

Brief Definitive Report

EFFECTOR T LYMPHOCYTES IN LYMPHOCYTIC CHORIOMENINGITIS VIRUS-INFECTED MICE

Cytolytic Activity of Lyt-23 Spleen Cells In Vitro Does Not Correlate with Elimination of Infectious Virus from Spleens*

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It has been known for a long time that it is predominantly the immune system which enables higher organisms to cope with viruses, and many of the events beginning with recognition of the invading agent and ending with its elimination have been successfully investigated. Others are still unknown and a clear description of the overall mechanism by which a viral infection is terminated has yet to be given. On the basis of experiments performed in vitro, it has been postulated that cytotoxic T lymphocytes (CTL) play a decisive role in host defenses against viruses by destroying infected cells *in situ*, thus controlling the spread of virus and aiding in its elimination (1).

We have examined this proposition by determining the ability of Lyt subsets of T cells to lyse lymphocytic choriomeningitis (LCM) virus-infected cells in vitro and to reduce the concentration of infectious virus in the spleens of infected mice. We found unselected T cells and selected Lyt-23 lymphocytes to be cytotoxic but only unfractionated cells to be able to eliminate virus.

Materials and Methods

Mice. Female C57BL/6J (B6) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine or from the Zentralinstitut für Versuchstiere, Hannover, Federal Republic of Germany. They were used when 8–12 wk old.

Virus. LCM virus, strain WE (2), had been triple plaque purified (3). It was propagated and usually titrated as plaque-forming units (PFU) in L cells (4). In a few experiments, virus concentrations were too low to allow their determination by a plaque assay. In these cases mice were employed (5) which are approximately 10 times more susceptible than L cell monolayers (F. Lehmann-Grube, unpublished observation). Mean infectious dose (ID_{50}) values thus obtained were numerically reduced 10-fold and all virus quantities are expressed as infectious units (IU).

Selection of T Lymphocyte Subsets. Anti-Lyt antisera were prepared according to the method of Shen et al. (6) as detailed in a previous communication (7). Specificities were ensured by determining the ability of selected T cell subsets to proliferate and to give rise to CTL upon allogeneic stimulation in a mixed lymphocyte culture. Several experiments were performed,

* Supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg. The Heinrich-Pette-Institut is financially supported by Freie und Hansestadt Hamburg and Bundesministerium für Jugend, Familie und Gesundheit, Bonn, and the Krebsforschungszentrum by Bundesministerium für Forschung und Technologie, Bonn.

each showing that Lyt-1 cells from B6 (H-2^b) mice infected 7 d previously by i.v. inoculation of 10² IU of LCM virus proliferated as well as unselected cells when stimulated by x-irradiated DBA/2 (H-2^d) spleen cells but did not develop cytotoxic activity against mouse mastocytoma P815 (H-2^d) cells, whereas Lyt-23 cells were completely nonresponsive. Mixtures of Lyt-1 plus Lyt-23 cells exhibited both activities, i.e., they proliferated and generated CTL (data not shown). These observations demonstrate that our anti-Lyt antisera had the desired specificities. Spleen cells, either untreated or enriched for T cells by use of nylon wool columns (8), were counted and 3×10^7 trypan blue-excluding (viable) cells/ml were incubated for 30 min at room temperature with either anti-Lyt-1.2 antiserum (final dilution 1:10) or anti-Lyt-2.2 antiserum (1:10) plus anti-Lyt-3.2 antiserum (1:40). The cells were washed, resuspended in the same volume of appropriately diluted (1:11) complement (C) (rabbit serum selected on the basis of high lytic activity in connection with anti-Lyt antisera and low toxicity for murine spleen lymphocytes), and incubated for 30 min at 37°C. Initially, this procedure was repeated once, but later it was realized that one such treatment sufficed to select for the appropriate Lyt subset. Volumes were adjusted to those of the C controls, meaning that the numbers of cells in subsets were kept equal to the numbers of corresponding cells in the untreated population. In mixtures of Lyt-1 and Lyt-23 lymphocytes the concentrations of both subclasses corresponded to the ones in unselected populations.

Adoptive Transfers. Mice were infected by i.v. inoculation of 10² IU of virus. 7 d later their spleen cells were dispersed and enriched for T cells on nylon wool columns (day-7-immune T cells). Groups of five mice were inoculated i.v. with 10² IU of virus and 16 h later by the same route with 5×10^5 untreated or corresponding numbers of selected immune T cells. After an interval of 40 h, individual spleens were homogenized and the virus concentrations per gram of tissue were determined (9).

LCM Virus-specific Cytotoxic T Cell Activity. Mice were infected i.v. with 10² IU of LCM virus and the cytotoxic activities of their spleen cells tested 8 d later. Target cells were cultivated LCM virus-infected B6 fetal fibroblastic cells that had been dispersed with trypsin and labeled with ⁵¹sodium chromate (Amersham Buchler, Braunschweig, Federal Republic of Germany). They were counted and dispensed into the flat-bottomed wells of tissue culture microtiter plates (Cooke Engineering, Alexandria, Va.), each cavity receiving 10⁴ cells in 0.1 ml of Eagle's minimum essential medium supplemented with nonessential amino acids and 5% fetal calf serum. Untreated spleen cells were added at indicated effector:target cell ratios. As in all these experiments, effector cells of selected populations were adjusted to volumes equivalent to the ones of C controls. The assay time was 12 h, and the specific release was calculated as $100 \times (a - b)/(c - b)$, where a equals ⁵¹Cr release from target cells in the presence of effector cells; b, release from target cells alone (spontaneous release); and c, release from target cells dissolved by the addition of detergent (maximum release).

Results

Lyt Phenotype of LCM Virus-specific CTL. Mice were infected with LCM virus and 7-8 d later their spleen cells or purified T cells were selected on the basis of Lyt surface antigens. The lymphocyte subsets were then tested for cytotoxic activity in vitro. One out of three experiments using day-8-immune T cells with similar results is depicted in Fig. 1. Virus-specific cytotoxic activity of selected Lyt-23 cells was reduced in comparison with unselected cells. As expected, essentially no cytotoxicity was associated with Lyt-1 cells. Mixtures consisting of Lyt-1 plus Lyt-23 cells were usually more active than Lyt-23 cells alone. Thus, considerable numbers of CTL in spleen cells of LCM virus-primed mice reside in the Lyt-23 T cell subset and their lytic activity is enhanced by Lyt-1 lymphocytes. Similar findings were obtained with day-7-immune T cells.

Dose-Response Relationship Between Transferred Immune Spleen T Lymphocytes and Elimination of Virus from Infected Mice. Treatment with cytotoxic antisera reduces the number of viable cells, and it could be argued that the inability of selected T cell

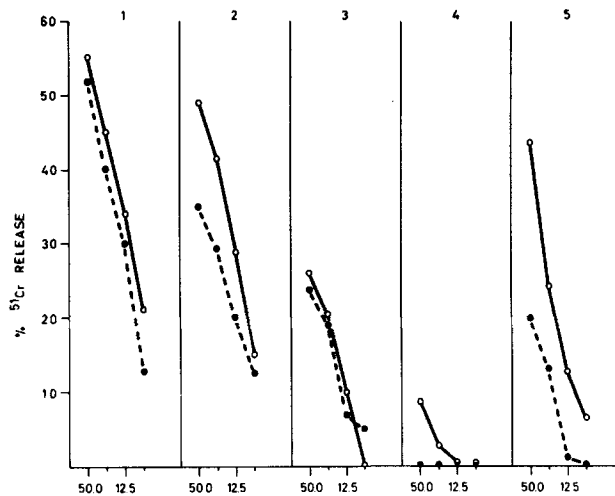


FIG. 1. Effect of anti-Lyt antisera plus C on cytotoxic activity in vitro of day-8-immune T cells from B6 mice on virus-infected syngeneic fibroblastic cells. Spleen cells enriched for T cells before treatment with anti-Lyt antisera, \circ ; spleen cells not enriched for T cells before treatment with anti-Lyt antisera, \bullet . Abscissa: effector:target cell ratio of cells calculated on the basis of numbers counted before treatment with antisera. 1, untreated cells; 2, C-treated cells; 3, cells treated with anti-Lyt-1.2 antiserum plus C (Lyt-23 cells); 4, cells treated with anti-Lyt-2.2 and anti-Lyt-3.2 antisera plus C (Lyt-1 cells); 5, mixture of Lyt-23 and Lyt-1 cells.

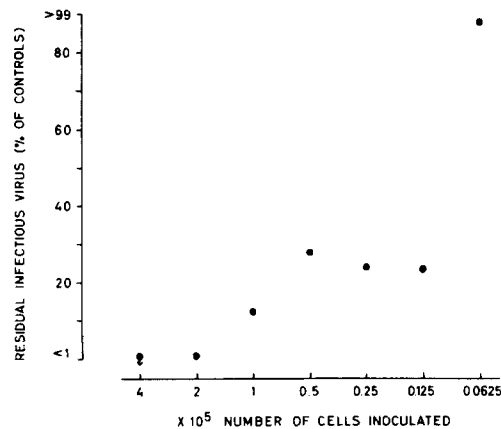


FIG. 2. Dose-response relationship in LCM virus-infected B6 mice between intravenously inoculated day-7-immune spleen T cells and concentration of infectious virus in spleens.

subsets to eliminate virus (see below) was a result of reduced absolute cell numbers rather than selective removal of particular effector cells. Therefore, diminished numbers of day-7-immune T cells were tested for their ability to affect virus concentrations in the spleens of mice. One of two experiments with essentially identical results is presented in Fig. 2. The results show that as few as 2×10^5 immune T lymphocytes reduced infectious virus by $>99\%$ and that even lower numbers were quite effective in this respect. Because participation of Lyt T cell subsets from immune mice in the elimination of virus from spleens of infected mice was examined with 5×10^5 unselected T cells or the corresponding numbers of Lyt-1 or Lyt-23 cells, the

TABLE I
*LCM Virus Concentrations in Spleens of B6 Mice Adoptively Immunized with Spleen T Lymphocytes from B6 Donor Mice, Unselected and Selected for Lyt Subpopulations**

Experiment	Unselected		Selected			Virus only
	Untreated	C treated	Lyt-23	Lyt-1	Lyt-23 + Lyt-1	
I	0.007 ± 0.004‡ (5)§	0.011 ± 0.018 (5)	4.879 ± 1.446 (5)	11.790 ± 6.797 (5)	3.867 ± 1.469 (5)	8.325 ± 1.688 (5)
II	0.079 ± 0.081 (5)	0.248 ± 0.339 (5)	9.709 ± 1.998 (5)	5.192 ± 3.462 (5)	5.618 ± 1.679 (5)	16.702 ± 8.785 (5)
III	0.026 ± 0.021 (5)	0.258 ± 0.451 (4)	3.722 ± 2.052 (4)	7.288 ± 3.004 (5)	4.479 ± 2.999 (5)	6.243 ± 1.594 (5)

* Donor mice were immunized 7 d before cell transfer by i.v. inoculation of 10^2 IU of LCM virus. Recipient mice were infected intravenously with 10^2 IU; they received 5×10^5 unselected or the equivalent of selected T cell-enriched spleen cells 16 h later, and spleen virus concentrations were determined after a further 40 h.

‡ IU/g of spleen; mean ± 1 SD × 10^6 .

§ Number of mice in sample is in parentheses.

concentrations of putative effector cells in these experiments should suffice to demonstrate marked reductions of virus titers.

Participation of Lyt T Cell Subsets in the Elimination of Virus from Spleens of Infected Mice. The ability of day-7-immune unselected T cells or selected T cell subsets to reduce virus concentrations in spleens of recipient mice was tested in three experiments (Table I). Whereas unselected as well as C treated spleen T cells were always markedly effective, the reduction of virus by selected Lyt-1 or Lyt-23 T cell subsets or mixtures of both was marginal and not preferentially associated with one of the two subpopulations. Thus, the CTL present in the Lyt-23 subset of spleen T cells did not contribute to the elimination of infectious virus from the spleen.

Discussion

For the control of viruses in mice T cells are required. Based on evidence obtained with a number of viral agents as dissimilar as ectromelia virus (10), influenza virus (11), and LCM virus (12), this statement is probably generally true. How T cells interfere with virus replication is not known and the question of whether they participate as effector cells or regulator cells or both can only be answered hypothetically. According to one such hypothesis, cytotoxic T lymphocytes recognize virus-infected cells and destroy them *in vivo* just as they do *in vitro*; destruction early in the viral growth cycle prevents maturation of progeny (1).

Our experiments show that in the LCM virus-infected mouse, unselected as well as selected Lyt-23 T lymphocytes are cytolytic for infected target cells *in vitro*, but that only the untreated population is able to eliminate virus from infected mice. Dose-response experiments demonstrate that the inability of selected T cell subsets to reduce virus concentrations in the spleens of recipient mice was not due to reduced cell numbers but to selective removal of effector cells. Thus, the expected correlation between cytolytic activity of virus immune T cells and their capacity to eliminate virus from the infected host could not be demonstrated. Yap et al. (11), who asked the same question, arrived at a different answer by concluding that cytotoxic T cells did protect mice by aiding in elimination of the virus. Their experimental protocol, however, deviated from the one employed by us in three major respects: (a) Their agent was the influenza virus; (b) the cytotoxic potential of the Lyt-23 cells shown to be effective in reducing virus concentrations in the lungs of recipient mice was not determined directly but was inferred from the requirement of H-2K and H-2D

compatibility with the donors; and (c) immune cells were raised in vitro by restimulating spleen cells from mice primed with the virus 21 d previously. Thus, a direct comparison between our results and those of Yap et al. is not possible.

In the present experiments it was apparently the Lyt-123 subset of T lymphocytes that suppressed the replication of LCM virus in the mouse. Whether this effect was achieved by noncytolytic Lyt-123 cells or by cytolytic elements contained in this subset is not revealed by our data. Perhaps Lyt-123 and Lyt-23 T lymphocytes represent two maturational stages of CTL, only one of which can elicit the effector function in vivo studied by us. There are other explanations. One is that isolated Lyt-23 CTL have altered homing properties and migrate to organs of the recipient mouse other than the spleen. It is also conceivable that the Lyt-23 T lymphocytes cannot express their cytolytic potential in the recipient mouse because of suppression by some mechanism attributable to the experimental conditions. Thus, our observations do not formally prove that CTL are not the last link in the chain of events resulting in termination of the viral infection. They do stress, however, the need for additional evidence to demonstrate that removal of virus from an organism is associated with destruction of tissue cells. The observation of Volkert and Hannover Larsen ([13]; and M. Volkert, personal communication), as confirmed by others ([14]; and F. Lehmann-Grube, unpublished observation), that adoptive immunization of congenital or neonatal LCM virus carrier mice results in the rapid elimination of infectious virus from probably all organs without causing pathological alterations either morphologically or functionally, points to a mechanism of virus elimination other than lysis of infected cells, at least in the LCM virus-infected mouse.

Summary

The T lymphocytes from mice recovering from infection with lymphocytic choriomeningitis virus were selected for subclasses by treatment with anti-Lyt antisera and complement. Lyt-23 cells and mixtures of Lyt-1 and Lyt-23 cells caused up to one-half the destruction of cultivated target cells as compared with untreated T lymphocytes; Lyt-1 cells alone were not cytotoxic. Selected and unselected spleen T cells were also inoculated intravenously into previously infected mice. Whereas unselected cells reduced infectious virus in the spleens of the recipients ~100-fold, only marginal effects, which were not preferentially associated with one particular subclass, were seen with selected Lyt-23 or Lyt-1 lymphocytes or a mixture of both. Apparently the Lyt-23 cells, shown to be cytolytic for infected cells in vitro, did not cause elimination of a measurable quantity of the virus from mice.

We thank Mrs. Magdalena Kallay and Mrs. Ursula Schaefer for expert technical assistance.

Received for publication 10 November 1980.

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