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*J Immunol* 1985; 135:597-602; ;
http://www.jimmunol.org/content/135/1/597
LACK OF CORRELATION BETWEEN CYTOTOXIC T LYMPHOCYTES AND LETAL MURINE LYMPHOCYTIC CHORIOMENINGITIS

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Adoptive transfer of lymph node and spleen cells from mice infected with LCM virus to similarly infected immunocompromised recipients has been the classic way to demonstrate the lethal role of T cells in the CNS disease caused by this virus. Isolation and adoptive transfer techniques are presented here which show that Thy-1+ cells isolated from the meningeal infiltrates (MI) of LCM virus-infected mice possess this property. We compared various T cell functions of MI cells from mice infected with two strains of LCM virus differing markedly in their pathogeneticities. One of these strains, termed aggressive, caused a typical, invariably fatal, CNS disease within 7 to 10 days after infection. The other virus, termed docile, killed few mice after the standard intracerebral inoculation, and could persist in the mice for 6 mo or more. The yields of MI leukocytes from mice infected with docile virus varied from 50 to 100% of those found in mice infected with aggressive virus (3 × 10^6 cells/brain). On a cell-to-cell basis, the CTL activity in the MI of mice infected with docile virus ranged from 50 to 100% of that found in the MI of mice infected with aggressive virus. MI cells from mice infected with aggressive virus consistently caused lethal disease by adoptive transfer into immunocompromised (irradiated) recipients infected with either strain of virus. All attempts to induce lethal disease by adoptive transfer of MI cells (or splenocytes) from mice infected with docile virus into irradiated recipients failed. The latter experiments with the docile-MI cells were performed with six times the number of aggressive MI cells needed to kill irradiated recipients by adoptive transfer. The possible reasons for this discordance between CTL and in vivo killer function are discussed.

Vertebrates possess a multitude of nonspecific and specific defense mechanisms which have the potential to protect against a myriad of invading microorganisms. Yet some microorganisms, specifically certain viruses, are more likely than others to cause disease. Comparison of closely related viruses which differ markedly in their pathogenesis has been a fruitful source of new concepts in our understanding and control of viral infections (1).

We have been studying two strains of lymphocytic choriomeningitis (LCM) virus, termed docile and aggressive, which differ markedly in the disease patterns they provoke after intracerebral (i.c.) injection into young adult C3H mice (2–5). At a standard dose of 300 plaque-forming units (pfu), the docile virus usually killed no more than 10 to 15% of the mice, whereas the aggressive virus induced a severe central nervous system (CNS) disease which was invariably fatal within 7 to 10 days. Although cytotoxic T lymphocyte (CTL) activity in the MI of mice infected 8 days previously with the nonlethal docile virus did not die, the infection nevertheless triggered clonal expansion of T cells capable of producing a fatal outcome. To resolve this seemingly paradoxical situation, we hypothesized that the lethal outcome was determined by a race between viral proliferation and induction of the killer T cell response. That is, because the docile virus replicated extremely rapidly in the viscera, the effectiveness of the killer T cell population was dissipated by migration to many different targets. On the other hand, because the aggressive virus grew so slowly in visceral organs, we suggested that a focused killer T cell assault on the brain resulted in a fatal outcome (8). The above hypothesis leads to the prediction that killer T cell activity should be low in the brains of mice infected with docile virus, and high in the brains of mice infected with aggressive virus. With the successful isolation by Saron and Guillot (9) of T cells with delayed-type hypersensitivity (DTH) function from the meningeal infiltrates (MI) of mice infected with an aggressive-type LCM virus, it became feasible to test this hypothesis. We report here that the in vitro CTL activity of the MI from docile virus-infected mice was close to that observed from mice infected with the aggressive strain. On the other hand, adoptive transfer experiments showed that lethal potential could be demonstrated only with MI from mice infected with the aggressive strain. Furthermore, these and other experiments led us to reinterpret data concerning the lethal potential of splenocyte
populations from mice infected with the docile LCM virus strain.

MATERIALS AND METHODS

Viruses and mice. The aggressive and docile viruses were isolated from the blood of a mouse persistently infected with the UBC strain of LCM virus (2). The viruses were cloned on the basis of distinctive plaque morphologies in Madin-Darby canine kidney cells (10). The viruses used in the present studies had been passed four times at low multiplicities of infection in Madin-Darby canine kidney cells with no apparent change in the responses they provoked in vivo. C3He/FeJ (H-2b) female mice, 3 wk of age, were purchased from The Jackson Laboratory, Bar Harbor, ME. Mice were routinely kept under ether anesthesia for 1 wk before use.

Isolation of MI cells. Under ether anesthesia, mice were exsanguinated and were then terminated by cervical dislocation. Skin was reflected over the skull and appropriate incisions were made so that the skull cap could be removed intact. Both the intact brain and skull cap were placed immediately in ice-cold Eagle's minimal essential medium.

Washing was performed by jetting ice-cold media over their surfaces with Pasteur pipets. The inside of the skull caps were intermittently scratched with the pipet as they were washed. Such washing was continued until the skull caps became translucent. The surface of the brain was not deliberately damaged, but the pipet tip was used to open and flush out crevices between the cerebral hemispheres and also between the cerebrum and cerebellum. The brain was jetted with medium about 10 to 15 times. Washing fluids were changed once and were filtered through nylon screening. The leukocyte content of pooled fluids was determined by hemacytometer before centrifugation at 4°C for 10 min at 400 X g. Cell pellets were resuspended with the appropriate medium to the desired volume. In some experiments, as designated in the text, leukocytes were purified by centrifugation on a cushion of Ficoll-sodium diatrizoate (4).

Depletion of Thy-1.2-sensitive cells with complement and antibody was carried out as described (4).

Cytotoxicity assay. L-929 (H-2b) cells were infected with the docile strain of LCM virus at a multiplicity of infection of 0.1. Two days later, these target cells were used in a CTL assay exactly as described previously (4), except for the following: the effector population in this case was the MI cells which had been purified by centrifugation through Ficoll-sodium diatrizoate (4).

Adoptive transfer of lethal disease: Use of MI cells. Five-week-old mice were given 400 rad whole body irradiation from a 137Cs source (Gammator, Model M-39-1, Isomedix, Parsippany, NJ) 24 hr before i.c. injection with 300 pfu of the LCM strain. Two days after infection, 4 X 10^6 MI cells in a volume of 0.3 ml were injected through the ophthalmic venous plexus. No less than 5 min and no more than 15 min before the adoptive transfer, mice were injected i.p. with 250 USP units of heparin. Mice were lightly etherized at the time of the adoptive transfer so that they became mobile shortly after the injection. Without these precautions, most mice died within 30 sec of the injection; even then, it was not unusual to find 10 to 20% of the mice dead within 2 hr.

USE OF SPLENOCYTES. Cells were purified through Ficoll-sodium diatrizoate exactly as described previously (4). Recipient mice were either infected 2 to 3 days before adoptive transfer with the docile strain of LCM virus (5) or were irradiated and infected as described above.

RESULTS

Cell recovery from MI. The yield of leukocytes from washed brains and skulls of mice infected i.e., 8 days previously with either the docile or aggressive viruses was directly compared in 14 individual experiments. The average yield in each experiment was based on hemacytometer counts of pools of cells harvested from 15 to 25 brains. In five of the experiments, the yields were about equal (3 X 10^6 leukocytes/donor ± 20%). In the majority of the remaining experiments, the MI yields from mice infected with docile virus were about 55% of those obtained from mice infected with aggressive virus (the lowest yield being 25%). In each of these experiments, control mice (five/group) were monitored to verify that the outcome of the infection followed the predicted pattern. If leukocytes were purified by using Ficoll-sodium diatrizoate, cell recoveries were about 50% of the input numbers (data not presented).

In vitro cytotoxicity of MI cells. The CTL activity in the MI of mice infected with docile or aggressive virus was compared side by side in six separate experiments. In four of these experiments, the total yield of viable leukocytes (based on trypan blue exclusion) from the MI of mice infected with either type of virus was about the same (±20%). In one of these four experiments, the CTL activities (percent 51Cr release at identical effector to target ratios) were similar, but in all other experiments, comparable percent 51Cr release required twice as many MI cells from mice infected with the docile virus as those obtained from mice infected with the aggressive virus. Typical data from these latter experiments are shown in Figure 1. The nature of these effector cells was repeatedly shown by their inefficient lysis of noninfected target cells and their sensitivity to antibodies against the T cell surface antigen Thy-1 (at 10:1 effector to target ratios, percent 51Cr release was in the 5 to 15% range). For the sake of clarity, only the Thy-1 sensitivity of aggressive MI cells is shown. The "enriched" nature of the MI effector population is shown in this figure by comparison with a
spleenocyte CTL population. As shown previously (4), spleenocyte populations display maximal cytolyis against identical virus-infected targets at effector to target ratios of 80:1. Figure 1 shows that at an effector to target ratio of 10:1, spleenocytes barely have the same activity as MI cells at a ratio of 1.25:1.

CNS disease after adoptive transfer of MI cells. The basic system for lethal adoptive transfer of MI cells into recipient mice was developed by using lymphocytes washed from the skull caps and brain dura matter of mice infected with the aggressive strain. As few as $1 \times 10^6$ MI cells were capable of inducing typical LCM-like symptoms (hunched posture, ruffled fur, blepharitis, and facial edema) terminating in convulsions and death (Table I). That this was a specific T cell function was shown as in Figure 1, this indicated the enriched nature of the adoptive transfer. Thus, anticipating a 30% reduction in (CTL) activity found that would consistently cause death. Again, as in Figure 1, this indicated the enriched nature of the T cell surface marker Thy-1.2. Recipient mice were also injected with various concentrations of splenocytes from mice infected with aggressive virus. In this case, a dose of $10^7$ cells/recipient (the only data shown) was the lowest concentration found that would consistently cause death. Again, as in Figure 1, this indicated the enriched nature of the T cells in MI preparations. In this and all succeeding adoptive transfer experiments (whether shown in table form or not), control mice were infected with virus and were simply kept for observation to ensure that the aggressive and docile virus behaved as expected (i.e., aggressive virus killed 100% of the mice, and docile virus killed no more than 15%).

Failure of MI cells from mice infected with docile virus to transfer lethal disease. By using protocols established above (Table I), we have been uniformly unsuccessful in adoptively transferring lethal disease with MI cells from mice infected with docile virus (docile MI cells). In four experiments in which each recipient was given $4 \times 10^6$ lymphocytes, none of 19 mice receiving docile MI cells succumbed. On the other hand, 14 of a total of 18 mice receiving aggressive MI cells died with typical LCM symptoms within 7 to 8 days—typical of previous experience. Although four of these mice did not die, they all exhibited the convulsive syndrome. In contrast, only two of the 19 mice receiving the docile MI cells displayed the (nonfatal) convulsive state. Data from one of these experiments are given in Table II. These experiments were performed "blind": the CTL activity of the docile and aggressive MI cells was not known until the day after the adoptive transfers took place. In each of these four experiments, the docile MI CTL activity was about 70% of that seen with the aggressive MI cells. Even though the number of docile MI cells used was four times higher than the number of aggressive MI cells needed to induce the LCM disease syndrome, an experiment was run using a higher concentration of docile MI cells for the adoptive transfers. Thus, anticipating a 30% reduction in (CTL) activity for no more than 3 days (5). Because the MI cells taken from the identical type of donor mice showed no such lethal potential (Table II), an experiment was performed not only to reestablish the potency of the splenocytes but also from the same pool of mice (in this case 15) to isolate and test the MI cells. The data from this experiment are presented in Table III. Once again, recipients of the docile MI cells failed to die with the aggressive-type syndrome. Because the splenocytes be-

### Table I

<table>
<thead>
<tr>
<th>Donor Cell Source</th>
<th>Cell Dose ($\times 10^6$/Recipient)</th>
<th>Treatment with Anti-Thy-1</th>
<th>No. Convulsing $^a$</th>
<th>No. Recipients $^a$</th>
<th>Days to Death (SD) $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI</td>
<td>$6 \times 10^6$</td>
<td>$+$</td>
<td>5/5</td>
<td>5</td>
<td>7.6 (1.1)</td>
</tr>
<tr>
<td></td>
<td>$6 \times 10^6$</td>
<td>$+$</td>
<td>5/5</td>
<td>5</td>
<td>7.4 (0.5)</td>
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<tr>
<td></td>
<td>$2 \times 10^6$</td>
<td>$+$</td>
<td>0/3</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^6$</td>
<td>$+$</td>
<td>3/3</td>
<td>3</td>
<td>7.0 (0)</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^6$</td>
<td>$+$</td>
<td>3/3</td>
<td>3</td>
<td>8.0 (1.4)</td>
</tr>
<tr>
<td></td>
<td>$0.5 \times 10^6$</td>
<td>$+$</td>
<td>3/5</td>
<td>5</td>
<td>7.0 (1)</td>
</tr>
<tr>
<td>Spleen</td>
<td>$10.0 \times 10^6$</td>
<td>$+$</td>
<td>4/4</td>
<td>4</td>
<td>6.7 (0.5)</td>
</tr>
</tbody>
</table>

Control Mice (unmanipulated):

- Donors infected with aggressive virus
- Recipients infected with aggressive virus

$^a$ Spleens and brains were removed from mice infected 8 days previously with the aggressive strain of LCM virus. Lymphocytes were collected ($2.4 \times 10^6$ MI cells/mouse) and were then purified through Ficoll-sodium dextran before i.v. injection into recipient mice infected 2 days previously with the docile LCM strain. CTL activity in these spleens was 44.7% at an effector to target ratio of 10:1.

$^b$ Beginning on the sixth day after adoptive transfer or 7 or 8 days after standard i.e. infection, mice become rigid when spun by the tail. The extended hind limbs briefly remained in that position after recovery of forelimbs in nonfatal cases (usually observed up through the 10th day after adoptive transfer).

$^c$ MI cells were $10^6$ docile MI cell donors were kept for observation to ensure that the control mice were infected with virus and were simply kept for observation to ensure that the aggressive and docile virus behaved as expected (i.e., aggressive virus killed 100% of the mice, and docile virus killed no more than 15%).

### Table II

<table>
<thead>
<tr>
<th>MI Cell Source</th>
<th>Cell Dose ($\times 10^6$/Recipient)</th>
<th>No. Convulsing $^a$</th>
<th>No. Recipients $^a$</th>
<th>Days to Death (SD) $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggressive virus-infected mice</td>
<td>$4 \times 10^6$</td>
<td>$7/7$</td>
<td>$7$</td>
<td>$7.7 (0.5)$</td>
</tr>
<tr>
<td>Docile virus-infected mice</td>
<td>$4 \times 10^6$</td>
<td>$0/4$</td>
<td>$0$</td>
<td>— (—)</td>
</tr>
</tbody>
</table>

Control Mice (unmanipulated):

- Donors infected with Aggressive virus
- Recipients Infected with Docile virus

$^a$ Brains were removed from mice infected 8 days previously with either the docile or aggressive LCM strains. Lymphocytes were collected ($2.8 \times 10^6$ MI cells/docile mouse) and were then purified through Ficoll-sodium dextran before i.v. injection into mice infected 2 days previously with the docile LCM strain. CTL activity at an effector to target ratio of 10:1 was 45.2% and 31.4% for the aggressive and docile MI cells, respectively.

$^b$ See footnotes b, c, and d for Table I.
handed according to previous experience, the results with MI cells could not be attributed to an unknown change in our system—neither virus nor mice.

**Failure of splenocytes from mice infected with docile virus to transfer lethal disease to irradiated recipients.** Explanations were sought for why killer T cells from docile virus-infected mice appeared to be found only by adoptive transfer of splenocyte populations but not by transfer of MI cells (Table III). The trivial explanation was that four times as many splenocytes as MI cells were adoptively transferred. The reasons for running the experiment this way were not random, but were based on the following. As is true with aggressive splenocytes (Table I), adoptive transfer of less than 1 x 10^7 docile splenocytes will not consistently kill recipients (data not presented). On the other hand, adoptive transfer of 2 x 10^7 MI cells was not considered because of the cost of the experiment in terms of the number of donors needed for each recipient, and the fact that by CTL activity the docile MI cells were easily four times as effective as splenocytes (Table I; Reference 4). However, it seemed that the system for demonstrating these differences was unnecessarily complex because any explanation had to deal with the fact that the recipient mice had inherently functional immune systems (Figure 1, Reference 4). Thus, it was decided to essentially repeat the experiment presented in Table III, except that the recipients were to be immunocompromised before adoptive transfer. Gamma irradiation was chosen as the means of rendering mice incapable of mounting an immune response against the virus. As reported previously (11), mice infected with LCM virus (in our case docile or aggressive) were more sensitive to irradiation than noninfected mice (data not presented). Four hundred rad/mouse was chosen as the standard dose because aggressive virus-infected mice receiving one-half that dose would generally not show symptoms of the disease nor mount any detectable CTL nor primary DTH footprint (12) responses, and would not eliminate virus during a standard 3-wk observation period (data not presented). Table IV clearly shows that such mice infected with aggressive virus will develop the fatal CNS disease within 6 to 7 days after adoptive transfer with splenocytes or MI cells from mice infected with docile virus. On the other hand, virtually none of the mice developed CNS disease when adoptively transferred with splenocytes or MI cells from mice infected with docile virus. It should be noted that almost one-half of the recipients receiving docile splenocytes died eventually.

However, they died without the convulsive syndrome and 14 to 15 days after adoptive transfer. They died in approximately the same time range and in the same numbers as irradiated, unmanipulated (no adoptive transfer) recipients infected with aggressive virus.

It could be argued that for some reason the splenocytes taken on day 8 from a mouse infected with docile virus could only express their lethal potential in a recipient infected with the same virus (Table III). Thus, an experiment was performed exactly as described in Table IV, except that the recipients were infected with docile virus 24 hr after irradiation. A primary concern was whether the docile virus could reach as high a titer in irradiated as in nonirradiated mice. An initial experiment indicated that this was likely to be the case (Table V). As expected, lymphocytes from mice infected with aggressive virus functioned in the traditional manner. In fact, the 4- to 5-day interval between adoptive transfer and death was decidedly shorter than our usual experience with recipients infected with aggressive virus. Once again, however,
lymphocytes from mice infected with docile virus failed to kill irradiated recipients (Table V).

Virus elimination resulting from adoptive transfer of splenocytes and MI cells. The data in Tables IV and V clearly indicated that splenocytes and MI cells from mice infected with docile virus lacked killing ability when adoptively transferred into irradiated recipients. On the other hand, these same splenocytes, but not MI cells, would kill unirradiated recipients infected with docile virus shortly before the adoptive transfer (Table III). Because we had known for some time that, regardless of which strain of virus was used, it was the rapidity and extent of virus growth which could determine the disease outcome (3, 5, 8), the effect of splenocyte and MI cells on the virus titers in recipients was examined. Under conditions identical to those used previously (Table III), the data in experiment 1 of Table VI demonstrate precisely what has been found with other LCM virus-mouse strain combinations (13, 14). That is, the T cell component of the docile splenocyte preparations had potent antiviral activity. By comparison, experiment 2 of Table VI shows that the MI cells used in the adoptive transfer (fourfold fewer than the number of splenocytes) appeared to have considerably less antiviral activity (less than one log_{10} unit compared to as much as four log_{10} units in virus reduction).

**DISCUSSION**

The LCM virus-mouse interaction stands as a milestone in viral immunopathology. After i.c. inoculation of LCM into adult mice, extensive virus replication is observed in the meninges, ependyma, and choroid plexus. Seven to 10 days later, the mice develop severe CNS disease and die. It is the immune response against the virus, specifically in the T cell compartment, which causes death (15). Until recently, it has been difficult not to conclude that the CTL is central to the disease process (6), for the following reasons. First, virus-specific CTL activity in the mouse parallels increasing severity of the disease (16, 17). Second, a greatly enhanced population of CTL can be found in the cerebrospinal fluid of mice shortly before death (18). Third, increasing CTL activity in splenocyte populations correlates with increasing ease in demonstrating (by adoptive transfer) their ability to induce fatal meningitis when injected into immunosuppressed recipients (19). Finally, both CTL and the population of cells with in vivo activity recognize virus-infected targets in the context of shared K or D major histocompatibility antigens (19).

Although these benchmark findings have been confirmed repeatedly, it is becoming somewhat unclear as to precisely what functions T cells must possess to cause death. If the CTL function of the virus-specific T cells is indeed the "thrust," it is difficult to understand why little or no damage is seen in the brain cells which support virus replication (20, 21). Furthermore, our results indicating a lack of correlation between CTL activity and in vivo lethal potential (Tables IV and V) also lead one to suspect that CTL activity alone may be insufficient to account for the disease syndrome. Other investigators have recently suggested a key role of the DTH T cell in lethal murine LCM infections (22, 23). At this time, we can neither refute nor support this suggestion.

Regardless of the possibility that T cells with lethal potential may have to possess additional functions beyond their CTL activity, one should consider the case for high virus suppression. Dunlop and Blanden (24) found that high virus environments could suppress LCM-specific CTL function both in vitro and in vivo. Although there is little indication of CTL suppression in our system (Fig. 1; Reference 4), except when exceptionally high doses of virus are used (23, 25; Pfau et al., unpublished observations), one could argue that the rapid and extensive replication of docile virus (5) selectively inhibits only killer T cell function. As yet, we are unable to support this attractive suggestion quite simply because the virus titers in adoptively transferred mice are not high. That is, splenocytes taken at the peak of CTL activity, from mice infected with either aggressive or docile virus, greatly suppress docile virus replication both in nonirradiated (Table VI) and irradiated data not presented) recipients recently infected with docile virus.

Because LCM virus variants are known which suppress CTL function (26), one could entertain the possibility that the docile virus, regardless of its ability to grow to high concentration in the mouse, suppresses (committed) killer T cell function. Again, this seems quite unlikely because splenocytes from mice infected with aggressive virus are perfectly capable of killing irradiated recipients infected with docile virus (Table V).

The potent antiviral activity of splenocytes from mice infected with docile virus (Table VI) may be intimately connected with their ability to cause a typical aggressive virus-like death when adoptively transferred into nonirradiated (but not irradiated) recipients recently infected with the same virus (Table III; Reference 5). Our previous publications showed that manipulation of virus doses could reverse disease outcomes. For example, very high i.c. doses of aggressive virus (10^9 pfu/mouse) spared while very low doses of docile virus (0.3 pfu/mouse) killed (5). Thus, the possibility exists that splenocytes from mice infected with docile virus caused rapid convulsive death of recipients not, as once thought, by their direct killing potential, but rather through their ability to modulate (by limiting virus growth) the recipient's own T cell response.

From another viewpoint, the potent antiviral activity of splenocytes taken from mice infected with docile virus would appear to be a paradox. One might think that such antiviral activity heralds virus clearance, yet virtually all
of these mice remain chronically infected for 6 mo to well over 1 yr (8). This situation is currently under investigation in view of both the changing nature of the virus in LCM persistently infected mice (2, 26), as well as the temporal functional changes in the antiviral activity of LCM-specific T cells (17, 27).

In summary, we have described a system in which LCM virus-specific CTL activity in lymphocyte populations is not accompanied by in vivo killing potential. Although it may still be true that CTL play a central role in the development of lethal CNS disease in mice, the data force us to consider the possibility that additional cell-mediated functions control pathogenesis.

Acknowledgment. We gratefully acknowledge the excellent technical assistance of Thomas Clark.

REFERENCES