Recovery from Experimental Rabies by Adoptive Transfer of Immune Cells

By BELLUR S. PRABHAKAR,1† HARVEY R. FISCHMAN1 AND NEAL NATHANSON2*

1Department of Epidemiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205, and 2Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, U.S.A.

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SUMMARY

The transient, sublethal infection produced by intracerebral inoculation of the Flury high egg passage (HEP) strain of rabies virus into adult mice was converted into a lethal one (approx. 80 to 100% mortality) by administering 150 mg/kg cyclophosphamide (CY) 2 days after infection. Immunosuppressed, infected animals showed no immunological response to rabies and died 15 to 20 days after infection. However, mortality was reduced to 12% when suppressed mice were adoptively immunized, 4 days after infection, with an intravenous injection of 60 x 10⁶ spleen cells from rabies-immune syngeneic donors. The lymphocytes obtained early after donor immunization (4 to 11 days) reduced mortality, whereas those obtained late (16 to 32 days after immunization) were not effective. The ability of donor cells to protect animals corresponded very closely with donor cytotoxic T lymphocyte (CTL) activity. Within 4 days after immune cell transfer, serum neutralizing antibody and CTL levels in recipients were comparable to those found in virus-infected control animals.

Immune donor cells were fractionated into thymus-derived (T-enriched) and bone marrow-derived (B-enriched) subsets. The T and B subsets reduced mortality to 32% and 34% respectively. CTL and serum neutralizing antibody responses could be detected in these animals, although they appeared later than in mice treated with unfractionated immune spleen cells. The present study demonstrates that both B and T lymphocytes are required for optimum clearance of rabies from the central nervous system (CNS) and suggests a functional role for rabies-specific CTL in vivo.

INTRODUCTION

Rabies infection is unique in that the virus replicates almost exclusively in the nervous system in the absence of a generalized viraemic phase. Although rabies infection is usually lethal, certain attenuated strains cause a sublethal infection and provide an excellent model to study the role of the immune response in recovery from a virus infection confined to the central nervous system (CNS).

The Flury high egg passage (HEP) strain of attenuated rabies virus is lethal when inoculated by the intracerebral (i.c.) route into suckling mice. However, even at large doses, HEP causes a sublethal, inapparent infection in adult mice (Koprowski et al., 1954) and virus is usually cleared by about 10 days. Recovery of adult mice is an immunological process,

† Present address: Laboratory of Oral Medicine, National Institute of Dental Research, Bethesda, Maryland 20205, U.S.A.

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since immunosuppression induced either by cyclophosphamide (CY) (Fischman & Strandberg, 1973) or by X-irradiation (Kaplan et al., 1975), results in prolonged and enhanced virus replication and death of the majority of animals. Selective depletion of B cells by treatment with anti-μ serum (Miller et al., 1977) or T cells, using AT-X-BM mice (Kaplan et al., 1975) also enhances the susceptibility of mice to HEP infection. However, the mortality rate in these partially immunodeficient animals is not as high as in animals that are completely immunosuppressed.

In this study, we have used the approach of adoptive cell transfer in order to evaluate the functional participation of bone marrow-derived (B) and thymus-derived (T) lymphocytes in recovery from experimental rabies. This approach provides an opportunity to choose various types of lymphocytes for transfer and to manipulate the numbers and sources of cells. Furthermore, both humoral and cell-mediated responses can be quantified by antibody titration and by determination of in vitro cytotoxic T lymphocyte (CTL) activity both before and after adoptive cell transfer.

METHODS

Animals. Female Balb/c and outbred Swiss mice, 6 to 8 weeks of age, were obtained from the Institute for Cancer Research, Philadelphia, Pa., U.S.A.

Virus. (i) The Flury HEP strain of rabies virus was prepared by inoculating 7-day-old embryonated hens' eggs via yolk-sac, and harvesting virus-infected embryos 10 days later. Stock virus was obtained from a clarified 20% suspension of emulsified, infected embryo heads in normal saline; the titre in suckling Swiss mice was $10^6.7$ i.e. LD$_{50}$/g tissue. A standard dose of 5000 i.e. LD$_{50}$ was used in pathogenesis experiments. (ii) HEP-BHK virus was prepared by serial passage of Flury HEP virus in BHK-21 cells (Sokol et al., 1968) and contained $10^8$ p.f.u./ml. Donor mice were immunized by i.c. injection of 5000 p.f.u. (iii) The ERA strain of rabies virus (Abelseth, 1964) was plaque-purified and propagated in BHK-21 cells (Kuwert et al., 1969). The titre of the stock virus was $10^8$ p.f.u./ml.

Immunosuppression. Mice were injected by the intraperitoneal (i.p.) route with CY (Mead Johnson, Evansville, Ind., U.S.A.) at a dose of 150 mg/kg body weight.

Virus isolation from the recipients. BHK-21 cells were inoculated with 0.1 ml of brain suspension, incubated for 3 days, trypsinized and transferred to eight-chambered slides (Lab-Tek) and further incubated for 24 h. Acetone-fixed cells or brain smears were stained with fluorescein-conjugated rabbit anti-rabies nucleocapsid antibody (Kissling, 1975).

Virus-neutralization test. The standard fluorescent focus-inhibition test (Smith et al., 1973) was used to measure neutralizing antibody titre.

In vitro cytotoxicity assay. A neuroblastoma cell line derived from A/J (H-2a) mice (Miller & Levine, 1974) was used for target cells. These cells were infected with ERA rabies virus at an input of 10 p.f.u./cell and incubated for 24 h at 37 °C. The labelling of cells and the CTL assay were carried out using procedures similar to those described by Wiktor et al. (1977a, b). An effector-to-target ratio of 100:1 was used, i.e. $2 \times 10^6$ effector cells and $2 \times 10^4$ target cells per well.

Results were calculated as percentage specific $^{51}$Cr release (Doherty & Zinkernagel 1976) using the formula:

$$\text{% Lysis} = \frac{I_i - N_i}{T_i - N_i} \times 100$$

where $I = $ immune spleen cells, $N = $ normal spleen cells, $T = $ total input ct/min and $i = $ ct/min associated with or released from virus-infected target cells. Similar calculations were made with control (uninfected) target cells, in which the $^{51}$Cr release was always less than 10%.
It seems clear that this assay detected cytolytic T lymphocytes (CTL) rather than natural killer (NK) cells or antibody-dependent cell-mediated cytotoxicity (ADCC) since (i) normal spleen cells failed to lyse infected targets; (ii) addition of antiviral antibody did not result in killing by normal spleen cells; (iii) the use of immune spleen cells from recombinant haplotype donors indicated restriction at K and D but not the I region of the H-2 complex (data not shown).

Adoptive transfer of donor cells. Donors were prepared by i.c. infection with HEP-BHK virus. Animals were sacrificed at intervals after infection and spleen cells were recovered. Unless otherwise specified $60 \times 10^6$ unfractionated T or B cells were obtained 7 days after donor immunization and injected intravenously (i.v.) into immunosuppressed recipients infected 4 days earlier.

Treatment of lymphocytes with anti-0 serum and complement. The splenic lymphocytes were suspended in anti-0 antibody (1/20000 final dilution) at a concentration of $20 \times 10^6$ cells/0.1 ml and incubated for 30 min at 4 °C. After washing once to remove the excess anti-0 antibody, an equal volume of fresh guinea-pig complement was added, and the suspension incubated at 37 °C for 45 min. Viability was determined by the trypan blue dye exclusion method.

Separation of T and B lymphocytes. T and B lymphocytes were separated by passage through nylon wool according to the method of Julius et al. (1973). Spleen cells were adsorbed to the column for 1 h at 37 °C and the non-adherent cells were obtained by washing the column with warm (37 °C) phosphate-buffered saline (PBS) containing 5% foetal calf serum (FCS). The adherent cells were eluted by flushing the column with cold PBS (4 °C) containing 5% FCS and repeatedly teasing and washing the nylon wool.

In the adoptive transfer experiments, T cells (non-adherent cells) were used without any further treatment, whereas B cells (adherent cells) were further treated with anti-0 antibody and complement to remove contaminating T cells. The T cell content of the T- and B-enriched populations was evaluated using anti-0 serum and guinea-pig complement. The T-enriched population contained more than 85% T cells, whereas the B-enriched population was essentially free of T cells.

RESULTS

Potentiation of HEP virus infection

Adult Balb/c mice inoculated intracerebrally with 5000 suckling mouse i.c. LD$_{50}$ of HEP virus had a totally inapparent infection. Administration of CY at a dose of 150 mg/kg, 2 days after virus infection, resulted in over 80% mortality (Table 1).

Sequential virus isolations from the brain, serum neutralizing antibody titres, and spleen CTL responses are shown in Fig. 1. In non-immunosuppressed mice, virus was cleared by day 8, while neutralizing antibody appeared on day 6, reaching a titre of 640 by day 12; CTL appeared on day 4 and peaked on day 6 to 8. In immunosuppressed mice, virus in the brain persisted until the time of death (day 15 to 20); both antibody and CTL failed to appear.

Adoptive transfer of protection with syngeneic immune spleen cells

When $60 \times 10^6$ immune cells were used to reconstitute HEP-infected recipient mice that were immunosuppressed, mortality was reduced from over 80% to 31 to 41%. Immune cells were effective in reducing mortality only when obtained 4 to 11 days after donor immunization; immune cells from donors sacrificed 16 to 32 days after infection had no protective effect, nor did normal spleen cells (Table 1). No significant difference was found in the survival time of animals that were untreated and treated unsuccessfully.
Table 1. Protection against rabies virus infection in immunosuppressed mice by syngeneic immune spleen cells*

<table>
<thead>
<tr>
<th>Virus</th>
<th>CY</th>
<th>Donor spleen cells</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>0/35 0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>30/36 83</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Normal cells</td>
<td>16/16 100</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Immune cells</td>
<td></td>
</tr>
<tr>
<td>4–5†</td>
<td></td>
<td></td>
<td>6/15 40</td>
</tr>
<tr>
<td>7–8†</td>
<td></td>
<td></td>
<td>5/16 31</td>
</tr>
<tr>
<td>9–11†</td>
<td></td>
<td></td>
<td>9/22 41</td>
</tr>
<tr>
<td>16–32†</td>
<td></td>
<td></td>
<td>18/20 90</td>
</tr>
</tbody>
</table>

*Balb/c mice were infected intracerebrally with HEP virus (5000 suckling mouse i.c. LD₅₀) and 2 days later were immunosuppressed with CY (150 mg/kg). Four days after infection, mice were injected intravenously with 60 × 10⁶ spleen cells. These animals were under observation for 3 months.
†Days between immunization and sacrifice of donors.

Adoptive immunization with fractionated immune spleen cells

Immune spleen cells obtained 7 days after donor immunization were fractionated into B and T cell-enriched subpopulations, and the protective effects of unfractionated and fractionated cells were compared (Table 2). T and B cells reduced mortality to 32% and 34% respectively, whereas unfractionated cells reduced mortality in these experiments to 12%.

The kinetics of infection in mice reconstituted with fractionated and unfractionated cells is represented in Fig. 2. Unfractionated cells reconstituted a rabies-specific immune response, but the onset was delayed by 3 to 4 days compared with non-suppressed infected mice (Fig.
Recovery from experimental rabies

Table 2. The effect of immune spleen cell transfer on rabies infection in immunosuppressed Balb/c mice*

<table>
<thead>
<tr>
<th>Virus</th>
<th>CY</th>
<th>Immune spleen cells</th>
<th>Mortality†</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>0/38 0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>64/78 82</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>Unfractionated cells</td>
<td>5/43 12</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>T lymphocytes</td>
<td>16/50 32</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>B lymphocytes</td>
<td>20/59 34</td>
</tr>
</tbody>
</table>

* All groups were infected with HEP virus and immunosuppressed with CY 2 days later. Unfractionated or fractionated cells obtained from (50 × 10⁶) immune spleen cells were given to recipients 4 days after infection. These animals were under observation for 3 months.
† Data are pooled from three similar experiments.

Fig. 2. Effects of adoptive immunization upon the course of HEP rabies virus infection in immunosuppressed mice. Comparison of reconstitution of rabies-specific immune responses in recipients treated with (a) unfractionated, (b) T-enriched and (c) B-enriched immune spleen cells. +, Presence of virus in brain; −, absence of virus in brain; ●, cytotoxic T lymphocyte activity; ×, antibody. Three mice were used for each data point. CY (150 mg/kg) was injected intraperitoneally on day 2 and spleen cells (60 × 10⁶) were injected intravenously on day 4.

1). The disappearance of virus antigen in immunosuppressed mice was also delayed several days.

Animals reconstituted with T cells (Fig. 2) generated a CTL response as effectively as did recipients of unfractionated cells. Although production of antibody was somewhat slower in T
cell recipients, virus was cleared by day 12 in both groups. B cells were least effective in reconstituting the immune response and virus was cleared only by day 15 (Fig. 2).

**Discussion**

This study is the first report of recovery from rabies infection of the CNS by adoptive transfer of immune lymphoid cells. Manipulation of the transfer system provides several lines of evidence to suggest that both antibody and rabies-specific CTL may be operative in virus clearance.

The time interval between immunization and collection of spleen cells from the donor was very critical for a successful adoptive transfer of protection. As has been noted in other experimental models of virus infection such as lymphocytic choriomeningitis (Johnson & Cole, 1975) and ectromelia (Gardner et al., 1974), the lymphocytes obtained from donors 4 to 11 days after their immunization were most effective. Furthermore, the protective ability of the transferred cells correlated closely with their ability to lyse infected target cells *in vitro*, suggesting a functional role for T cells.

The importance of T lymphocytes is also supported by previous studies using T cell-deficient mice. Turner (1976) has shown that the nude mice (nu/nu) were not readily immunized by inactivated HEP rabies virus, whereas their normal littermates (nu/+ ) were. Adult-thymectomized X-irradiated bone marrow-reconstituted (AT-X-BM) mice, or adult mice treated with anti-thymocyte serum have also been shown to be susceptible to HEP virus infection (Kaplan et al., 1975).

It is likely that B lymphocytes are also involved in clearance of rabies virus from the CNS. This assumption is based on an earlier study (Miller et al., 1977) in which mice depleted of B cells by treatment with anti-μ antiserum exhibited delayed virus clearance. In the present study, the transfer of B lymphocytes was found to confer protection comparable to that provided by T cells. However, the appearance of CTL activity in B cell-reconstituted mice precluded any definite conclusions about the role of lymphocytes. The late development of CTL activity in these mice probably represents recovery from the single dose of CY (Turner, 1979).

In conclusion, optimum clearance of attenuated HEP rabies virus from the CNS probably requires the participation of both T and B cell virus-specific effector mechanisms. To participate in recovery, both cell types must migrate into the CNS. Based on the available information, the destruction of virus-infected target cells would involve cytotoxic T lymphocytes or complement in the presence of antibody. Released virus would be neutralized by the antiviral antibody and eventually cleared by the phagocytic cells. The kinetics and mechanisms by which virus-specific lymphocytes migrate into the CNS are poorly understood and require further study.

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**References**


Recovery from experimental rabies


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