0.005-ml aliquots i.e. into groups of ICR mice.

Anti-<i>θ</i> Serum. The procedure used to both prepare and evaluate the specificity of the anti-<i>θ</i> serum used in these studies has been described previously (6, 14). The method of treating lymphoid cells with this reagent and guinea pig C is given in detail elsewhere (6).

Light Microscopy. Mice were perfused with isotonic saline followed by 1% acetic acid in 10% formalin. Brains were sectioned in paraffin at 8 μm in a parasagittal plane, and stained with hematoxylin and erythrosin.

Immunofluorescent Staining. The indirect method was used employing antiserum to LCM virus obtained from neonatally inoculated virus-carrier mice 2 wk after their receiving immune lymphoid cells as adults (7), and a fluoresceinated goat antimouse IgG (Meloy, Springfield, Va.).

**Results**

**Generation of Virus-Specific Cytotoxic Lymphocytes.** Spleen donors were groups of 3-4 BALB/c mice which were immunized i.p. with either 30 x 10<sup>3</sup> or 3 x 10<sup>3</sup> LD<sub>50</sub> of LCM virus and then sacrificed at different times after immunization. The kinetics of effector cell generation were essentially identical using either virus dose. The amounts of isotope released by uninfected targets after exposure to either normal or immune lymphocytes were the same.

As shown in Fig. 1, in the spleens of mice given 30 x 10<sup>3</sup> LD<sub>50</sub> of virus, specific cytolytic activity increased from a barely detectable level on day 4 postinfection to a maximum of 25-30% on day 8. For the next 2-3 days this effector activity remained relatively unchanged but, thereafter, it gradually decreased to a threshold level of about 3% by day 30 postinfection. As first suggested by our earlier but more limited studies (7), the lytic activity of immune lymphoid cells
obtained at any time during this period was abolished by treatment with anti-θ serum and C.

*Spleen Cell Division after LCM Virus Infection.* The kinetics of [125I]UDR uptake by spleen cells of BALB/c mice immunized with a single dose of LCM virus varied little from one experiment to the next (Fig. 2). A sharp, transient radioactive peak was regularly observed 7 days after immunization with either of the two virus doses employed. Correspondingly, the total number of nucleated cells in day 8 immune spleens had increased between two- and threefold when compared to cell numbers in spleens from mice given an appropriate dilution of normal brain.

Two interesting observations can be made from comparing the data in Figs. 1 and 2. First, maximal cell division in the spleens of infected mice preceded the time when peak cytoltyic activity in this organ was reached and, second, the observed decrease in cell division appeared to occur more rapidly than the subsequent fall in effector cell activity. Undoubtedly related to the decline in both isotope retention and effector cell activity was the disappearance of detectable infectious virus from the spleens of immunized animals, occurring regularly between the 8th and 9th days.
Although not shown in Fig. 2, this same pattern of DNA synthesis was also seen in the spleens of C3H × C57BL F1 mice, after primary LCM virus immunization. Furthermore, i.p. inoculations with virus rendered noninfectious either by heating at 56°C for 1 h or by a 15 min exposure to ultraviolet light results in levels of splenic DNA synthesis which were not significantly different from those obtained after the inoculation of diluted uninfected brain.

Production of Disease in CY-Induced Virus-Carrier Mice. In order to correlate the ability of immune lymphocytes to lyse cultures of virus-infected target cells in vitro with their ability to mediate fatal acute CNS disease in CY-induced virus carriers, groups of adult BALB/c donor mice were immunized i.p. with either a single dose or four weekly dose of $3 \times 10^3$ LD$_{50}$ of virus. At selected times after immunization, aliquots containing $100 \times 10^6$ viable nucleated pooled donor spleen cells were adoptively transferred to recipient virus-carrier mice and also assayed for lytic activity in vitro.

As shown in Table I, $100 \times 10^6$ nonimmune donor lymphocytes, or lymphocytes obtained between 6 and 10 days after primary immunization, were largely
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TABLE I

Production of Acute CNS Disease in CY-Induced LCM Virus-Carrier Mice with Adoptively Transferred Immune Splenic Lymphocytes*

| Donor lymphocytes | Cell dose (× 10⁶) | Cytotoxicity in vitro | Disease production |
|-------------------|------------------|----------------------|-------------------|
|                   |                  | %                    | D/T†             | Mortality % |
| Nonimmune         | 100              | 0                    | 0/15             | 0          |
| 1° + 6§           | 100              | 20 ± 3               | 1/6              | 17         |
|                   | 10               |                      | 0/6              | 0          |
| 1° + 8            | 100              | 28 ± 3               | 1/7              | 14         |
| 1° + 10           | 100              | 29 ± 3               | 1/6              | 17         |
|                   | 10               |                      | 0/6              | 0          |
| 1° + 18           | 100              | 13 ± 2               | 5/6              | 83         |
|                   | 10               |                      | 0/6              | 0          |
| 1° + 30           | 100              | 3 ± 1                | 6/6              | 100        |
| 4° + 6            | 100              | 12 ± 3               | 6/6              | 100        |
|                   | 10               |                      | 5/6              | 83         |
| 4° + 18           | 100              | 3 ± 2                | 6/6              | 100        |
|                   | 10               |                      | 5/6              | 83         |
| 4° + 18 (after anti-B+C) | 100       | 0/6                  | 0                |            |

* Adult BALB/c mice inoculated i.c. with virus, given CY 3 days later, and subsequently adoptively immunized with spleen cells from nonimmune or immune donors.
† Proportion of mice developing fatal CNS disease during a 14 day observation period. Mortality between 1° + 10 and 1° + 18 significant at P < 0.025 (χ², 5,32).
§ Spleen cells from immune donors were obtained 6, 8, 10, 18, and 30 days after one i.p. virus dose or 6 and 18 days after the last of four weekly doses. The percentages of specific cytotoxicity are the same as shown in Fig. 1.

ineffective in mediating fatal disease even though it was during this same period that the immune cells exhibited maximum cytotoxicity in vitro. All surviving mice remained virus carriers, as determined by immunofluorescent staining of their brains, and none showed any histologic evidence of choriomeningitis. However, at 18 and 30 days and even up to 90 days (not shown) when their cytotoxic capabilities were either diminishing or had disappeared, donor splenic lymphocytes were able to produce fatal choriomeningitis in almost all virus-carrier recipients.

The disease-producing ability of lymphocytes from mice given multiple injections of LCM was markedly greater than lymphocytes from singly immu-
nized animals and appeared to be independent of when the cells were obtained after the last immunizing virus injection. Transfers of either $100 \times 10^6$ or $10 \times 10^6$ lymphocytes produced fatal disease in practically all carrier mice and pretreatment of these cells with anti-θ serum and C ablated their ability to mediate disease. Of interest in these and similar experiments were the consistently lower levels of cytolytic activity in spleens obtained at various intervals after multiple immunization as compared with levels present at corresponding times after primary immunization.

Protection of Susceptible Mice From Lethal LCM. Since the cytolytically active spleen cell population generated during at least the first 10 days after primary immunization with LCM virus was unable to effect a change in the course of an established carrier infection, it was of interest to determine whether this population could prevent or alter the initiation of infection. Accordingly, spleen cells from nonimmune donors and those obtained at various times after primary infection were transferred i.p. to groups of normal syngeneic recipients at a dose containing $100 \times 10^6$ viable nucleated cells. After an 18–24 h interval, the recipients were challenged i.c. with 50–100 LD₅₀ of LCM virus and thereafter observed daily for signs of CNS disease over a 2 wk period.

As shown in Table II, cytolytically active donor spleen cell populations obtained only at 6 and 8 days postimmunization were able to significantly prevent the clinical expression of LCM. Cells obtained at 4 days and at 10 days conferred no protection to recipients despite the fact that the latter population exhibited a degree of cytolytic activity in vitro which was comparable to that of cells which did protect. The protective capacity of day 8 immune cells was

| Donor lymphocytes (100 × 10⁶) | Cytotoxicity in vitro | Protection | Mean survival times$§$ |
|-----------------------------|----------------------|------------|------------------------|
|                             | %                    | S/T‡       | Survival               |
| Nonimmune                   | 0                    | 0/30       | 0                      | 8.5 |
| 1° + 4                      | 3 ± 1                | 0/8        | 0                      | 8.0 |
| 1° + 6                      | 20 ± 3               | 9/15       | 60                     | 7.0 |
| 1° + 8                      | 28 ± 3               | 22/31      | 71                     | 7.0 |
| 1° + 8 (after anti-θ + C)   | 0                    | 29         | 22                     | 7.0 |
| 1° + 10                     | 29 ± 3               | 0/14       | 0                      | 8.0 |
| 1° + 30                     | 3 ± 1                | 0/15       | 0                      | 6.0 |

*Adult BALB/c mice challenged i.c. with LCM virus 24 h after adoptive immunization with nonimmune spleen cells or cells obtained from donors 4, 6, 8, 10, and 30 days after primary immunizing infection.

‡ Proportion of animals surviving at 14 days after virus challenge.

§ Mean number of days before death of nonprotected mice.
markedly reduced after treatment with anti-θ serum and C. In addition, lymphocytes obtained 30 days after primary immunization, and having barely detectable cytolytic activity, failed to prevent LCM and also hastened disease onset.

From additional observations (Table III), it appeared that protection against fatal disease required that there be a certain minimum interval between spleen cell transfer and virus challenge. Both day 6 and day 8 immune lymphocytes failed to significantly protect recipients when given 1 h before challenge.

More direct evidence bearing on the mechanism by which day 8 immune cells protect mice against LCM was obtained by histologically examining brains from

**Table III**

*Protective Ability of Immune Lymphoid Cells Adoptively Transferred at 1 or 24 h before Virus Challenge*

| Donor lymphocytes (100 x 10⁶) | Time of challenge | Protection S/T† | Survival |
|-----------------------------|-------------------|-----------------|----------|
|                             | h                 |                 |          |
| 1° + 6                      | 1                 | 0/5             | 0        |
|                             | 24                | 4/5             | 80       |
| 1° + 8                      | 1                 | 1/14            | 7        |
|                             | 24                | 5/5             | 100      |
| 1° + 10                     | 1                 | 0/5             | 0        |
|                             | 24                | 0/5             | 0        |
| Nonimmune                   | 1                 | 0/5             | 0        |
|                             | 24                | 0/5             | 0        |

* Adult BALB/c mice challenged i.e. with LCM virus either 1 or 24 h after adoptive immunization with spleen cells from immune or nonimmune donors.
† Proportion of animals surviving at 14 days after virus challenge.

these animals as well as measuring their virus content. Two groups of 14 mice were each given either nonimmune or day 8 immune spleen cells followed by i.e. virus challenge (50–100 LD₅₀) 24 h later. On the 6th day of infection, when virus levels in the brains of nonimmunes were maximal (1), a comparison of individual brain virus titers in five mice from each group revealed approximately 100-fold less virus in the brains of animals given immune cells (Table IV). Although histological choriomeningitis was present in all animals, it was generally less severe in the group which had received immune cells. After 6 days, 100% of mice which were recipients of nonimmune lymphocytes before virus challenge had already developed the typical convulsive diathesis preceding fatal LCM, whereas, only about 30% of immune spleen cell recipients showed some overt evidence of mild disease. Brains from surviving animals in this latter group were free of infectious virus.

It should be noted that no significant contribution to the protective capacity of
LYMPHOCYTIC CHORIOMENINGITIS VIRUS-SPECIFIC T LYMPHOCYTES

TABLE IV
Reduction of Brain Virus Titers Mediated by day 8 Immune Splenic Lymphocytes*

| Donor lymphocytes (100 x 10^8) | Brain virus titers | Protection |
|-------------------------------|-------------------|------------|
|                               | (mean = 3.0)      | S/T § Survival |
| 1° + 8                        | 2.0, 2.3, 2.4, 3.6, 4.8 | 6/9        | 67 |
| Nonimmune                     | 4.5, 5.0, 5.1, 5.3, 5.5 (mean = 5.1) | 0/9        | 0 |

* Adult BALB/c mice challenged i.c. with LCM virus 24 h after adoptive immunization with spleen cells from day 8 immune or nonimmune donors.

§ Log_{10} LD_{50}/0.03 g brain (mice killed 6 days after challenge).

Prevention of Virus-Carrier State Induction in AT x BM Mice. The i.c. inoculation of LCM virus into adult mice which have been depleted of T lymphocytes by neonatal thymectomy (15) or by repeated administration of heterologous antilymphoid sera (16), usually produces neither clinical nor histological evidence of LCM but, instead, leads to the establishment of the chronic virus-carrier state. Similarly, congenitally athymic mice (G. A. Cole and R. A. Prendergast, unpublished observations) or mice thymectomized as adults, then lethally irradiated and reconstituted with syngeneic bone marrow (17), almost invariably develop persistent infections after i.c. virus challenge.

In order to assess the ability of day 8 immune donor lymphocytes to prevent or abort the initiation of a persistent LCM virus infection in recipients possessing little or no endogenous T lymphocytes, groups of C3H x C57BL F1 AT x BM mice were injected i.p. with either 100 x 10^6 normal or immune splenic lymphocytes 24 h before i.c. challenge with 50-100 LD_{50} of LCM virus. For comparison, AT x BM mice given no lymphoid cells and their unmanipulated normal littermates were also challenged. During the following 18 days, all mice developing convulsive disease were killed and their brains examined both histologically and by immunofluorescent staining. At the end of this period the remaining mice were treated in the same fashion. The various outcomes are presented in Table V.

As anticipated, AT x BM mice given virus not preceded by lymphoid cells all developed an asymptomatic carrier infection with immunofluorescent viral antigen readily visualized in their neural membranes, and with a total lack of inflammatory cells at these sites. The administration of nonimmune donor lymphocyte before virus challenge led to the production of clinical disease in
100% of mice between 8 and 10 days postinfection. Brains from all such animals showed convincing microscopic evidence of mild to moderate choriomeningitis as well as viral persistence. In contrast, none of the AT × BM mice given virus preceded by day 8 immune donor lymphocytes developed overt disease nor was immunofluorescent viral antigen detectable in neural membranes. However, histologically, all presented with a mild choriomeningitis indicating that the transferred immune cells were not only able to mediate some degree of immunopathology but that they were also capable of preventing the establishment of the virus-carrier state, apparently without the cooperative benefit of recipient T lymphocytes. All normal littermates developed clinical LCM on the 7th or 8th day postinfection accompanied by a characteristic moderate to severe choriomeningitis and virus-specific immunofluorescence in cells of the meninges and choroid plexus.

**Discussion**

The present data have confirmed our earlier evidence (9, 10) for the existence of at least two different virus-specific T-lymphocyte subsets generated at different times in the spleens of mice after immunizing infections with LCM virus. These subsets are identified by differences in their functional capabilities both in vitro and in vivo. Lymphocyte populations obtained between 6 and 8 days postinfection possess high levels of cytolytic activity as measured in vitro and, when transferred to syngeneic nonimmune normal and AT × BM mice prevent, respectively, the development of acute lethal CNS disease and the establishment of the virus-carrier state when these animals are challenged i.c. with LCM virus 18–24 h later. In both situations, CNS infection is transient and virus appears to be cleared as the direct result of the transferred cells. However, these cells will elicit neither clinical nor histological evidence of immune-mediated CNS disease in CY-induced virus-carrier mice. Lymphocytes obtained 18 days after primary infection, at a time when cytolytic activity is diminished, or at 30 days when it is practically nil, have no protective effect but, instead, actually
hasten disease onset when administered to normal mice before virus challenge. Moreover, these cells regularly produce fatal LCM when transferred to virus-carrier mice.

The generation of each of these functionally different T-lymphocyte populations, hereafter referred to as either effector or memory subsets, bears a definite temporal relationship to the periods after immunizing infection when virus initially replicates in visceral tissues and then is subsequently cleared. Thus, effector activity reaches maximal levels 1 or 2 days before infectious virus in the spleen becomes undetectable, after which it gradually diminishes. In contrast, the development of a virus-specific memory subset, as defined by its disease-producing potential in virus carriers, occurs well after virus clearance and then persists for months.

An important observation made during these studies is that the protective role played by day 6 or day 8 immune lymphocytes cannot be explained simply on the basis of their effector activity as measured in vitro. Although these cells and day 10 cells have similar levels of activity, only the former are able to prevent the establishment of CNS infection. What appears to be the determining factor is whether or not the effector subset is obtained during the time when viral antigen-induced DNA synthesis is taking place in donor spleens. It could be argued, therefore, that under the conditions of our experiments, it is a cell population comprised of the immediate precursors of the effector subset which, in vivo, actually mediates both the inflammation of neural membranes and the elimination of virus. The failure of day 10 effector lymphocytes to perform similarly may be due to their lack of this proliferative component, thereby restricting their capacity to expand with sufficient rapidity to engage in the elimination of an increasing number of virus-infected cells. Alternatively, differences in the in vivo migratory patterns between effector lymphocytes and their progenitors may influence their respective efficiencies in homing to sites of antigen (18) which, in this study, is represented by virus in the CNS. Zinkernagel and Doherty (19) have shown that LCM virus-specific effector T lymphocytes accumulate in the cerebrospinal fluid of lethally infected mice just before death. However, it is not clear as to whether the majority of these cells arise after differentiation locally or whether they migrate to the CNS after being generated in distant lymphoid tissues.

The inability of day 6 and day 8 immune lymphocytes to protect normal mice if administered 1 h before virus challenge (Table III) suggests that the donated cells require some critical period of time to reach either relevant CNS sites or peripheral lymphoid tissues where cooperation with endogenous lymphoid elements may occur. If some early cooperative event is indeed necessary for the expression of donor cell protective function, it apparently does not require the participation of recipient T lymphocytes since protection occurs in AT × BM mice. The same can be said for the ability of a memory subset to elicit fatal choriomeningitis in virus-carrier AT × BM mice (G. A. Cole and R. A. Prendergast, unpublished observations). The question still remains as to which cell type(s) mediates cytopathology and virus clearance in vivo. However, it is becoming increasingly evident from several recent in vitro studies (20, 21) that although the lysis of cultured virus-infected target cells can be attributed solely to T lymphocytes, the elimination of virus from the cultures is macrophage dependent. Furthermore, careful electron micro-
scopic examination of brains from mice (F. A. Murphy, personal communication) and rats during acute LCM virus-induced CNS disease has revealed little if any direct interaction between virus-containing cells and lymphocytes although macrophages are abundant. We would therefore favor the notion that, while the hypothetical "kiss of death" by the effector lymphocyte may be a reality in vivo, the resolution of the infectious process is accomplished by recruited macrophages. This position is defended in greater detail by Blanden in a recent review (22).

Our data do not permit any conclusive statement as to whether or not the early appearing proliferating cell population derives from the same pool of antigen-reactive precursors which eventually differentiate into either the effector or the memory subset. Obviously, the precursors of both subsets must arise during a circumscribed period of viral replication. Since the full expression of memory probably occurs about a week after peak effector activity is reached, it is tempting to speculate that the development of memory begins when virus falls to levels insufficient to drive a common antigen-reactive precursor to its more (and perhaps final) differentiated state, i.e., the effector lymphocyte. Supporting this idea is the observation that, in comparison with primary immunizing LCM virus infection, reinfection leads to a marked restriction in viral replication (5) and to reduced and more transient levels of effector activity (Table I). Nevertheless, spleens from reinfected donor mice show an increased memory potential as reflected by the fewer number of cells required to elicit CNS disease in virus carriers.

We have emphasized that the various outcomes of LCM virus infections can be explained on the basis of the kinetic relationships between viral replication and immune induction (23). The present study supports this view. The protection afforded a normal mouse by a proliferating effector subset does not come from preventing the initiation of infection in its CNS but is, instead, the result of a more efficient elimination of virus and/or infected cells after infection is established. In comparison, the lack of protection by a memory subset could be attributed to its less differentiated state and therefore a longer period would be required for the development of effector activity. The accelerated death resulting from the transfer of a memory subset to normal mice may actually represent the "too little-too late" expression of this activity.

A totally different set of conditions exists in virus-carrier recipients. These animals are given immune donor lymphocytes at a time when virus in the CNS has already reached maximal levels, and the infection has spread to extraneural tissues resulting in a persistent viremia (1). Under these circumstances, it is not surprising that only a memory subset, with its inherently greater potential for proliferation and differentiation, can alter the course of infection, especially since these cells, or their progeny, must travel a virus-laden course to the CNS.

Some of the findings presented in this report are supported in related studies by other workers in this field who used different strains of mice and/or virus. Thus, a carefully documented study by Hannover Larsen (24) provided strong evidence for the increasing ability of donor lymphoid cells, obtained at successive intervals after primary LCM virus infection, to permanently reduce viremia when transferred to syngeneic C3H adult mice with carrier states induced by neonatal infection. For reasons discussed elsewhere (3, 23), these animals, unlike CY-induced carrier mice, are susceptible to the CNS disease-producing capacity of immune lymphocytes so that reduction of circulating virus probably

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2 Del Cerro, M. and A. A. Monjan. Manuscript in preparation.
represents the analogue of immune-mediated CNS disease occurring at extraneural tissue sites.

Using CBA mice, a significant antiviral effect was noted by Mims and Blanden (25) when animals were transfused with day 8 immune donor spleen cells 48 h after an i.c. or i.p. challenge with the viscerotropic WE3 strain of LCM virus. Under these conditions, no protective effect was demonstrated but virus titers in the liver and spleen were reduced about 100-fold 24 h after cell transfer when compared to titers in these organs from recipients given nonimmune cells. The antiviral effect was almost completely eliminated if the immune cells were pretreated with anti-θ serum. Interestingly, levels of virus in the brain were unaffected and it is likely that the donated cells were unable to traverse a gradient of viral antigen favoring extraneural tissues. The transfer of lymphocytes obtained from donors 95 days after primary infection resulted in no diminution of virus, suggesting that some differentiating step may have been required before these cells could exert any antiviral activity.

In basic agreement with our results are those of a recent study by Volkert et al. (26) who showed that a radioresistant, cytolytically active "early" immune cell population, obtained from C3H donors 9 days after infection with another viscerotropic virus strain (Traub), protected normal mice from fatal LCM if transferred at the time of virus challenge or 2 days after infection. In confirmation of Hannover Larsen's findings (24), these same cells had little ability to affect the viremia present in adult mice with carrier infections initiated shortly after birth. "Late" immune cells, obtained 30 days postimmunization, were not protective but were capable of reducing viremia in carrier mice. This capability was considerably reduced if the cells were X irradiated or treated with anti-θ serum and C before transfer. The demonstrable protective effect of "early" immune cells given as late as 2 days postinfection, and the relative resistance of these cells to X irradiation in vitro are observations which appear to be at variance with our interpretation that the conditions for protection require the transfer of a proliferating effector subset before the initiation of infection. However, the lymphocyte populations used in these investigations consisted of both spleen and lymph node cells obtained from donors immunized with a strain of virus which persists for long periods in visceral tissues (27). These variables and possibly others based on genetically controlled differences in the immunologic reactivity of different mouse strains to LCM virus (28) may have influenced a particular experimental result.

Employing somewhat different experimental conditions, Doherty and Zinkernagel (29) have recently demonstrated that cytolytically active splenic T lymphocytes obtained between 7 and 9 days postinfection of donors with the WE3 strain of LCM virus could dramatically shorten the interval to fatal CNS disease in recipient mice previously inoculated i.c. with the neurotropic Armstrong strain and immunosuppressed with CY. The donor cells were transfused at a time when the infection was well established in the neural membranes of recipients but before any extensive invasion of their extraneural tissues. Under these circumstances the recipients developed a precocious LCM which appeared as early as 3 days after immune cell transfer, in striking comparison to the 8- to 10-day period preceding the death of animals which received nonimmune cells. Lending further support to our findings, these investigators found that the transfer of donor spleen cells taken 11 and 13 days after infection gave the same results as nonimmune cells.

Collectively, the experimental results discussed above illustrate a marked functional heterogeneity of virus-specific T-lymphocyte subsets generated at different times after LCM virus infection. The precise nature of their interaction with infected cells in vivo, leading to viral clearance and/or immunopathology, is still unclear. It appears likely that both of these outcomes are mediated by an
Summary
At varying intervals after immunizing infections of adult BALB/c mice with lymphocytic choriomeningitis (LCM) virus, splenic lymphocytes were tested for their ability to either elicit acute LCM or protect against lethal intracerebral LCM virus challenge when transferred to syngeneic recipients that were, respectively, virus carriers induced by cyclophosphamide-induced immunosuppression and normal susceptible mice. These lymphocytes were also assayed for their capacity to lyse, in vitro, LCM virus-infected BALB 3T3 cells labeled with ⁵¹Cr.

Only lymphocytes obtained from donor mice between 6 and 8 days postimmunization significantly protected normal recipients from the development of lethal central nervous system (CNS) disease when transferred 18-24 h before virus challenge. At 10 days they were not protective even though their cytolytic activity in vitro either exceeded or approximated that of protective lymphocytes. The capacity to protect more closely corresponded with a period of virus-induced DNA synthesis in donor spleens as measured by the incorporation of [5-³²P]2'-deoxyuridine. However, none of these cytolytically active lymphocyte populations were effective in mediating acute CNS disease when transferred to virus-carrier mice. In contrast, lymphocytes obtained 18 days or later after primary immunization, although having no protective capacity and exhibiting minimal cytolytic activity in vitro, were able to regularly produce acute disease in virus-carrier mice. The ablation by anti-θ serum treatment of these in vivo and in vitro virus-specific immune functions established that the relevant lymphoid cells were T lymphocytes.

Similarly, reconstitution of C3H × C57BL F₁ adult mice, depleted of lymphocytes by thymectomy and lethal irradiation, with syngeneic day 8 immune donor lymphocytes rendered them resistant to the development of the carrier state after intracerebral virus challenge; reconstitution of such animals with nonimmune lymphocytes restored their ability to develop typical LCM.

Collectively, these data indicate that immunogenic stimulation with LCM virus leads to the development of at least two different virus-specific T-lymphocyte subsets: (a) an early appearing transient effector population of cells and, perhaps, their immediate precursors whose generation requires the presence of virus, and (b) a stable memory population that appears well after virus clearance and which has little or no cytolytic activity. The possible mechanism by which these functionally different subsets can influence the outcome of acute or chronic LCM virus infections is discussed.

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880 LYMPHOCYTIC CHORIOMENINGITIS VIRUS-SPECIFIC T LYMPHOCYTES

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