INFECTION AND IMMUNITY, Nov. 1972, p. 695-698

Vol. 6, No. 5
Printed in U.S.A.

Antiviral Action of Immune Lymphocytes in Mice Infected with Lymphocytic Choriomeningitis Virus

C. A. MIMS[1] AND R. V. BLANDEN

Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia

Received for publication 31 July 1972

Spleen cells from immunized mice were shown to have a strong antiviral action after transfusion into infected recipients. Spleen, liver, and lung titers in recipients were reduced within 24 hr, but there was no detectable effect on brain titer. Spleen cells were active if taken 6 days after infection of donors, when no antibody was detectable. Spleen cell activity was diminished, but by no means abolished, by treatment with potent anti-theta antibody. Immune spleen cells transferred to mice infected 3 days earlier induced early signs of sickness but no change in average survival time. Normal mice injected intracerebrally with a mixture of immune spleen cells plus virus showed unusually early illness and death.

It has long been recognized that it is the immune response that is responsible for pathological lesions and death in adult mice infected with lymphocytic choriomeningitis (LCM) virus, the virus infection itself being nonpathogenic for mouse cells and tissues (5, 7). The relative importance of humoral and cell-mediated immunity in this classical example of viral immunopathology is still in question; results in vivo have often been difficult to interpret, and both antibody and sensitized cells have been shown to be cytotoxic for infected cells in vitro (8, 11; M. B. A. Oldstone, K. Habel, and F. J. Dixon, Fed. Proc., **28**: 429, 1969).

Immunopathological considerations have overshadowed the possible antiviral ("useful") role of the immune response in LCM virus infection. The present study was initiated after Blanden had clearly shown that lymphocytes have an important antiviral function in mice infected with ectromelia virus (1-3).

MATERIALS AND METHODS

Mice. Adult 8-week-old mice of the CBA strain were used in most experiments. Adult mice of the multicolored outbred Walter and Eliza Hall Institute (WEHI) strain were used in some experiments.

Virus. The WE_3 strain of LCM virus was used as the superantant fraction of a lung suspension obtained from guinea pigs 6 days after subcutaneous infection. The virus stock contained $10^{8.5}$ plaque-forming units (PFU)/ml. The Armstrong mouse-adapted strain of LCM virus was maintained by mouse intracerebral passage and was assayed intracerebrally in WEHI mice.

Antiserum to LCM virus. After primary footpad infection with LCM virus, mice were given a subsequent intravenous or intraperitoneal injection of virus and were bled 7 days later. The pooled serum was not heat-inactivated before use; it neutralized at 0.5 but not at 0.1 dilutions (see below).

LCM virus neutralizing antibody tests. A sensitive in vivo technique was used, involving the inhibition of primary footpad swelling in mice (6). Fresh serum was added to an equal volume of diluent containing virus, and after incubation at 37 C for 0.75 hr, 0.05 ml (containing 50 PFU virus in controls) was injected into the right, hind footpads of groups of five mice. A control injection of normal mouse serum plus virus was made into the left, hind footpad. Footpad swelling was determined with dial-gauge callipers (H. C. Kröplin, Schluchtern Hessen, Germany) 24 hr later, and readings in test and control feet were compared.

Titrations. Plaque assays were made on BHK21 cell monolayers. After infection of monolayers in 60-mm diameter Falcon plastic petri dishes, a 5.0-ml overlay (1% calf serum in Eagle salt solution plus 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 0.1% diethylaminoethyl [DEAE]-dextran, 0.1% bovine serum albumin [BSA], antibiotics, and tris-(hydroxymethyl)aminomethane [Tris] buffer) was added. Plates were incubated at 36 C in a humidified, but not gassed, incubator. A further 0.5-ml overlay was added on day 3 or 4, a neutral-red staining overlay was added on day 6, and plaques were counted on day 7. Plaques were not always easy to see, but reproducible counts could usually be made after observation against a suitable light background. Organ homogenates were made in ice-cold 0.5% gelatin saline by a method described previously (1). Organs from individual mice were homogenized and

[1] Present address: Department of Microbiology, Guy's Hospital Medical School, London Bridge, London, S.E.I. England.

assayed separately: mean log titers, standard error of the mean, and values of t and P were calculated. Titers are expressed as $\log_{10}$ PFU/whole organ.

Spleen cell suspensions. Spleens were passed through stainless-steel sieves into cold Pucks A saline containing 1% calf serum. After dissociation of clumps by pipetting, suspensions were filtered once through tightly packed cottonwool columns (3 by 0.5 cm), washed three times in Pucks A saline, and suspended for injection in Pucks A saline containing 1% calf serum and 5 IU of heparin/ml. Cell viability was determined by 0.05% trypan blue exclusion and was usually 70 to 85%.

Anti-theta ascitic fluid. Ascitic fluid from AKR mice immunized with CBA mouse thymocytes was used. It was prepared, tested, and used exactly as described by R. V. Blanden and R. E. Langman (Scand. J. Immunol., *in press*). Cells were not exposed to complement after anti-theta treatment.

RESULTS

Transfer of antiviral activity by immune spleen cells. Spleen cell suspensions were made from normal CBA mice and from CBA mice 8 to 9 days after intravenous infection with 5,000 PFU of WE_3 strain LCM virus; 2×10^7 to 2×10^8 viable cells were injected intravenously to recipients. Cell recipients had been infected intravenously or intracerebrally with 10^5 PFU of virus 48 hr earlier. Tissues for virus assay were taken from 4 to 5 mice just before cell transfer and from each group 24 hr after cell transfer.

Results from two experiments in which 1.3×10^8 viable cells were transfused are shown in Table 1. There was a highly significant reduction of spleen titers by immune cells, a significant reduction of liver and lung titers, but no demonstrable effect on brain titers.

In a similar experiment, 2×10^7 viable spleen cells from immune or from normal donors were transfused into groups of infected mice, while those in a third group received 0.75 ml of immune mouse serum. Spleen virus titers 24 hr later were $10^{4.9} \pm 0.3$ in mice given immune cells, compared with $10^{6.2} \pm 0.7$ in mice given normal cells ($P < 0.01$). Mice given antibody showed a striking reduction in spleen titers to $10^{3.4} \pm 0.4$ ($P < 0.001$). Pooled sera from donors of spleen cells and pooled sera from recipients of spleen cells 24 hr after cell transfer were tested for neutralizing antibody. Neutralizing antibody could not be detected in these sera.

In another experiment, spleens were taken from CBA donors 4, 6, 8 and 95 days after infection, and 10^8 viable cells were transfused into groups of four CBA mice that had been infected with 10^5 PFU of virus intravenously 2 days earlier. Spleen cells from congenitally infected carrier mice of the WEHI strain were also transfused, and spleens of individual recipients were assayed 24 hr later, as in the preceding experiments. The results (Table 2) show that the antiviral action of spleen cells was demonstrable at 6 and 8 but not 4 or 95 days after infection of donors and that spleen cells from carrier mice were inactive.

To determine whether thymus-derived cells were responsible for the antiviral activity of immune spleen cells, the spleen cells obtained 8 days after infection in the last experiment were treated with either normal or anti-theta ascitic fluid: 3×10^8 cells were incubated in 10 ml of a 1:2 dilution of ascitic fluid for 30 min at 36 C and then washed once before intravenous in-

TABLE 1. *Effect of immune and nonimmune spleen cells on target organ titers in CBA mice infected intracerebrally with LCM virus*[a]

Time (days)	Treatment	Titers[b]			
		Liver	Spleen	Brain	Lung[c]
2	Nil	3.2 ± 0.2	5.5 ± 0.2	5.7 ± 0.07	3.81 ± 0.1
3	Nil	4.3 ± 0.2	5.9 ± 0.2	6.9 ± 0.1	4.85 ± 0.05
	Immune cells	2.8 ± 0.1[d]	3.0 ± 0.2[e]	6.8 ± 0.04	4.05 ± 0.3[f]
—	Normal cells	4.2 ± 0.3	5.8 ± 0.2	6.8 ± 0.05	ND[g]

[a] Viable cells (1.3×10^8 per mouse) given 48 hr after infection.
[b] Titers expressed as mean log PFU/organ ± standard error of the mean in groups of four mice.
[c] Data from experiment with intravenously infected recipients.
[d] Significantly less than the other 3-day liver titers ($P < 0.01$).
[e] Significantly less than the other 3-day spleen titers ($P < 0.001$).
[f] Significantly less than the other 3-day lung titers ($P < 0.05$).
[g] Not done.

TABLE 2. *Antiviral action of spleen cells taken at different times after LCM virus infection, and the effect of anti-theta antibody*

Material	Cell transfer	Spleen virus content mean[a]	*P* value compared with 3-day control
Spleen, 2 days		5.21 ± 0.1	
Spleen, 3 days	Nil	5.76 ± 0.2	
	4-day immune cells	5.56 ± 0.1	0.45
	6-day immune cells	4.01 ± 0.1[b]	<0.001
	8-day immune cells plus normal ascitic fluid	3.24 ± 0.1[b]	<0.001
	8-day immune cells plus anti-theta ascitic fluid	4.09 ± 0.1	<0.001
	95-day immune cells	5.49 ± 0.1	0.3
	Carrier immune cells	5.45 ± 0.2	0.3

[a] Titers expressed as mean log PFU/spleen ± standard error of the mean in groups of four mice.
[b] Difference between these values significant at $P < 0.01$.

jection (7×10^7 viable cells per mouse) into infected mice as before. The antiviral action of immune spleen cells was significantly inhibited by anti-theta antibody (Table 2), but considerable antiviral activity remained.

Attempts to induce disease in infected mice by immune cell transfer. Two groups of eight mice were infected intracerebrally with 100 median lethal doses of LCM virus of the Armstrong strain so that visceral infection should be minimal (12). Sixty-four hours later, mice were transfused with spleen cells from normal mice or from donors infected 10 days earlier. Half the recipients were transfused intravenously (10^8 cells) and half intracerebrally (2×10^6 cells), and they were observed thrice daily for sickness or death. Those given immune cells became clinically sick 6 to 24 hr after transfer, sitting inactive, hunched and ruffled but not undergoing LCM-type convulsions or tail-twirling; those given normal cells were not sick until 2–3 days after transfer. Average survival time and mortality were the same in each group, and there was no difference between mice receiving cells intravenously or intracerebrally.

Early signs of disease after intracerebral injection of immune cells plus virus. Tosolini (*personal communication*) has studied the disease picture in normal mice injected intracerebrally with immune spleen cells mixed with LCM virus. Mice became sick after 24 hr, showed LCM-type convulsions on spinning by the tail on the third day, and died after 3 to 6 days. Mice receiving virus plus normal cells, or virus plus mouse antiserum, did not become sick until 5 days and died after 6 to 8 days; immune cells alone had no effect. Further experiments were done to confirm and extend the findings of Tosolini.

Groups of six, normal adult WEHI mice were injected intracerebrally with 10^7 normal or immune spleen cells mixed with $10^{6.5}$ PFU of LCM virus. Immune cells were taken from WEHI mice 8 days after intravenous infection with 1,000 PFU of virus. Mice given immune cells plus virus were sick from days 2 to 3, and on day 4 some showed LCM-type convulsions by twirling of the tail; mice given normal cells plus virus were not detectably sick until days 5 to 6. There were no differences in average survival time. Another group of mice received heat-inactivated (20 min at 56 C) virus plus immune cells, and another group received the same number of viable immune cells which had been washed after in vitro incubation with virus for 20 min at 37 C. There were no signs of illness in either group of mice. It was concluded that for the unusually early pathological effect immune cells plus infectious virus were needed, and that immune cells were not themselves pathogenic after exposure to infectious virus.

DISCUSSION

The above experiments show that immune spleen cells have a clear antiviral effect within 24 hr of transfer to infected recipients. Cells became active 4 to 6 days after primary infection of donors, were highly active at 8 days, and inactive by 95 days. In a series of passive transfer experiments with ectromelia virus, Blanden showed that immune spleen cells also possessed antiviral activity 4 to 6 days after primary infection of the donor; activity had almost disappeared by 20 days (2). Cole et al. (*personal communication*) have shown that donor spleen cells taken 5 to 6 days after primary infection with LCM virus caused lesions and death when transferred into cyclophosphamide-induced adult mouse carriers of LCM virus; cells taken at 25 days were ineffective. Activity was prevented by treating cells with anti-theta serum. In these last two systems, there is compelling evidence that cell-mediated immunity is more important than

antibody in causing the antiviral or pathogenic effect. One might expect that the antiviral effect of immune spleen cells harvested 6 days after primary LCM infection is also due in large part to cell-mediated immunity; circulating antibodies are difficult to detect even after 10 days, whereas the histological accompaniments of the immune response are visible in spleens after 4 days (10). This idea received support from the fact that anti-theta treatment of immune spleen cells significantly impaired their antiviral effect. However, a considerable antiviral effect was still conferred by cells which survived anti-theta treatment, in contrast to Blanden's ectromelia experiments, where anti-theta treatment of cells completely abolished the antiviral effect. It remains a possibility that in the LCM experiments enough T cells escaped anti-theta effects to transfer significant activity. Alternatively a significant contribution to the LCM antiviral effect is made by cells which are not thymus derived and which presumably act by secreting antibody. However, no neutralizing antibody was detected in the serum of the immune cell donors, nor in recipients 24 hr after cell transfer. Conceivably, small amounts of antibody could be produced locally by immune cells lodging in foci of infection. Transfusion of immune serum had a dramatic antiviral effect, but this is of uncertain relevance for the question of antiviral mechanisms in the mouse 6 days after primary infection, when no antibody is detectable. Also, to some extent the reduction in spleen titers could have been due to in vitro viral neutralization after homogenization by antibody present in splenic blood vessels, rather than to a genuine inhibition of virus growth in the spleen.

The clinical disease induced in infected mice by transfusion of immune cells was less dramatic than in the experiments of Gilden et al. (4) who produced persistently infected mice by cyclophosphamide treatment and induced lesions and death by immune cell transfusion. In the above experiments, the adoptive cell transfer needed careful timing; if it came too early, virus growth would be terminated before it had proceeded far enough, and if too late the immune response of the infected animal would produce a disease. The transfused cells nevertheless had a distinct pathogenic effect, with accelerated onset of sickness but no difference in survival time.

The mechanism of the pathogenic effect of intracerebrally injected immune cells plus virus is presumably as follows. The large dose of virus causes early and extensive infection of meninges, ependyma, and choroid plexus epithelium, and the immune cells remaining at these sites after the injection (9) then react immunopathologically with the infected target cells. A similar local effect of LCM virus mixed with immune cells is seen in the footpad. Immune cells plus LCM virus injected into the footpad of normal mice gave a delayed footpad swelling of almost the same magnitude as that seen after the injection of virus by itself into sensitized mice (12).

LITERATURE CITED

1. Blanden, R. V. 1970. Mechanisms of recovery from a generalised viral infection: mousepox. I. The effects of antithymocyte serum. J. Exp. Med. **132**:1035–1054.
2. Blanden, R. V. 1971. Mechanisms of recovery from a generalised viral infection: mousepox. II. Passive transfer of recovery mechanisms with immune lymphoid cells. J. Exp. Med. **133**:1074–1089.
3. Blanden, R. V. 1971. Mechanisms of recovery from a generalised viral infection: mousepox. III. Regression of infectious foci. J. Exp. Med. **133**:1090–1104.
4. Gilden, D. H., G. A. Cole, and N. Nathanson. 1972. Immunopathogenesis of acute central nervous system disease produced by lymphocytic choriomeningitis virus. II. Adoptive immunization of virus carriers. J. Exp. Med. **135**:874–889.
5. Hotchin, J. 1962. The biology of lymphocytic choriomeningitis infection: virus-induced immune disease. Cold Spring Harbor Symp. Quant. Biol. **27**:479–499.
6. Hotchin, J. 1962. The foot pad reaction of mice to lymphocytic choriomeningitis virus. Virology **17**:214–216.
7. Lehmann-Grube, F. 1971. Lymphocytic choriomeningitis virus, p. 28–78. *In* Virology monographs, vol. 10. Springer-Verlag, Vienna.
8. Lundstedt, C. 1969. Interaction between antigenically different cells. Virus-induced cytotoxicity by immune lymphoid cells in vitro. Acta Pathol. Microbiol. Scand **75**:139–152.
9. Mims, C. A. 1960. Intracerebral injections and the growth of viruses in the mouse brain. Brit. J. Exp. Pathol. **41**:52–59.
10. Mims, C. A., and F. A. Tosolini. 1969. Pathogenesis of lesions in lymphoid tissue of mice infected with lymphocytic choriomeningitis (LCM) virus Brit. J. Exp. Pathol. **50**:584–592.
11. Oldstone, M. B. A., and F. J. Dixon. 1970. Tissue injury in lymphocytic choriomeningitis viral infection: virus-induced immunologically specific release of a cytotoxic factor from immune lymphoid cells. Virology **42**:805–813.
12. Tosolini, F. A., and C. A. Mims. 1971. Effect of murine strain and viral strain on the pathogenesis of lymphocytic choriomeningitis infection and a study of footpad responses. J. Infect. Dis. **123**:134–144.