

Brief Definitive Reports

CYTOTOXIC THYMUS-DERIVED LYMPHOCYTES IN CEREBROSPINAL FLUID OF MICE WITH LYMPHOCYTIC CHORIOMENINGITIS

BY R. M. ZINKERNAGEL* AND P. C. DOHERTY

(From The Department of Microbiology, The John Curtin School of Medical Research,
Canberra A. C. T., Australia)

(Received for publication 6 August 1973)

The fatal neurological disease occurring in adult mice injected intracerebrally (IC) with lymphocytic choriomeningitis (LCM) virus is apparently induced by massive invasion of leukocytes (1, 2) into the central nervous system (CNS). Dosing with antithymocyte serum or cyclophosphamide (2, 3) prevents cellular infiltration: mice survive as asymptomatic carriers. Adoptive induction of this inflammatory process, by injecting immune spleen cells into immunosuppressed recipients, depends on presence of specific thymus-derived lymphocytes (T cells) in the transferred cell population (4). Such lymphocytes can now be assayed *in vitro* by measuring release of radioactivity from ^{51}Cr -labeled target cells infected with LCM virus (5, 6). The present paper gives evidence that these cytotoxic T cells are found in cerebrospinal fluid (CSF) of mice with clinical LCM. This is, to our knowledge, the first direct demonstration of specifically sensitized lymphocytes in a virus-induced inflammatory exudate.

Materials and Methods

Experimental.—8–10-wk old CBA/H male mice were injected IC with 300 mouse IC LD₅₀ of the WE3 strain of LCM virus (1). This represents a 10^{-5} dilution of infected guinea pig lung. Two experiments were done: (a) sequential development of cytotoxicity in spleen was assessed by inoculating groups of three animals each for 7 days. All mice were killed on the same day and activities of spleen cell suspensions (7) were determined simultaneously in one assay; (b) 60 mice were injected with virus and killed, by ether anesthesia and exsanguination, when clinically affected 7 days later. Blood was defibrinated by rotation in the presence of glass beads and leukocytes were separated (8) on a Ficoll (Pharmacia, Uppsala, Sweden) gradient. Spleens were removed and samples of CSF were withdrawn from the cisterna magna (9), yielding 5–10 μl (containing approximately 100,000 inflammatory cells) from each mouse. Cells from CSF were pooled, washed once in 30.0 ml of medium (see below), and pelleted. Aliquots were treated with AKR anti- θ ascitic fluid, or normal AKR ascitic fluid, and agarose absorbed guinea pig complement (7). Cell viability was determined by Trypan blue exclusion. All counts cited are for viable cells, viability of untreated cells from all sources being invariably greater than 85%.

^{51}Cr Release Assay.—The medium used throughout was Eagle's minimal essential medium plus nonessential amino acid (F15, Grand Island Biochemical Co., Grand Island, N. Y.)

* Dr. Zinkernagel is supported by the Schweizerische Stiftung fuer Biologisch Medizinische Stipendien.

incorporating, except for dilution of ^{51}Cr , 10% heat inactivated fetal calf serum. Monolayers of L929 cells (L cells) were grown in flat bottomed plastic tissue culture trays (Linbro Chemical Co., New Haven, Conn.). Two types were used, one with a well diameter of 16 mm (3×10^5 target cells) and the other of 6 mm (4×10^4 target cells). Use of the 6 mm wells was necessitated by the small numbers of cells obtained from CSF. Target cells were grown for 24 h, infected with a high dose ($1.5 \times 10^6 \text{ LD}_{50}$) of WE3 LCM virus in guinea pig spleen suspension, and labeled with 2 μCi of ^{51}Cr (C. A. E., Gif-sur Yvette, France) after a further 24 h. Control monolayers were either left uninfected, or infected with 5×10^7 PFU of the Moscow strain of ectromelia virus at 6 h after labeling.¹ All monolayers were washed thoroughly (four times) to remove excess ^{51}Cr and layered with the cell suspension to be tested, dispensed in either 1.5 ml or 0.3 ml of medium. Assays were incubated for 17 h at 37°C in an atmosphere of 10% CO_2 and 7% O_2 in N_2 . Supernates were then gently aspirated and monolayers were lysed with water. Percent of ^{51}Cr release was determined as, $(\text{Counts in supernate} \times 100) / (\text{Counts in supernate} + \text{counts in water lysate})$. This value was corrected by a factor of 100/75, which takes into account that the mean amount of label maximally released by water lysis of infected target cells is 75%.

RESULTS AND DISCUSSION

Only a small proportion of an IC inoculum (<10%) remains in the CNS (10). The majority of virus injected escapes directly into the blood. Splenic lymphoid tissue should thus be rapidly exposed to viral antigen, the initial low dose being greatly reinforced by viral replication within the organ (1). Significant cytotoxic activity was first detected in spleen at 6 days after inoculation and reached a very high level by the following day (Fig. 1), when all mice showed obvious signs of neurological impairment. Onset of symptoms thus parallels development of cytotoxic cells in lymphoid tissue.

CSF from clinically affected mice contained cells which were cytotoxic for L cells infected with LCM virus (Table I). Activity was destroyed by treatment with AKR anti- θ ascitic fluid and complement (which killed 60% of cells), but not by normal AKR ascitic fluid and complement. Release of ^{51}Cr was apparently due to presence of activated thymus-derived lymphocytes. These T cells are specific (Table II) insofar as they had no activity against either normal L cells or L cells infected with ectromelia virus, which are readily lysed by lymphocytes from ectromelia immune mice.¹

Presumably cytotoxic T cells are generated in spleen² and circulate in blood (Table II). At least some of these lymphocytes localize in the CSF. Many more sensitized cells probably enter other organs e.g., lung and liver (11). Cell populations from CSF exhibit greater cytotoxic activity than do blood leukocytes (Table I), an indication that specifically committed lymphocytes may be concentrated in the inflammatory exudate. Also, the minimal contamination with blood (<5%) that may occur during sampling (9) would have no significant effect on activity of CSF cells.

¹ Gardner, I., N. A. Bower, and R. V. Blanden. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. Manuscript in preparation.

² Doherty, P. C., R. M. Zinkernagel, and I. A. Ramshaw. Cell-mediated cytotoxicity in lymphocytic choriomeningitis. II. Development and specificity of thymus-derived effector lymphocytes. Manuscript in preparation.

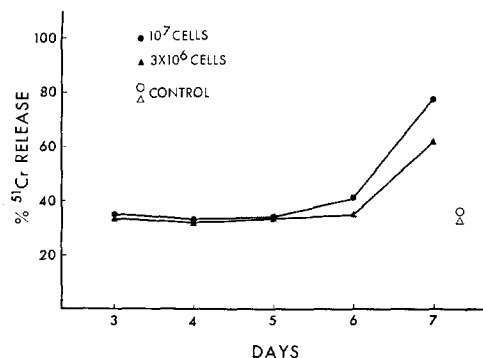


FIG. 1. Sequential development of cytotoxic activity in spleens from mice injected IC with 300 LD₅₀ of WE3 LCM virus. Spleen suspensions were assayed on 3×10^5 target L cells infected with LCM virus. Levels of ⁵¹Cr release by immune cells were significantly greater than those caused by normal spleen cells on days 6 ($P < 0.01$) and 7 ($P < 0.001$), for 1×10^7 cells, and day 7 ($P < 0.001$) for 3×10^6 cells.

TABLE I

Cytotoxic Activity of 1.5×10^5 Viable Cells for 4×10^4 Target L Cells Infected with LCM Virus

Source	Inoculum	Treatment	% ⁵¹ Cr release	P values
	LCM	Nil	49.0 ± 0.5	
CSF	LCM	Anti-θ*	27.7 ± 1.3	<0.001
	LCM	N ascitic*	43.7 ± 0.5	<0.001
Blood	LCM	Nil	36.5 ± 1.1‡	
Spleen	LCM	Nil	41.2 ± 1.5	<0.01
	Nil	Nil	29.5 ± 0.8	

* Treated with AKR anti-θ ascitic fluid and complement, or normal AKR ascitic fluid and complement.

‡ This value is not significantly different from background release caused by 1.5×10^6 blood cells (Table II).

TABLE II

Specificity of Cytotoxic Cells

Source	Cells		% ⁵¹ Cr release from 4×10^4 L cells		
	Inoculum	No.	LCM	Ectromelia	Normal
CSF	LCM	1.5×10^5	49.0 ± 0.5*	22.5 ± 0.4	24.5 ± 1.1
Blood	LCM	1.5×10^6	68.2 ± 1.1*	—	36.1 ± 1.1‡
Spleen	LCM	1.5×10^6	56.7 ± 0.5*	19.0 ± 0.3	22.5 ± 0.7
	Nil	1.5×10^6	23.7 ± 0.5	—	—

* Significantly greater ($P < 0.001$) than release from normal targets, or by control spleen cells on infected targets.

‡ This high release from normal targets may be due to presence of large numbers of contaminating red blood cells.

Most virus in CNS is localized, by immunofluorescence, in cells of the choroid plexus, ependyma, and meninges (2). Direct cell-mediated damage to cells expressing LCM viral antigen would offer some explanation for the pathogenesis of acute LCM (1). There is, however, no rigorous proof that such a process occurs in vivo.

The data presented here support the following conclusions: (a) sensitized thymus-derived lymphocytes are invasive; (b) capacity to release ^{51}Cr in vitro is not conferred on non- θ -bearing cells in the inflammatory exudate; (c) cytotoxic potential is not readily exhausted, either by the process of entry into CSF or by the large amounts of viral antigen present in cells lining the CSF spaces (2).

REFERENCES

1. Lehmann-Grube, F. 1971. Lymphocytic Choriomeningitis Virus. *Virol. Mongr.* **10**:1.
2. Gilden, D. H., G. A. Cole, A. A. Monjan, and N. Nathanson. 1972. Immunopathogenesis of acute central nervous system disease produced by lymphocytic choriomeningitis virus. I. Cyclophosphamide-mediated induction of the virus-carrier state in adult mice. *J. Exp. Med.* **135**:860.
3. Lundstedt, C., and M. Volkert. 1967. Studies on immunological tolerance to LCM virus 8. Induction of tolerance to the virus in adult mice treated with anti-lymphocytic serum. *Acta Pathol. Microbiol. Scand.* **71**:471.
4. Cole, G. A., N. Nathanson, and R. A. Prendergast. 1972. Requirement for θ -bearing cells in lymphocytic choriomeningitis virus-induced central nervous system disease. *Nature (Lond.)*. **238**:335.
5. Marker, O., and M. Volkert. 1973. Studies on cell-mediated immunity to lymphocytic choriomeningitis in mice. *J. Exp. Med.* **137**:1511.
6. Cole, G. A., R. A. Prendergast, and C. S. Henney. 1972. Proceedings of the symposium on lymphocytic choriomeningitis and other arenoviruses. F. Lehmann-Grube, editor. Hamburg. In press.
7. Blanden, R. V., and R. E. Langman, 1972. Cell-mediated immunity to bacterial infection in the mouse. Thymus-derived cells as effectors of acquired resistance to *Listeria monocytogenes*. *Scand. J. Immunol.* **1**:379.
8. Thorsby, E., and A. Bratlie. 1970. A rapid method for preparation of pure lymphocyte suspensions. In *Histocompatibility Testing*. P. I. Terasaki, editor. Munksgaard, Copenhagen. 655.
9. Doherty, P. C. 1973. Quantitative studies of the inflammatory process in fatal viral meningoencephalitis. *Am. J. Pathol.* In press.
10. Mims, C. A. 1960. Intracerebral injections and the growth of viruses in the mouse brain. *Br. J. Exp. Pathol.* **41**:52.
11. Mims, C. A., and R. V. Blanden, 1972. Antiviral action of immune lymphocytes in mice infected with lymphocytic choriomeningitis virus. *Infect. Immun.* **6**:695.