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Immunological tolerance to lymphocytic choriomeningitis virus in neonatally infected virus carrier mice: evidence supporting a clonal inactivation mechanism

J. CIHAK & F. LEHMANN-GRUBE *Heinrich-Pette-Institut für experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse* 52, 2000 *Hamburg* 20, *Federal Republic of Germany*

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Summary. Previous studies have shown that no cell-mediated immunity against LCM virus-infected cells can be detected in neonatally established LCM virus carrier mice suggesting that they are immunologically tolerant to virally-altered cell membrane antigens. In this communication experiments are described aimed at analyzing the mechanism. Virus-specific cell-mediated immunity was assessed by ^{51}Cr release and target cell reduction assays. Attempts to demonstrate cells in spleens of CBA/J carrier mice able to suppress in syngeneic recipients the induction or the effector phase of the cytotoxic T-cell response against LCM virus-infected cells were unsuccessful. Also, no factors were detected in CBA/J and C57BL/6J carrier mice, either spleen cell-associated or free in the circulation, which would block the activity of cytotoxic T-lymphocytes against LCM virus-infected syngeneic target cells. The results indicate that inability of LCM virus carrier mice to act immunologically against virus-infected target cells is due to deletion or irreversible inactivation of T lymphocytes carrying receptors for virally altered cell membrane antigens.

Correspondence: Dr F. Lehmann-Grube, Heinrich-Pette-Institut für experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany.

INTRODUCTION

When an adult mouse is infected with lymphocytic choriomeningitis (LCM) virus, it may die. If it survives, the virus is eliminated and resistance against re-infection and disease develops. In contrast, when a mouse is infected *in utero* or shortly after birth, it remains healthy and the virus persists life-long (Traub, 1973). In such an animal both cell-mediated and humoral immune responses to most other antigens are normal (Lehmann-Grube, 1971; Oldstone, Tishon, Chiller, Weigle & Dixon, 1973).

The general belief that persistence of the virus is made possible by immunological tolerance (Hotchin, 1971; Lehmann-Grube, 1971; Volkert & Lundstedt, 1971) was questioned (Oldstone & Dixon, 1969) when detection of virus-specific antibodies (Benson & Hotchin, 1969; Oldstone & Dixon, 1969) and cell-mediated immunity (Oldstone & Dixon, 1971) in carrier mice was reported. More recent data, however, fully support the original hypothesis. Volkert *et al.* (Volkert, Bro-Jørgensen, Marker, Rubin & Trier, 1975) have shown that in C3H LCM virus carrier mice, T helper or B lymphocytes sensitized against LCM virus complement-fixing antigen were absent. We found that spleen cells from neonatally-infected LCM virus carrier NMRI

colony-bred and CBA/Ca inbred mice were free of cytotoxic activity against LCM virus-infected target cells, whereas spleen cells from virus-immune mice were highly active. Also, spleen cells from carrier mice were not stimulated to take up tritiated thymidine *in vitro* by incubating them with syngeneic LCM virus-infected and mitomycin-treated cells, which again contrasted with the stimulating effect virus-infected cells had on spleen cells from immunized mice (Cihak, Cihak & Lehmann-Grube, 1974; Cihak & Lehmann-Grube, 1974). Other authors also failed to detect virus-specific cell-mediated immunity in LCM virus carrier mice (Cole, Prendergast & Henney, 1973; Marker & Volkert, 1973; Zinkernagel & Doherty, 1974).

While all these data favour the interpretation that virus persistence is a consequence of immunological tolerance, they do not explain the underlying mechanism. According to the clonal selection theory Burnet (1959), deletion of cell clones is the basis of immunological tolerance, and many examples have become known which are best explained by assuming such a mechanism (Howard & Mitchison, 1975). However, there are other possibilities. In some forms of tolerance suppressor cells have been detected suggesting that tolerance may be the consequence of a particular type of cell-to-cell interaction resulting in active inhibition of the immune response (Gershon & Kondo, 1971; Basten, Miller, Sprent & Cheers, 1974; Kölsch, Stumpf & Weber, 1975). Other cases have become known in which inhibition of potentially active immune cells by blocking factors appears to be decisive (Hellström & Hellström, 1974). Experiments have been conducted to find out whether LCM virus-specific immunological tolerance in carrier mice is brought about by elimination or inactivation of cell clones or whether blocking factors or suppressor cells play a role.

In our previous studies, cytotoxicity as an expression of cell-mediated immunity was measured by incubating spleen cells for 48 h with target cells and then counting the latter. This method was also employed here. However, in order to make the data directly comparable with the chromium release test, most experiments were also done employing this more widely used assay.

MATERIALS AND METHODS

Virus

LCM virus, strain WE (Rivers & Scott, 1936), was used throughout. LD_{50} and ID_{50} were determined in weanling mice (Lehmann-Grube, 1964a).

Mice

Inbred CBA/J, SJL/J, and DBA/1 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, CBA/Ca mice from Animal Suppliers, London, and C57BL/6J (C57) mice from Gl. Bomholdgård, Ry, Denmark. BALB/c mice were bred and kindly provided by Dr E. Kölsch from this institute.

Persistent infection was induced by infecting mice at the age of less than 24 h intraperitoneally with 10^4 ID_{50} of virus (Lehmann-Grube, 1964b). Carrier mice were always used when older than 2 months. Viremia was ascertained for each individual mouse never longer than 14 days prior to its experimental use.

Unless stated otherwise, immunization of adult mice was done by inoculating subcutaneously or intravenously 100 ID_{50} of virus.

Methods for measuring cytotoxic activity of lymphocytes

Target cell reduction assay. Target cells were LCM virus-infected secondary mouse foetus fibroblastic cells grown in 3·5 cm petri plates with medium consisting of Eagle's minimum essential medium (MEM) plus non-essential amino acids (Lockart & Eagle, 1959) and 10% heated calf serum. They were incubated at 37° in a CO_2-gassed and humidified incubator and were infected at multiplicities of 0·01–0·1 ID_{50} per cell. Cultures were used 48 h later when they contained approximately 2×10^5 cells. Spleen cells, syngeneic with target cells, were obtained from immune mice, carrier mice, and control mice by teasing the organs in MEM. Clumps were allowed to settle and the cells in 4 ml of MEM supplemented with 10% heated foetal calf serum were added to duplicate cultures at ratios of 100 erythrosin-excluding nucleated spleen cells per one target cell. After 48 h of co-cultivation at 37° the remaining target cells were determined by counting their nuclei set free by treatment with 0·1% tween 80 in 0·1 M citric acid.

^{51}Cr release assay. Target cells were monolayer-grown L 929 (H-2^k) cells infected for 48 h with LCM virus at multiplicities 0·1–0·01. For labelling with ^{51}Cr-chromate (Amersham Buchler, Braunschweig,

Fed. Rep. Germany) they were dispersed with trypsin, washed, and resuspended in assay medium consisting of MEM supplemented with nonessential amino acids and 10% foetal calf serum. Two $\times 10^6$ cells together with 25 μCi ^{51}Cr in 0·25 ml of assay medium were incubated for 30 min at 37°. The cells were washed three times and dispensed into wells of microtitre plates, surface treated for cell culture purposes (Greiner, Nürtingen, Federal Republic of Germany), each well receiving $2{\cdot}5 \times 10^4$ cells in 100 μl of medium. Spleen cells from CBA/J (H-2^k) mice, prepared as for the target cell reduction assay, were then added to wells in triplicate, each one receiving 8×10^5 cells in 100 μl of assay medium, which corresponds to a ratio spleen cells to target cells of 32 : 1. The plates were incubated for 16 h at 37° under 5% CO_2, whereupon 100 μl supernatant medium were removed from each well to be counted in a gamma scintillation spectrometer (Packard, Downers Grove, Ill., U.S.A.). Total release was determined by hypotonic lysis of target cells in four microcultures, and spontaneous release in four cultures containing target cells but no spleen cells. Calculation of percentage ^{51}Cr release was based on the formula:

$$\% \, ^{51}\text{Cr release} = \frac{\text{release in the presence of spleen cells} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

^{51}Cr release from virus-infected target cells in the presence of normal spleen cells was between 0 and 19%.

Removal of T lymphocytes, B lymphocytes, or macrophages from spleen cell suspensions

Anti-theta antiserum was prepared according to Reif & Allen (1966). Absorption with CBA/J mouse brain completely abolished its activity against thymus cells, which confirms its specificity. Use of anti-theta antiserum to eliminate theta-bearing cells has been described (Cihak & Lehmann-Grube, 1974).

Immunoglobulin (Ig) bearing cells were eliminated by treatment with anti-mouse Ig antiserum and complement. Mouse Ig was prepared from serum by precipitation with ammonium sulphate. Rabbits were immunized by intramuscular inoculation of 8 mg Ig emulsified with Freund's complete adjuvant, followed after 2 months by inoculation of 5 mg Ig precipitated with alum (Kabat & Mayer, 1961). Two weeks later the animals were bled. The antiserum was extensively absorbed with thymocytes from CBA/J mice, as was a normal rabbit serum for control purposes. Spleen cells were dispersed and 8×10^7 nucleated cells were suspended in 0·8 ml anti-mouse Ig antiserum, diluted twofold with Hanks' balanced salt solution (BSS) to be incubated for 30 min at 37°. Cells were then centrifuged and resuspended in 2 ml of agarose-absorbed five fold diluted guinea-pig serum (Cohen & Schlesinger, 1970). Incubation was continued for 30 min, cells were washed twice, and were used at ratios 100 : 1 in the target cell reduction test. Treatment with antiserum and complement resulted in loss of viability (as judged from uptake of erythrosin) in 40% of the cells, while treatment with normal rabbit serum and complement had no effect. Viability of CBA/J thymocytes was not affected by incubation with antiserum plus complement.

The methods of removing adhering and phagocytic cells have been described (Cihak & Lehmann-Grube, 1974).

Cell transfer

Suspensions of spleen or thymus cells were prepared as for the cytotoxicity assay. They were washed three times with BSS or MEM. Cells considered viable from their ability to exclude erythrosin B (Merchant, Kahn & Murphy, 1964) were counted and suspensions injected into recipient mice by the intravenous route. Cells were always kept at 0–4°.

Pre-incubation of spleen cells in vitro

Spleen cells were suspended in culture medium at a concentration of 5×10^6 cells/ml and 5 ml were incubated in 5 cm petri plates for 20 h under an atmosphere humidified and CO_2-gassed such that the pH was maintained at 7·2. They were then washed and resuspended in medium to be used in the cytotoxicity assay.

RESULTS

Identification of cells responsible for cytotoxic activity against LCM virus-infected target cells

During this work two different procedures were employed. For the chromium release test it has been shown that target cell killing is done solely by T cells; B cells or macrophages do not participate to a measurable degree (Doherty, Zinkernagel & Ram-

Table 1. Effect of treatment with anti-θ antiserum and complement of spleen cells from LCM virus-immune CBA/Ca mice upon their cytotoxicity for LCM virus-infected CBA/Ca fibroblastic cells, measured with the target cell reduction assay

Treatment of spleen cells	% Reduction of target cell number* Days after immunizing infection 9	18	45	188
Normal AKR serum +complement	84	96	86	27
AKR anti-θ serum +complement	−29	0	−35	−24
AKR anti-θ serum (no complement)	n.d.†	97	n.d.	n.d.

* Percentage reduction of number of target cells after cultivation together with spleen cells from experimental mice as compared with number of target cells after cultivation with spleen cells from normal control mice. Minus signs denote higher number of target cells in experimental cultures than in control cultures.

† Not determined.

shaw, 1974). Extending our previously published results, the data reported here show that the specific cytotoxic reduction of the number of LCM virus-infected target cells by incubation for 48 h with LCM virus-immune syngeneic spleen cells is abolished by treatment with anti-theta antiserum plus guinea-pig complement (Table 1) and is not influenced by removing macrophages or B lymphocytes (Table 2). Thus, the cytotoxic activity of immune spleen cells against LCM virus-infected syngeneic target cells is in both types of assay a function of T lymphocytes.

Table 2. Effect of removal of macrophages or of immunoglobulin bearing cells on cytotoxic activity of spleen cells from LCM virus-immune CBA/Ca mice, measured with the target cell reduction assay

Exp. no.	Treatment of spleen cells	% Reduction of target cell number
1	None	77
	Iron + magnet	80
	Adsorption on glass wool	89
	Adsorption on petri plates	83
2	Normal rabbit serum +complement	60
	Rabbit anti-mouse Ig anti-serum + complement	55

Table 3. Effect of spleen cells from normal, LCM virus-immune, and persistently infected CBA/J mice on LCM virus-infected L 929 cells, measured with the ^{51}Cr release assay

Spleen cells	% ^{51}Cr release Exp. 1	Exp. 2	Exp. 3	Exp. 4
Normal	11	17	n.d.	n.d.
Immune*	94	96	100	98
Carrier†	3	8	6	3

* Pooled spleen cells from two mice immunized by intravenous infection with 10^2 ID_{50} of LCM virus 7 days (experiments 1, 3, 4) and 9 days (experiment 2) prior to the test.

† Pooled spleen cells from two virus carrier mice 8–12 weeks old. In experiment 3 carrier mice were challenged by intravenous inoculation of 10^2 ID_{50} LCM virus 7 days prior to the test. In experiment 4 carrier mice were challenged with 10^7 ID_{50} 6 days prior to the test.

Search for specific cytotoxic T lymphocytes in the spleens of LCM virus carrier mice

We have previously shown that spleen cells from CBA/Ca and NMRI carrier mice are free of cytotoxic activity against LCM virus-infected cells as determined by the target cell reduction assay (Cihak & Lehmann-Grube, 1974). The same was found to be true with CBA/J and C57 carrier mice when cytotoxic activity was measured by chromium release and target cell reduction assays. Data illustrating this statement are presented in Tables 3 and 6.

Search for blocking factors

Attempts were made to inhibit the effect of spleen cells from LCM virus-immune C57 and CBA/J mice on LCM virus-infected syngeneic target cells with serum from carrier mice. Regardless of concentration of serum and whether target cells or effector cells were treated, the results were negative in both the target cell reduction test (Table 4) and the ^{51}Cr release test (Table 5). The moderate reduction of cytotoxicity after treatment of target cells or immune spleen cells with serum from virus carrier mice in some experiments was obviously nonspecific because normal serum had the same effect.

Next we tried to remove a hypothetical blocking factor from the spleen cells of C57 and CBA/J carrier mice by washing them repeatedly or by

Table 4. Effect of treatment of either LCM virus-infected target cells or spleen cells from LCM virus-immune mice with serum from syngeneic carrier mice on cytotoxic activity, measured with the target cell reduction assay

Exp. no.	Treatment of: Target cells Serum	Dilution	Spleen cells Serum	Dilution	% Reduction of target cell number
1*	None		None		98
	Normal	1 : 8			97
	Normal	1 : 2			98
	Carrier	1 : 8			97
	Carrier	1 : 2			96
2†	None		Normal	1 : 2	60
	None		Carrier	1 : 2	58

* Serum from C57 mice was diluted with MEM and 2×10^5 LCM virus-infected C57 fibroblastic cells were incubated with 0·4 ml for 60 min at 37° in a 5% CO_2 atmosphere. The serum was not removed when immune C57 spleen cells were added.

† Serum from CBA/J mice was diluted with BSS and 2×10^7 immune CBA/J spleen cells were incubated with 0·4 ml for 30 min at 37°. The cells were washed once before their addition to LCM virus-infected CBA/J fibroblastic cells.

Table 5. Effect of treatment of either LCM virus-infected target cells or spleen cells from LCM virus-immune mice with serum from syngeneic virus carrier mice on cytotoxic activity, measured with the ^{51}Cr release assay

Treatment of: Target cells*	Spleen cells†	% ^{51}Cr release Exp. 1	Exp. 2
None		99	100
Carrier serum		74	88
Normal serum		76	82
	None	93	100
	Carrier serum	71	97
	Normal serum	75	n.d.‡

* Twenty µl of serum from CBA/J mice were added to each of three wells containing $2{\cdot}5 \times 10^4$ LCM virus-infected L 929 target cells and 100 µl medium. After incubation for 60 min at 37° under 5% CO_2, 8×10^5 immune spleen cells per well were added.

† One hundred µl of serum from CBA/J mice were added to 4×10^6 immune CBA/J spleen cells suspended in 0·5 ml culture medium. After incubation for 30 min at 37° under 5% CO_2 this suspension was directly employed to perform the cytotoxicity test on LCM virus-infected L 929 target cells.

‡ Not determined.

incubating them for extended periods of time in culture medium prior to their interaction with target cells. The results (Table 6) show that neither treatment led to cytotoxicity of spleen cells from virus carrier mice. We conclude that blocking of receptors on cytotoxic T-cells or blocking of antigenic sites on target cells is not responsible for the tolerance to LCM virus in carrier mice.

In further experiments unwashed spleen cells from C57 and CBA/J carrier mice were added to spleen cells from immune mice of the corresponding strains, and these mixtures were tested for the effect they had on syngeneic virus-infected target cells. The results (Tables 7 and 8) show that the activity of immune spleen cells was not affected by the presence of carrier spleen cells.

The cytotoxic T-cell response in CBA/J mice infected with LCM virus was considerably reduced if heated serum from LCM virus-immune CBA/J mice was inoculated 2 h after the virus. In contrast, if the immunizing infection with LCM virus was followed by an inoculation of heated serum from CBA/J carrier mice, the development of cytotoxic activity as tested with the ^{51}Cr release test was not affected (Table 9). Thus, antibody with the ability

to prevent the induction of virus-specific cell-mediated immunity in infected mice does not seem to be present in the serum of CBA/J carrier mice.

Search for suppressor cells

Spleen or thymus cells from virus carrier mice were transferred intravenously to syngeneic normal recipients whose spleen cells were then tested for ability to reduce the number of target cells *in vitro*. Initially, recipient mice were given 100 ID_{50} of cell culture-grown LCM virus 8 days after cell transfer, which was thought necessary to establish an immunizing infection. This step was omitted, however, when it was found that LCM virus, which

Table 6. Effect of either pre-incubation *in vitro* or extensive washing of spleen cells from LCM virus carrier mice on their effect upon LCM virus-infected syngeneic fibroblastic cells, measured with the target cell reduction assay

Treatment of carrier spleen cells*	% Reduction of target cell number†	
	Mouse strain	
	C57	CBA/J
None	−20	−35
Washed five times	−14	−35‡
Pre-incubation for 20 h at 37°	−9	−19§

* After treatment cells were added to cultures at ratios of 100 erythrosin-excluding nucleated cells to one target cell.
† For explanation, see Table 1.
‡ Spleen cells from immune mice reduced the number of target cells 97% before washing as well as after washing five times.
§ Spleen cells from immune mice pre-incubated *in vitro* for 20 h at 37° reduced the number of target cells by 91%.

Table 7. Effect *in vitro* of spleen cells from LCM virus carrier mice on cytotoxic activity of spleen cells from LCM virus-immune syngeneic mice, measured with the target cell reduction assay

Spleen cells	% Reduction of target cell number		
	Exp. 1*	Exp. 2†	Exp. 3‡
Immune	98	n.d.§	n.d.
Immune + normal	n.d.	83	90
Immune + carrier	96	86	91

* C57 mice. Ratio of cells from immune mice to cells from carrier mice 4 : 1.
† CBA/J mice. Equal number of spleen cells from immune mice and carrier mice; thus the ratio of immune spleen cells to target cells was 50 : 1.
‡ Mice and ratio as in experiment 2. The spleen cell mixtures were pre-incubated for 30 min at 37° before their addition to syngeneic target cells.
§ Not determined.

Table 8. Effect *in vitro* of spleen cells from CBA/J virus carrier mice on cytotoxic activity of spleen cells from LCM virus-immune CBA/J mice, measured with the ^{51}Cr release assay

Spleen cells	% ^{51}Cr release	
	Ratio effector to target cells	
	32 : 1	6 : 1
Immune	100	59
Immune + carrier*	95	62

* Spleen cell mixtures were pre-incubated for 2 h at 37° before their addition to target cells. Ratio of immune cells to carrier cells 1 : 1.

Table 9. Effect of treatment with serum from virus carrier mice or immune mice on the development of cellular immunity in LCM virus-infected CBA/J mice, measured with the ^{51}Cr release assay

Serum*	^{51}Cr release†		
	Exp. 1	Exp. 2	Exp. 3
Carrier‡	99	96	n.d.¶
Immune§	31	n.d.	13
Normal	94	100	99

* Serum, 0·2 ml, was inoculated intraperitoneally 2 h after the intravenous inoculation of 100 ID_{50} of LCM virus. All sera were heated at 56° for 30 min.
† Cytotoxic activity of spleen cells was tested 7 days after the immunizing infection.
‡ Pooled serum from CBA/J carrier mice 7 (experiment 1) and 11 (experiment 2) weeks old.
§ Pooled serum from CBA/J mice infected intravenously 3 months previously with 10^7 ID_{50} LCM virus. In an *in vitro* neutralization test this serum pool had a titre of 320.
¶ Not determined.

Table 10. Cytotoxic effect upon LCM virus-infected C57 fetal fibroblasts of spleen cells from C57 mice, which had received intravenously 5×10^7 spleen cells from syngeneic carrier mice or normal mice and had been inoculated 8 days later with LCM virus; cytotoxic activity was measured with the target cell reduction assay

Spleen cells at day 0	Virus at day 8	Day of test†	% Reduction of target cell number
Carrier	+*	22	22
Normal	+	22	95
Carrier	−	14	24
Carrier	−	25	19

* 10^2 ID_{50} of LCM virus inoculated subcutaneously.
† Pooled spleen cells from two mice were used.

Table 11. Cytotoxic effect upon LCM virus-infected CBA/J fibroblasts of spleen cells from CBA/J mice inoculated previously with 5×10^7 untreated or treated spleen or thymus cells from syngeneic carrier mice; cytotoxic activity was measured with the target cell reduction assay

Cell inoculum	% Reduction of target cell number	
	Exp. 1*‡	Exp. 2†‡
Carrier spleen	25	17
Carrier spleen sonicated	17	29
Carrier thymus	30	n.d.§
Carrier thymus sonicated	43	n.d.
Normal spleen	−1	n.d.
Normal thymus	−5	n.d.

* The cytotoxicity test was performed 13 days after transfer of cells.
† Cytotoxicity test 10 days after cell transfer.
‡ Pooled spleen cells from two mice were used.
§ Not done.

is inevitably transferred together with carrier spleen or thymus cells, is as immunogenic as the virus from cell cultures; thus, the immunizing antigen is always given to recipient mice together with the cell inoculum.

When spleen cells from C57 carrier mice had been transferred, the cytotoxic T cell response of recipient mice was greatly reduced in comparison with the response in mice which had received spleen cells from normal syngeneic mice and virus (Table 10). When spleen cells or thymus cells (plus associated virus) from CBA/J carrier mice had been transferred, the cytotoxic activity of the recipients' spleen cells was also low (Table 11), which contrasts with more than 90% destruction of target cells by spleen cells from control mice infected with 100 ID_{50} of LCM virus obtained from carrier mice. At first sight these observations seemed to indicate that the cells from persistently infected donors indeed transmitted a suppressive effect on the cell-mediated immune response of the recipient mice. However, when cells from CBA/J carrier mice were sonicated before being transferred (more than 99% disrupted), the response remained as low as after transfer of intact cells (Table 11), indicating that active suppression

Table 12. Cytotoxic activity of spleen cells from CBA/J mice inoculated 8 days previously with 5×10^6 treated or untreated spleen cells from CBA/J carrier mice and infected 1 day later with 10^2 ID_{50} of LCM virus, measured with the ^{51}Cr release assay

Cell inoculum	% ^{51}Cr release	
	Exp. 1*	Exp. 2*
Carrier spleen cells	27	31
Disrupted carrier spleen cells	17	37
Carrier spleen cells disrupted and heated for 30 min at 56°	91	96
Normal spleen cells	95	95

* Pooled spleen cells from two mice were used.

Table 13. Blood virus titre and cytotoxic activity, measured with the target cell reduction assay, of spleen cells of CBA/J carrier mice at intervals after their intravenous inoculation with $2{\cdot}5 \times 10^7$ normal spleen cells from C57 mice

Days after inoculation*	Virus titre†	% Reduction of target cell number‡	
		Exp. 1	Exp. 2
4	4·2	−45	n.d.§
7	4·5	−31	n.d.
11	3·5	−25	−25
14	4·5	−26	9
18	n.d.	n.d.	0

* Mice were inoculated with allogeneic spleen cells at different times and cytotoxicity was tested on two occasions.
† Log_{10} LD_{50} per 0·03 ml of blood. Blood virus titres in untreated CBA/J carrier mice varied from $10^{3\cdot5}$ to $10^{4\cdot5}$ LD_{50} per 0·03 ml.
‡ For explanation, see footnote Table 1.
§ Not done.

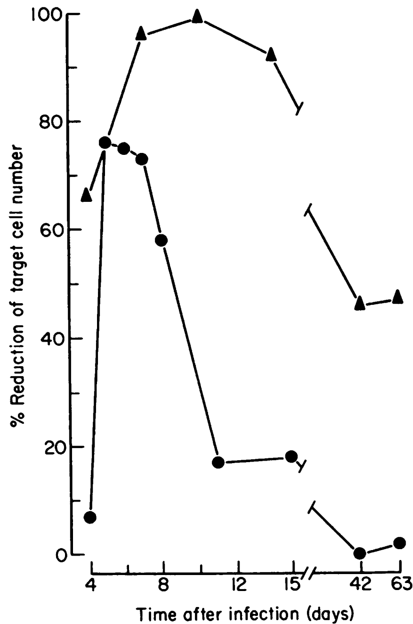

Figure 1. Development with time of cytotoxic activity of spleen cells, as expressed by their ability to reduce numbers of target cells, of mice infected with high or low doses of LCM virus. Data are from two independent experiments. In each, groups of CBA/J mice were infected at intervals intravenously with 10^2 or 10^7 ID_{50} of LCM virus and cytotoxic activity of spleen cells against LCM virus-infected CBA/J fibroblastic cells was measured in one target cell reduction test. Data points were always obtained from two spleens pooled for the assay. (●) 10^7 ID_{50}; (▲) 10^2 ID_{50}.

by the transferred cells was not the mechanism. This conclusion was supported by results from further experiments. Untreated or variously treated spleen cells from CBA/J virus carrier mice were transferred intravenously to normal syngeneic mice. One day later the recipient mice were given 100 ID_{50} LCM virus intravenously and after 7 more days the cytotoxic activity of recipients' spleen cells was tested with the ^{51}Cr release test (Table 12). Again, untreated carrier spleen cells suppressed the cytotoxic T cell response of recipient mice. Disruption of carrier spleen cells by freezing and thawing, a procedure known to have no significant effect on the virus, did not alter their suppressive activity. In contrast, when frozen-thawed carrier spleen cells were heated for 30 min at 56°, thereby completely inactivating the associated virus, the suppressive activity was abolished.

These results we interpreted to mean that suppression was caused by a direct effect of the virus which is present in all organs of carrier mice and is thus inevitably transferred in high concentrations together with the spleen cells. Confirmation came from observations on the cytotoxic T-cell response in CBA/J mice after their intravenous infection with as large a quantity of cell culture-grown LCM virus as is present in the spleen cell inoculum derived from carrier mice. Independent experiments employing both our tests revealed that the response was considerably lower and of shorter duration when mice were infected with 10^7 ID_{50} rather than with the usual immunizing dose, i.e. 10^2 ID_{50} (Figs 1 and 2).

Attempts to reduce the spleen cell-associated virus to low levels by repeated washings, treatment with rabbit anti-LCM virus hyperimmune serum plus complement or removal of phagocytic cells, which are known to actively participate in virus replication, were unsuccessful (data not presented).

We further investigated whether carrier spleen cells could suppress by *in vivo* contact the effector

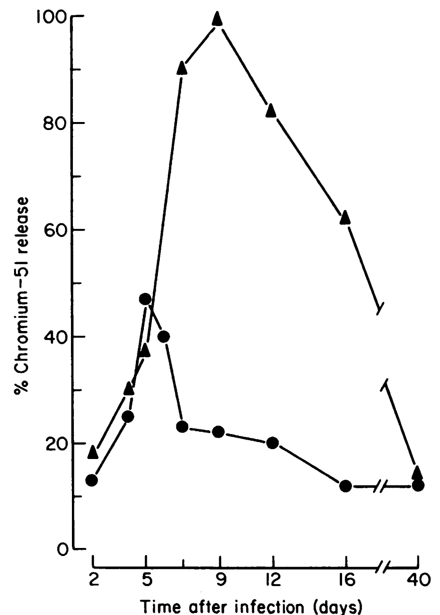

Figure 2. Development with time of cytotoxic activity of spleen cells, as expressed by their ability to release chromium from target cells, of mice infected with high or low doses of LCM virus. Groups of CBA/J mice were infected at intervals intravenously with 10^2 or 10^7 ID_{50} of LCM virus and cytotoxic activity of spleen cells against LCM virus-infected L cells was measured in one ^{51}Cr release test. Data points were always obtained from two spleens pooled for the assay. (●) 10^7 ID_{50}; (▲) 10^2 ID_{50}.

Table 14. Virus titres in blood of CBA/J (H-2^k) carrier mice injected intravenously with spleen cells from various allogeneic normal mice

Cells transferred (H-2)	No. of cells transferred	Virus titre*	
		Day 11	Day 14
SJL/J (s)	1×10^7	≥ 3.5	≥ 3.5
	4×10^7	≥ 3.5	≥ 3.5
DBA/1 (q)	1×10^7	≥ 3.5	≥ 3.5
	4×10^7	≥ 3.5	≥ 3.5
BALB/c (d)	1×10^7	≥ 3.5	≥ 3.5
	4×10^7	≥ 3.5	≥ 3.5

* Log_{10} LD_{50} per 0·03 ml blood. Blood virus titres in untreated CBA/J carrier mice varied from $10^{3.5}$ to $10^{4.5}$ LD_{50} per 0·03 ml.

phase of cell-mediated immunity against LCM virus. Five $\times 10^7$ spleen cells from CBA/J carrier mice were transferred intravenously to CBA/J mice which had been immunized by intravenous injection of 100 ID_{50} LCM virus 7 days previously. Control mice were immunized but did not receive cells. Ten days after the infection, i.e. 3 days after cell transfer, cytotoxic activity of spleen cells of recipient mice as measured by the ^{51}Cr release test was 95%; in control mice it was 94%. Thus, *in vivo* contact with carrier spleen cells had no effect on the cytotoxic activity of T cells from immune mice.

The results were different when the donor-recipient relationship was reversed. CBA/J mice were immunized by infection with 100 ID_{50} and 7 days later 5×10^7 spleen cells were transferred intravenously to each of two syngeneic carrier mice. The same number of cells from the same pool was given to normal control mice. After 3 more days, spleen cells from the control mice released 92% of ^{51}Cr from target cells. In contrast, cytotoxicity of spleen cells from carrier mice which had received immune spleen cells 3 days previously was much lower, corresponding to a ^{51}Cr release of only 6%.

Attempts to abrogate tolerance to LCM virus by allogeneic interaction were unsuccessful. CBA/J virus carrier mice were inoculated intravenously with spleen cells from normal C57 mice, and at intervals thereafter the cytotoxic activity of the spleen cells of recipient mice as well as their blood virus titres were determined. No significant effect on either parameter was observed (Table 13). We then tested other allogeneic combinations, and again no depression of blood virus titres was observed (Table 14).

DISCUSSION

Formal proof of the assumption that persistent infection of the mouse with the LCM virus is made possible by immunological tolerance depends on the demonstration that the part of virus-specific immunity which is responsible for virus elimination is absent in these animals. Elimination of LCM virus from the mouse is a cell-mediated immune function, and in the case of abrogation of the virus carrier state by adoptive immunization transfer of T lymphocytes alone is essential (Volkert *et al.*, 1975). However, the precise mechanism by which T cells participate in removal of the virus is unknown. It is assumed that virus elimination from an infected host is the consequence of a direct interaction between cytotoxic T-lymphocytes and infected cells (Pang & Blanden, 1976; Zinkernagel & Althage, 1977); hence, we have chosen to assess virus-specific cell-mediated immunity by measuring *in vitro* cytotoxic T-cell activity against virus-infected target cells.

In previous studies performed in this laboratory (Cihak *et al.*, 1974; Cihak & Lehmann-Grube, 1974) and elsewhere (Cole *et al.*, 1973; Marker & Volkert, 1973; Zinkernagel & Doherty, 1974) no cell-mediated immunity against LCM virus-infected cells was demonstrated in carrier mice, which was interpreted to mean that inability to eliminate the virus is a consequence of immunological tolerance. Immunological tolerance is an operational term. The present study is concerned with the mechanism of unresponsiveness of carrier mice against the LCM virus. A search for blocking factors either in the serum or the spleens of carrier mice was unsuccessful. Other authors have also failed to detect blocking factors in the circulation of persistently LCM virus-infected mice (Marker & Volkert, 1973). Nor did we succeed in demonstrating suppressor cells in carrier mice. The marked suppressive effect of transferred carrier spleen cells on the cytotoxic T-cell response in syngeneic normal recipients, also seen by Zinkernagel & Doherty (1974), was apparently caused by the high quantity of virus carried over to the recipients and not by the cells themselves.

Suppressor cells from mice unresponsive to picryl-

chloride have been shown to block the effector phase of contact sensitivity (Zembala & Asherson, 1973). Spleen cells from carrier mice transferred to syngeneic LCM virus-immune mice did not interfere with the expression of cytotoxic activity of the recipients' spleen cells. However, when cells from immune mice were transferred to carrier mice, cytotoxic activity was not found in their spleens. Marker & Volkert had reported a similar finding (Marker & Volkert, 1973). In carrier C3H mice adoptively immunized with lymphoid cells from hyperimmune syngeneic mice the levels of cytotoxic T-cell activity against LCM virus-infected L cells were low, although infectious virus was effectively eliminated. Rather than speculating on an otherwise undetectable suppressor mechanism, we think that disappearance of cytotoxic activity of the transferred spleen cells in carrier mice results from the encounter of immune spleen cells with the great number of infected cells in all organs of the recipients, thus leading to insufficient homing of the transplanted cells or to their immunological exhaustion.

Allogeneic confrontation has been shown to be effective in breaking suppressor cell-mediated tolerance to sheep red blood cells in rats (McCullagh, 1970). Zinkernagel & Doherty (1974) observed appearance of cytotoxic activity and a more than 1000-fold reduction of virus titres in spleens of CBA/H carrier mice 11 days after the inoculation of 5×10^7 normal allogeneic spleen cells. However, they themselves were unable to reproduce this finding in repeat experiments (Zinkernagel & Doherty, 1974 and personal communication). Our attempts to abrogate the tolerance to LCM virus in carrier mice by inoculation of normal spleen cells from H-2 different mouse strains have failed.

Further support for the conclusion that unresponsiveness to LCM virus in carrier mice is not maintained by suppressor cells is derived from experiments of Volkert and co-workers (Volkert, Hannover Larsen & Pfau, 1964; Hannover Larsen & Volkert, 1967). They observed that the LCM virus carrier state could be terminated not only by transfer of lymphoid cells from syngeneic LCM virus-immune mice but also by transfer of lymphoid cells from normal mice, though very high numbers of the latter were required. Thus, neither manifestation nor even induction of immunity to LCM virus was suppressed.

Since neither blocking factors nor suppressor cells were detected in LCM virus carrier mice, the most likely explanation for their unresponsiveness is deletion or irreversible inactivation of T-cell clones involved in the cell-mediated immune reaction against virus-infected cells. *Mutatis mutandis*, the same mechanism is thought to maintain transplantation tolerance in mice induced by neonatal inoculation of allogeneic cells (Brooks, 1975; Silvers, Elkins & Quimby, 1975) and tolerance to histocompatibility determinants in tetraparental bone marrow chimeras (von Boehmer, Sprent & Nabholz, 1975). Tolerance against LCM virus-altered major histocompatibility antigens (Zinkernagel & Doherty, 1975) is probably initiated and maintained in much the same way as tolerance to alloantigens.

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