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Cytotoxic T Cells Are Induced in Mice Infected with Lymphocytic Choriomeningitis Virus Strains of Markedly Different Pathogenicities

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The ability of two lymphocytic choriomeningitis virus substrains to induce cytotoxic T-lymphocyte (CTL) responses in intracerebrally infected mice was examined. One strain, designated A (aggressive), provoked a convulsive type of death in 100% of the mice within 8 to 9 days, whereas the other strain, designated D (docile), killed less than 10% of the mice during 28-day observation periods. CTL activity was assessed by the capacity of partially purified splenocytes to lyse ^{51}Cr-labeled L-cell targets infected with either type of lymphocytic choriomeningitis substrain. The CTL population was identified by its sensitivity to anti-Thy-1 serum and its inability to lyse uninfected target cells or infected target cells with which it differed at the level of antigens controlled by the major histocompatability gene complex. A strong CTL response developed in mice infected with either lymphocytic choriomeningitis substrain, although the activity provoked by substrain D was somewhat less than that seen after substrain A infection. Peak CTL activities induced by both strains occurred at about the same time. Even though docile virus replicated more extensively in the brain than did aggressive virus and fluorescent antibody staining revealed similar distributions of viral antigen, no inflammatory response was noted in the brains of mice infected with docile virus. These results are discussed with regard to the role of CTLs in mediating classic central nervous system pathology.

Lymphocytic choriomeningitis (LCM) virus infection of mice has gradually been recognized since its discovery over 45 years ago by Traub (18) as a model without equal for studying viral immunobiology and immunopathology and cell-cell recognition (1). Whereas transplacental or neonatal infection of mice can lead to lifelong, persistent infection, intracerebral (i.c.) infection of adult mice can cause a rapidly convulsive central nervous system disease. It has been known for some time that these responses are quite dependent on the strain of LCM used. Shortly after Traub discovered murine LCM, he observed (19) that naturally passed virus isolated from the thoracic and abdominal organs differed markedly in pathogenicity for adult mice from the same virus which had been modified by multiple serial mouse brain-to-mouse brain transfers. More recently, Suzuki and Hotchin (17), using plaque-purified virus, reestablished and extended these findings by showing that brain-passed, or what is called "aggressive" virus, killed neonatal mice and produced early, convulsive death in adult mice. On the other hand, liver-passed, or "docile," virus induced persistent infection in neonates and either a late, wasting death or persistent infection in adult mice. Both virus populations, exhibiting a remarkably clear organ specificity, could in fact coexist in the same persistently infected mouse (9).

We began to examine these viruses recently with regard to their evolutionary significance in persistent infection. Both types could be readily cloned from the blood of a persistently infected mouse (11). The aggressive virus was found to be resistant to defective interfering particles that it generated shortly after neonatal infection (11) and to induce interferon after i.c. infection of adult mice (10), properties which the docile virus lacked. Because of the profound effect interferons can have on the immune system (16), the ability of these viruses to provoke cytotoxic T-lymphocyte (CTL) responses in adult mice was determined. CTLs are widely considered to be critical in eliminating virus from peripherally infected mice as well as in causing the fatal neurological disease in mice infected i.c. (4, 24). The data in this communication show that the CTL response is similar in mice infected with

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either virus. The significance of these findings with regard to viral pathogenesis is discussed.

MATERIALS AND METHODS

Cells. L-929 cells (H-2k) and MDCK cells were maintained as previously described (7, 22). Target cells for the CTL assay were prepared in the following manner. After medium was decanted from a freshly confluent monolayer of L-929 cells, virus at a multiplicity of infection of 0.1 was added. Incubation was continued, with intermittent agitation, for 1.5 h at 37°C. The inoculum was removed, new medium was added to the original volume, and incubation was continued for 48 h at 37°C. The cells were then dispersed and pelleted at 500 × *g* for 5 min just before labeling with radioactive isotope.

Viruses. The UBC strain of LCM with a long history of tissue culture passage was used to infect neonatal ICR outbred Swiss mice (Blue Spruce Farms, Altamont, N.Y.). From the blood of a 10-month-old ICR persistently infected mouse, virus excised from large clear or pinpoint plaques (11) was passed no more than twice in MDCK cells at a multiplicity of infection of 0.1. Virus was harvested before maximal cytopathic effects, 48 to 72 h postinfection (7). Virus stocks giving rise to pinpoint plaques had aggressive (A) characteristics, whereas the clear-plaque virus induced the docile (D) disease syndrome (10).

Mice-effector cell preparation. Three-week-old female C3HeB/FeJ (H-2k) and four-week-old female BALB/cByJ (H-2d) mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Mice were injected i.c. with 300 MDCK PFU of virus in a volume of 0.03 ml. Spleens were harvested from two mice infected with a given virus on day 7 or 8 after infection (1 day before death induced by A virus). Spleens were prepared by standard procedures (8) and adjusted to 2×10^7 nucleated cells per ml of minimal essential medium (MEM). Samples (4 ml) of this suspension were then placed over 3 ml of sodium diatrizoate-Ficoll (LSM-Litton Bionetics, Inc., Kensington, Md.) and spun for 40 min at 400 × *g*. The cells at the interfaces were removed, combined, and diluted to a final volume of 15 ml with MEM. After the cells were centrifuge-washed twice at 300 × *g* for 10 min, they were suspended in MEM with either 10% fetal calf serum or 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 0.3% bovine serum albumin.

Lymphocytotoxicity assays. Lymphocyte-mediated killing was carried out essentially by the procedure of Welsh and Oldstone (21). Target L-929 cells were labeled with sodium chromate (^{51}Cr) (ICN, Irving, Calif.) at a concentration of 90 μCi per pellet of 3×10^6 cells for 1 h at 37°C. Spleen cells were added to 0.32-cm^2, flat-bottomed wells of 96-well tissue culture cluster plates (Costar, Cambridge, Mass.) to give various effector-to-target multiplicities (80:1, 40:1, 20:1, 10:1, 5:1, 1:1). Each test sample was plated in triplicate. For the spontaneous lysis control, MEM was used in place of the effector spleen cells. For the 100% lysis control, 0.1 N HCl was added before the target cells. Data are expressed as: percent specific ^{51}Cr release = {[(counts per minute of test sample) − (counts per minute of MEM control)]/[(counts per minute of HCl control) − (counts per minute of MEM control)]} × 100. Background ^{51}Cr release from L cells in 16-h assays was usually about 15%.

Depletion of T cells in splenocyte populations. Lymphocytes prepared by centrifugation through sodium diatrizoate-Ficoll were suspended at a concentration of 10^7/ml in MEM-HEPES-bovine serum album medium. Anti-mouse T-cell serum (rabbit anti-mouse brain-associated Thy-1 antiserum; Cedarlane Laboratories, Hornby, Ontario, Canada) was added to a final dilution of 1:20, followed by incubation for 60 min at 4°C. The cells were then centrifuge-washed at 500 × *g* for 10 min, resuspended to the original volume with fresh medium, and incubated for 60 min at 37°C with rabbit complement (Cedarlane) diluted 1:12. The cells were centrifuge-washed three times at 500 × *g* for 10 min, using 15-ml volumes of MEM-HEPES-bovine serum albumin for the first two washes and MEM plus fetal calf serum for the final resuspension. The cells were used at an effector-to-target ratio of 40:1 (using pretreatment counts) for the ^{51}Cr assay.

RESULTS

Capacity of LCM substrains to generate cytotoxic splenocytes and target L cells. Preliminary data with L-cell targets infected with tissue culture-passed LCM virus indicated a strong CTL response in mice infected with either A or D LCM viruses. Splenocytes from A virus-infected mice were consistently found to be somewhat more effective lysing targets than comparable cells from D virus-infected mice. Since effector function and target recognition could be critical in determining in vivo pathogenic patterns, ^{51}Cr release assays were carried out with L-cell targets produced by infection with either the A or D substrains. Figure 1 shows data from one experiment which is representative of the five experiments performed. The consistent pattern was that effector cells generated by substrain A (A effectors) were more active than those generated by substrain D (D effectors), and effector cells were always more active against target cells infected with homologous virus than they were against those infected with heterotypic virus.

Replication of LCM substrains in L-cell targets. Expression of viral antigens on target cell membranes could influence recognition by effector cells. Since LCM viruses bud exclusively from the cell membrane, cell-associated infectious virus determined by sonic disruption of washed cells during the early stages of infection (21, 22) was considered to reflect the viral antigens on the cell surface. At the time the ^{51}Cr assays were performed, L cells infected with the D substrain (D targets) contained four times more infectivity (10^7 PFU/ml) than cells infected with substrain A (A targets). The characteristic plaque morphologies of each virus (11) were maintained during this passage.

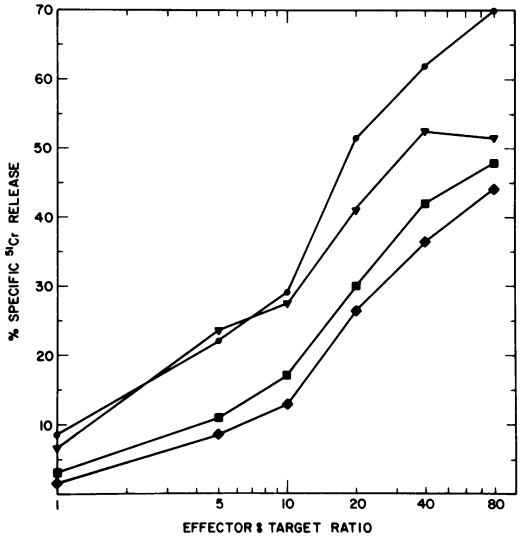

FIG. 1. Effect of LCM virus substrains on the induction of murine splenocytes and L-cell targets in an in vitro recognition assay. Three-week-old female C3H mice in groups of three were infected i.c. with 300 PFU of the A or D substrains of LCM virus. On day 8 after infection, the spleens from two mice in each group were processed for use against ^{51}Cr-labeled L cells infected with either the D or A substrains. Uninfected effector or target cells were run as controls in each of the four combinations, with results similar to those shown in Table 1. Symbols: ●, A effector, A target; ▲, A effector, D target; ■, D effector, D target; ◆, D effector, A target.

Role of thymus-derived lymphocytes and histocompatability antigens in target recognition. The ability of both A and D effectors to destroy target cells infected with homologous virus was abrogated by treatment with anti-Thy-1 serum (Table 1). Furthermore, cell-cell recognition at the level of the major histocompatability (H-2) complex was evident by the inability of splenocytes from a haplotype d mouse (BALB/c) infected with one or the other virus substrain to lyse L cells (H-2k) infected with homologous virus.

Kinetics of effector cell generation in mice infected with LCM substrain D. Splenocytes harvested on day 7 after infection of C3H mice infected with either substrain A or D had relatively poor cytotoxic activity (20 to 30% ^{51}Cr release). Measurement of cytotoxic activity of A effectors could not be made after day 8 after i.c. infection, since even at that time the mice were near death. However, mice infected with substrain D died, if at all, 14 to 28 days after i.c. infection. The activity of splenocytes taken from substrain D-infected mice between days 8 and 13 after infection was as follows. Three-week-old female C3H mice in groups of three were infected i.c. with 300 PFU of LCM substrain D. On day 13, 12, 11, 10, 9, and 8 postinfection, spleens from two mice in each group were removed, and the cells were processed for use against ^{51}Cr-labeled L cells infected with homologous virus. On days 8, 9, 10, 11, 12, and 13 postinfection, ^{51}Cr release was 57, 63, 50, 38, 40, and 34%, respectively (effector/target cell ratios of 5:1, 10:1, 20:1, and 40:1 were used, but only representative data at the 20:1 ratio is given). As previously shown (2, 12, 14) with CTLs induced under immunizing conditions, maximum ability to lyse targets appeared on days 8 and 9 after infection. A pronounced decrease in activity occurred between days 9 and 11, after which no further reduction was seen.

Ability of LCM virus substrains to replicate in the mouse brain. Histologically, the fatal murine central nervous system disease provoked by i.c. infection with LCM virus has been characterized by striking mononuclear infiltration in the choroid plexus, leptomeninges, and ependyma, the sites of preferential virus replication (12). Since a vigorous CTL response was made in mice infected with substrain D which was almost comparable to that made in mice infected with substrain A, it was possible that a difference existed in the targets for these cells. Specifically, virus replication in the brains of mice

TABLE 1. Restricted cytotoxic splenocyte activity against LCM virus-infected L cells[a]

Effector cell inducer (virus strain)	Effector cell origin (mouse strain)	Anti-T-cell treatment	Target cell inducer (virus strain)	% ^{51}Cr release
A	C3H	−	A	72
A	C3H	+	A	12
A	BALB/c	−	A	11
A	C3H	−	—	9
—	C3H	−	A	17
D	C3H	−	D	56
D	C3H	+	D	6
D	BALB/c	−	D	10

[a] Three-week-old female C3H and four-week-old female BALB/c mice in groups of three or six were infected i.c. with 300 PFU of the D or A substrains of LCM virus. On day 8 after infection, the spleens from two to four mice in each group were removed, and the cells were processed for use against ^{51}Cr-labeled L cells either infected with homologous virus or uninfected. Effector/target ratios were 40:1, and paired groups of cells used in the T-cell specificity part of the experiment were physically handled identically except for the presence or absence of complement and anti-T-cell serum. —, Uninfected mice injected i.c. with the virus diluent medium (MEM plus fetal bovine serum).

infected with either substrain was examined. When equal amounts of either virus were injected i.c., docile virus could be detected earlier, rose more rapidly, and reached higher titers (as much as 300-fold) than the aggressive virus (Table 2). Yet under normal conditions, all mice infected with A virus died between days 8 and 9 after injection, whereas all mice receiving D virus lived through a 21-day observation period (at this time, all six mice were virus carriers, with average serum titers of 3×10^5 PFU/ml).

TABLE 2. Progression of virus replication in the brain and disease outcome in mice infected with different LCM strains[a]

Days post-infection	Replication with following virus strain: A		D	
	No. of PFU/g × 10^{-4}	% Survival[b]	No. of PFU/g × 10^{-4}	% Survival[b]
1	<0.0045	100	<0.0045	100
2	<0.015	100	0.042	100
3	0.27	100	22.0	100
4	0.95	100	26.0	100
5	0.44	100	110.0	100
6	4.5	100	1,400.0	100
7	4.0	100	180.0	100
8	—[c]	50	—	100
9	—	0	—	100
21			—	100

[a] Two groups of 20 C3H mice were injected i.c. with 300 PFU of either the A or D strain of LCM virus. At daily intervals for the first 7 days after infection, brains were removed from two mice infected with each type of virus. One brain was homogenized and plaque-titrated (10), whereas the other was kept frozen in case duplicate titrations were thought necessary (in these cases, the virus titers were never found to differ by more than twofold).

[b] Not including deliberately sacrificed animals.

[c] —, Not tested.

DISCUSSION

Comparison of the immune response in adult mice infected i.c. with LCM virus substrains markedly different in their lethality can be a powerful tool for defining the requisites of neurological disease. Until now, low virulence strains have received relatively little attention, probably because the mouse-virus strain combinations employed thus far have led to 50 to 60%, rather than 100%, mortality (17, 19). Deaths usually occurred 4 to 5 days later than in mice receiving highly lethal virus. In contrast, uniformity of the response in the present system is striking. With the D virus used here, more than 10% mortality was rarely observed during 28-day observation periods in all inbred mice thus far examined from Jackson Laboratories (C3HeB/FeJ, C3H/HeJ, CBA/CaJ, BALB/cByJ, BALB/cJ). This low virulence was usually observed after i.c. doses ranging from 3×10^2 to 3×10^4 PFU. However, from other commercial sources of inbred and outbred mice, erratic, sometimes seasonal mortality patterns have been found with the D virus.

The results presented here show that, although two LCM virus substrains can differ markedly in the disease patterns they induce, both provoke vigorous CTL responses. These observations are central to defining pathogenic mechanisms, since there is compelling evidence for the idea that CTLs are involved rather directly in causing lethal disease. It is well established that the T-cell response against LCM virus inoculated i.c. is solely responsible for the acute central nervous system disease induced by infection (4, 13). Are these cells and CTLs one and the same subgroup of lymphocyte? The following indicates to us that it is reasonable to assume that the same population of T cells is involved in in vivo and in vitro responses. The critical role of T cells has been shown by their ability to induce fatal disease after adoptive transfer into syngeneic, immunosuppressed recipients infected with the virus. The shortest interval between adoptive transfer and death of the recipient correlates with peak CTL activity (3, 5). Moreover, both in vitro and in vivo systems for measuring lymphocyte activity share a restriction at the major histocompatability complex (H-2 antigens) level (5, 6). Furthermore, meningeal inflammatory exudate may be isolated by tapping the cisterna magna (23) and constitutes a very potent source of LCM-specific CTLs. Although there is no rigorous proof that CTLs are localized in the actual lesions, this has been shown for poxvirus-induced meningitis (15).

Not only has D virus been found to replicate well in the brain (Table 2), its distribution, as demonstrated by fluorescent-antibody staining of thin sections of 6-day-postinfection brain, was comparable to that of A virus (M. J. Buchmeier, personnel communication). Since D virus induced a strong CTL response (Fig. 1) with little subsequent mononuclear cell infiltration into the brain (R. M. Friedman, personnel communication), other events must be critical in the virus-host interaction which lead to central nervous system disease. We have suggested that, since A viruses induce interferon (10), expression in the brain of either H-2 or viral antigens on target cell membranes could be enhanced. Four-way cross-adoptive transfer experiments in which CTLs and recipient mice infected with either substrain are used, currently under way, should reveal any target defect (as well as determining whether D virus-induced CTLs have in vivo activity).

It could also be that D virus, unimpeded by interferon, spreads and replicates extensively throughout the body, with the result that the effector T-cell population is recruited to many infected tissues. This putative dilution effect has been proposed (8, 24) as the explanation for the inability of adoptively transferred T cells to kill LCM persistently infected mice (20).

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