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Shared determinants between virus-infected and trinitrophenyl-conjugated H-2-identical target cells detected in cell-mediated lympholysis*

Infection of H-2-identical mice with either lymphocytic choriomeningitis (LCM) virus, vaccinia virus, or paramyxo (Sendai) virus resulted in the generation of specifically sensitized cytotoxic T lymphocytes (CTL). CTL generated *in vitro* against 2,4,6-trinitrophenyl (TNP)-conjugated syngeneic stimulator cells were specifically cytotoxic for TNP-conjugated H-2K(D) region identical targets. Both LCM and vaccinia-induced CTL, however, were found to be strongly cytotoxic towards TNP-conjugated, H-2K(D) region-identical target cells. In contrast, Sendai virus-induced CTL did not lyse TNP-conjugated, syngeneic target cells. Inhibition experiments using cold targets suggested that shared antigenic determinants can be detected on either LCM virus-infected and TNP-conjugated targets, which are not present on the cell surface of normal target cells.

1. Introduction

Immune T lymphocytes of mice infected with either lymphocytic choriomeningitis (LCM) virus [1, 2] vaccinia virus [3] or paramyxo (Sendai) virus [4, 5] require H-2 compatibility with virus-infected target cells for lytic interactions to occur. Similarly, cytotoxic T cell responses to 2,4,6-trinitrophenyl (TNP)-"modified" syngeneic mouse lymphocytes [6, 7], to minor histocompatibility antigens [8] and to the male Y antigen [9] appear to be H-2-restricted.

In analyzing the specificity of cytotoxic T lymphocytes (CTL) generated against virus-infected or TNP-"modified" syngeneic targets, effective lytic interactions between virus-induced CTL and TNP-"modified" target cells were repeatedly observed. We now describe experimental evidence suggestive of the existence of shared determinants in cell-mediated lympholysis (CML) between virus-infected and TNP-conjugated H-2-identical targets.

2. Materials and methods

2.1. Mice

The origin and breeding of the H-2-congenic and recombinant mice used has been described [10]. CBA/J, BALB/c and C57BL/6 mice were purchased from G.I. Bomholtgard, Ry, Denmark, and used experimentally at an age of 6–10 weeks.

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* This work was supported by the Sonderforschungsbereich 107 (Mainz) and the Deutsche Forschungsgemeinschaft (grant to U.K.).

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Abbreviations: **CTL:** Cytotoxic T lymphocytes **EID:** Egg infective dose **LCM:** Lymphocytic choriomeningitis **LD:** Lethal dose **LPS:** Lipopolysaccharide **TCID:** Tissue culture infective dose **TNP:** 2,4,6-Trinitrophenyl **TNBS:** 2,4,6-Trinitrobenzene-sulfonic-acid **PEC:** Peritoneal exudate cells **PBS:** Phosphate buffered saline **A/I ratio:** Attacker-to-inhibitor ratio **K/T ratio:** CTL-to-target ratio

2.2. Virus immunization and preparation of infected target cells

The viruses used (LCM (WE-3 strain) [2, 11], vaccinia (WR strain) [3] and paramyxo (Sendai) [5]) have been described. Mice were infected by intraperitoneal (i.p.) injection of either 1000 lethal dose (LD_{50}) of LCM virus [2] or 10^6 tissue culture infective dose $(TCID)_{50}$ of vaccinia virus [3] or 10^7 egg infective dose $(EID)_{50}$ of Sendai virus [5]. Eight days after injection, splenic lymphocytes of individual mice were prepared [11] and tested for the presence of CTL in a ^{51}Cr release assay [2, 11]. The target cells used were syngeneic macrophages obtained from peritoneal exudate cells (PEC). The adherent PEC were infected with either LCM virus (at a multiplicity of about 20 mouse LD_{50}/cell) or vaccinia virus (at a multiplicity of 10 $TCID_{50}$/cell) or Sendai virus (at a multiplicity of 10 EID_{50}/cell) over a 24 h period, and simultaneously labeled with ^{51}Cr-chromate (Amersham/Buchler, Frankfurt, Fed. Rep. Germany). In some experiments the continuous mouse fibroblast line L_{929} ($H\text{-}2^k$) was also used as target cell.

2.3. Induction of TNP-specific CTL

The method described by Shearer et al. [6] was used. In brief, x-irradiated (3000 r) splenic lymphocytes (10^7) were incubated for 10 min at 37 °C in phosphate buffered saline (PBS) containing 10 mM 2,4,6-trinitrobenzene-sulfonic-acid (TNBS) (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.); 1 x 10^6 TNP-modified cells (stimulator cells) were cultured over 5 days together with 4 x 10^6 syngeneic splenic lymphocytes (responder cells). The preparation of lymphoid cells, the culture media, the culture system and the culture conditions have been described in details elsewhere [10].

2.4. Preparation of TNP targets

Replicate cultures of splenic lymphocytes (4 x 10^6) were cultured in the presence of 5 μg lipopolysaccharide (LPS)/ml (Difco Laboratories, Detroit, Mich.) over a period of 48 h–72 h [10]. The cells were harvested, labeled with ^{51}Cr-chromate and, if necessary, incubated for 10 min at 37 °C in PBS containing 10 mM TNBS. Cells were washed and used as targets.

2.5. Cytotoxicity assay

Various numbers of viable "attacker cells" (either splenic lymphocytes from virus-infected mice or *in vitro* sensitized effector cells) were incubated with a constant number (2×10^4) of ^{51}Cr-labeled target cells in round-bottom plastic tubes (Fa. Greiner, Nürtingen, Fed. Rep. Germany, Cat. No. 175) as described in detail [10]. The percentage specific lysis was calculated according to the formula

$$\% \text{ specific lysis} = \frac{\text{^{51}Cr release by immune cells} - \text{^{51}Cr release by normal cells}}{\text{maximal ^{51}Cr release} - \text{^{51}Cr release by normal cells}} \times 100$$

Each assay was performed in triplicate. The standard deviations (SD) of the experimental samples were calculated in a Wang computer. The data in the tables are given without SD, since in the ^{51}Cr assay the SD of specific lysis varied less than 5 %.

2.6. Cold inhibition experiments

The cytotoxicity inhibition assay was performed according to Herberman et al. [12], the only modification being that the assay was performed in tubes instead of plastic dishes. The experimental results are expressed as:

$$\% \text{ inhibition of specific lysis} = \frac{\text{\% specific lysis in immune control} - \text{\% specific lysis in group with cold inhibitory cells}}{\text{\% specific lysis in immune control}} \times 100$$

The percentage of inhibition values obtained with the various concentrations of inhibitor cells are plotted against the log of the attacker-to-inhibitor (A/I) ratio.

3. Results

3.1. H-2 compatibility requirements for lytic activity of LCM virus-induced and TNP-induced CTL

In agreement with the work of Shearer et al. [6], Forman [7], Doherty and Zinkernagel [1], the data given in Table 1 and previous work [2] clearly demonstrate that LCM-induced and TNP-induced CTL are highly cytotoxic against either LCM virus-infected or TNP-conjugated, H-2K(D) region-compatible targets. I region compatibility was not sufficient for lytic interactions to occur.

Table 1. H-2 restriction of the cytotoxic activity of TNP-induced CTL[a]

Target cells: Strain	Identity with stimulating H-2 regions	Specific lysis[b] (%)
B10.A(4R)	K, IA, IB, IC, S, D	50
B10.A(2R)	K, IA D	55
ATL	IA	10
C57BL/6	IB, IC, S, D	4
DBA/2	none	−1

a) Spleen cells of B10.A (4R) mice were cocultivated with TNP-conjugated syngeneic splenic lymphocytes (stimulator cells). After 5 days, the cultured cells were harvested and tested for cytotoxicity against a variety of LPS-induced, ^{51}Cr-labeled blast lymphocytes. Background lysis of the target cells was less than 21 %. Assay time was 3 h.
b) ^{51}Cr release assay; effector-to-target cell ratio 20:1.

3.2. Specificity of the CTL generated in H-2-identical mice infected with different virus strains

Next it was tested whether CTL derived from H-2-identical mice infected with LCM virus, vaccinia virus, or Sendai virus would lyse only syngeneic target cells infected with the virus used for the induction of the CTL. The results given in Table 2 indicate the specificity of the CTL generated. Thus CTL induced in LCM virus-infected CBA mice effectively lysed CBA mouse-derived, LCM-infected macrophages, but were ineffective against Sendai virus, or vaccinia virus-infected syngeneic targets (and vice versa).

3.3. Cytotoxicity of virus-induced CTL towards TNP-conjugated syngeneic targets

Since CTL from virus-immune mice were cytotoxic only for syngeneic targets infected with the virus strain that was used for the induction of the CTL, the cytotoxic activity of virus-immune CTL towards TNP-"modified" syngeneic targets was tested. Surprisingly it was found that CTL derived from both LCM virus-immune and vaccinia virus-immune mice exhibited strong cytotoxicity towards TNP-conjugated syngeneic targets (Table 3). CTL derived from Sendai virus-immune mice, however, failed to do so.

In reciprocal experiments the cytotoxic activity of TNP-specific CTL towards LCM and vaccinia virus infected targets was tested. No cytotoxicity against LCM virus or vaccinia virus-infected targets was detectable (Table 4).

Table 2. Specificity of CTL generated during infection with different virus strains[a]

Splenic lymphocytes (effector cells)	Specific lysis of macrophage targets (%): Noninfected 50:1[b]	Noninfected 5:1	LCM-infected 50:1	LCM-infected 5:1	Vaccinia-infected 50:1	Vaccinia-infected 5:1	Sendai-infected 50:1	Sendai-infected 5:1
Normal	2	0	0	0	5	1	0	−1
LCM-immune	5	3	**43**	**18**	6	4	5	2
Vaccinia-immune	0	−3	2	0	**34**	**20**	4	3
Sendai-immune	2	2	4	3	4	0	**19**	**8**

a) Spleen cells of CBA mice (H-2^k), infected with LCM, vaccinia or Sendai virus 8 days previously, were tested in a ^{51}Cr release assay for cytotoxicity against normal and virus-infected macrophage target cells. Background lysis was less than 27 %; assay time was 8 h.

Table 3. Cross-reactivity of virus-induced CTL towards TNP-conjugated targets[a]

H-2^k splenic lymphocytes (effector cells)	Specific lysis of H-2^k target cells (%) TNP-conjugated 50:1[b]	5:1	Normal 50:1	5:1	Virus-infected 50:1 LCM	Sendai	Vaccinia
Normal	5	1	2	−3	−3	0	5
LCM-immune	47	12	−2	0	38	5	1
Vaccinia-immune	25	5	−3	−3	6	4	32
Sendai-immune	3	−1	1	−1	n.d.[d]	19	−1
TNP-specific CTL[c]	52	24	4	2	5	n.d.	3

a) Splenic lymphocytes from either normal CBA mice or from CBA mice infected 8 days previously with either LCM, vaccinia or Sendai virus, or *in vitro*-induced TNP-specific CTL, were assayed for cytotoxicity against either TNP-conjugated, or virus-infected syngeneic macrophages. Background lysis was less than 32 %. Assay time against TNP-conjugated, normal and infected macrophages was 8 h. Similar results were obtained in 3 independent experiments.
b) Effector-to-target cell ratio 50:1.
c) *In vitro*-induced.
d) n.d. = not done.

Table 4. H-2 restriction of the cross-reactivity between LCM virus-induced CTL and TNP-conjugated syngeneic targets[a]

Effector cells (H-2^k)	Specific lysis of ^{51}Cr-labeled targets (%)[b] B10.BR-TNP	B10.A.-TNP	B10.A(4R)-TNP	ATL-TNP	C57BL/6 TNP	L929 LCM virus-infected
TNP-specific[c] CTL	63	50	39	6	2	2
LCM-induced[d] CTL	59	52	35	4	6	49

a) *In vitro*-induced TNP-specific CTL and *in vivo*-induced LCM-specific CTL were tested for cytotoxicity against a variety of TNP-conjugated target cells and against LCM virus-infected L929 fibroblasts. Background lysis was less than 30 %. Assay time was 6 h. In control experiments it could be demonstrated that both ATL-TNP and C57BL/6 targets can be lysed effectively by specifically sensitized CTL (data not given).
b) Effector-to-target cell ratio 30:1; LPS-induced blast lymphocytes were used as targets.
c) *In vitro*-induced.
d) *In vivo*-induced.

3.4. Role of H-2 in determining cross-reactivity

Evidence has been presented (Tables 1 and 2) that K(D) region compatibility between virus-induced and TNP-specific CTL and targets was required for lysis to occur. The results given in Table 4 suggest that the same compatibility requirements must be met to achieve lysis of TNP-conjugated targets by syngeneic LCM virus-specific CTL.

3.5. Influence of LCM virus infection on TNP-conjugated target cells upon the cross-reactivity observed

The observation that LCM virus-specific CTL are able to lyse TNP-conjugated syngeneic targets may be explained by the activation of two clones of CTL during LCM virus disease. If so, one would expect that LCM-infected targets, which in addition have been TNP-conjugated, would be lysed to a much greater extent than either LCM-infected or TNP-conjugated targets. The results given in Table 5 clearly indicate that TNP conjugation of LCM-infected targets did not result in an increased cytolytic activity.

3.6. Cold target competition experiments

The magnitude of cytotoxicity observed in the ^{51}Cr assay has been shown to be proportional to the log of the CTL to target (K/T) ratio [13]. Thus a decrease of the K/T ratio by addition of unlabeled targets to a fixed number of CTL and labeled targets will result in a reduction of percentage of cytotoxicity [12]. The criteria used to define positive

Table 5. Lysis of TNP-conjugated LCM-infected targets by LCM- specific CTL

Effector cells (H-2^k)	Specific lysis of ^{51}Cr-labeled targets (%)[a] L929 20:1	2:1[b]	L929-TNP 20:1	2:1	L929-LCM 20:1	2:1	L929-LCM-TNP 20:1	2:1
TNP-specific CTL	3	−1	71	46	2	−1	62	37
LCM-induced CTL	27	4	73	29	84	29	79	33

a) Assay time 15 h.
b) Effector-to-target cell ratio.

results in the inhibition assays performed were (a) a clear dose-response relationship between inhibition and numbers of added inhibitor cells and (b) the slope of the inhibition curve similar to that produced by the positive inhibition control. The results given in Fig. 1a show that the cytotoxicity of LCM virus-induced CTL towards LCM-infected targets could be partially blocked by cold TNP-conjugated syngeneic targets. However, the inhibition capacity of cold LCM-infected targets was significantly stronger. On the other hand both cold LCM-infected and cold TNP-conjugated targets blocked equally well the cross-reactivity of LCM-specific CTL towards TNP-conjugated targets (Fig. 1b). These results suggested that the antigenic determinants recognized by LCM-specific CTL on TNP-conjugated syngeneic targets are also present on LCM-infected syngeneic target cells. Thus the cross-reactivity noted appeared to be due to specific recognition of shared cell surface determinants rather than to unspecific cytotoxic activity.

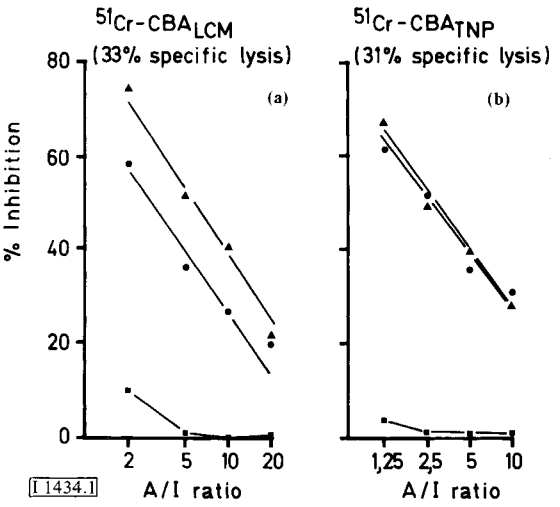

Figure 1. (a) Percentage inhibition of the cytotoxicity of LCM virus-induced CTL (H-2^k) towards a constant number (2 x 10^4) of LCM virus-infected ^{51}Cr-labeled CBA macrophages (H-2^k) obtained with graded numbers of cold LCM virus-infected CBA macrophages (▲—▲), cold TNP-conjugated CBA macrophages (H-2^k) (●—●) or normal, cold CBA macrophages (■—■). Cytotoxicity of LCM-induced CTL towards LCM-infected targets was 33 %.
(b) Percentage inhibition of the cytotoxicity of LCM virus-induced CTL (H-2^k) towards a constant number (2 x 10^4) of TNP-conjugated ^{51}Cr-labeled CBA macrophages obtained with graded numbers of cold LCM virus-infected CBA macrophages (▲—▲), cold TNP-conjugated CBA macrophages (●—●) or with normal cells (■—■). Cytotoxicity of LCM-induced CTL towards TNP-conjugated syngeneic CBA macrophage targets was 31 %.

4. Discussion

The aim of this study was to characterize the cross-reactivity observed between virus-induced CTL and TNP-"modified" syngeneic targets. Of the three virus-induced CTL populations tested, LCM-induced CTL cross-reacted strongly with TNP-"modified" target cells. No cross-reactivity was noted with Sendai virus-induced CTL, whereas the cross-reactivity of the vaccinia virus-induced CTL was intermediate. On the other hand, TNP-induced CTL failed to exhibit cytotoxicity towards LCM and vaccinia virus-infected syngeneic targets. Studies of the reactivity of LCM virus-induced CTL towards TNP-"modified" targets revealed that H-2K(D) compatibility of the target cells was required for lytic interactions to occur, a feature characteristic for T cell-mediated lysis of both virus-infected and TNP-"modified" cells [1, 6, 7]. Finally, cold target inhibition experiments suggested that the cross-reactivity noted was due to shared antigenic determinants between LCM-infected and TNP-conjugated target cells.

Monolayer absorption studies of CTL have convincingly demonstrated the existence of clonally restricted, antigen-specific CTL populations [14]. The lack of cross-reactivity of CTL generated in H-2-identical mice infected with three different virus strains (Table 3) indicated the activation of clonally restricted, virus-specific CTL. Thus the fact that LCM and vaccinia virus-immune CTL were also reactive against TNP-conjugated targets (third-party cells) may be explained by at least two mechanisms. First, LCM virus infection of mice may result in the specific activation of CTL precursors reactive to antigens present on LCM virus-infected cells. In addition, activation may occur of CTL precursors not specific for LCM, some of which are able to recognize antigens present on TNP-"modified" targets, thereby causing the cross-reactivity observed. Alternatively, it is conceivable that CTL activated during LCM virus infection are able to lyse TNP-conjugated targets because come antigenic determinants present on LCM-infected cells are also present on TNP-conjugated cells.

The blocking studies would support the second mechanism. Cold TNP-modified targets were found to block the lytic activity of LCM-induced CTL towards labeled LCM targets less well than cold LCM targets. On the other hand, the cross-reactivity of LCM-induced CTL towards TNP-modified targets was blocked equally well by both cold LCM-infected cells and TNP-modified cells. Therefore, these findings are most readily explained by the assumption that the antigenic determinants recognized on TNP targets are also present on LCM-infected cells. In other words, the cross-reactivity noted appears to be due to shared antigenic determinants between LCM-infected targets and TNP-conjugated targets. This conclusion is further supported by the finding that the cross-reactivity of LCM virus-induced CTL towards TNP-conjugated targets is similarly H-2-restricted as the lytic interactions with LCM virus-infected syngeneic targets. Thus H-2K identity between effector cells and targets was sufficient for cross-reactivity to occur (Table 4). We have previously shown that the H-2 restriction of cytotoxic T cell responses to viral infections (or hapten-conjugated cells) is only demonstrable when syngeneic "modified" stimulator cells induce CTL precursors to differentiate into CTL [15]. Since in LCM virus-infected mice only autologous infected cells trigger the differentiation of CTL, the LCM-induced CTL cross-reactive to TNP-conjugated targets are most likely triggered *in vivo* by antigenic determinants present on syngeneic, LCM-infected cells. Moreover LCM-induced cytotoxicity against TNP-conjugated LCM-infected targets was found to be almost equal when compared to LCM-infected targets (Table 5). Altogether these findings, therefore, strengthen the view of shared antigenic CML determinants between LCM virus-infected and TNP-conjugated target cells.

We also observed that murine CTL sensitized against allogeneic transplantation antigens are cytotoxic towards TNP-

conjugated syngeneic targets (unpublished results). Moreover, CTL sensitized against minor histocompatibility antigens [8] also appear to be cytotoxic to syngeneic TNP-conjugated targets (unpublished results). These data therefore suggest that the receptors of a variety of specific CTL appear to cross-react with syngeneic, TNP-modified cell surface antigens. However, since so far no reactivity of TNP-specific CTL towards virus-modified targets was detected (Table 3), the cross-reactivity described here appears to be of a "one-way" type.

So far studies of cytotoxic responses to viral infections [16] and hapten-conjugated syngeneic cells [6] indicated that the CTL generated are triggered by specific "modifications" of structures coded for in the H-2K(D) gene region. At present we explain the distinct cross-reactivity characteristic for LCM virus-induced CTL towards TNP-conjugated targets with the assumption that the "modification" of H-2K(D) gene products obtained by TNP conjugation mimics, in part, that present on LCM virus-infected targets. If this conclusion is correct, then it is conceivable that the number of "specific" modifications of a given H-2K(D) gene product may be rather limited. Therefore one explanation for the polymorphism of the H-2K(D) region products in the mouse [17] may be that for any infectious (or reactive) agent there is a high degree of possibility for the induction of different "modifications" of the H-2K(D) phenotypes in order to trigger different antigen-reactive clones of CTL precursors.

We gratefully acknowledge the technical assistance of Ms. L. Ptschelinzew, C. Hard, and G. Lavaud.

Received May 17, 1976.

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Functional half-life of virgin and primed B lymphocytes

The functional half-life, a measure of the persistence of lymphocytes in an antigen-free environment, has been estimated and found to be about 7 days for virgin 2,4,6-trinitrophenyl (TNP)-reactive mouse B cells, and 2–3 times longer for TNP-primed B cells. Using allotype-congenic mice, lymph node cells were transferred from virgin or primed CBA/Ig^b donors to normal CBA/Ig^a recipients, and the proportion of donor B cells estimated at intervals. This was done by making a further transfer to irradiated recipients and challenging with a TNP conjugate. The donor contribution declined with time in approximately exponential fashion to give the functional half-life. The experiment with primed cells necessitated mixing them with virgin cells, and thus allowed the possibility of interactions. However, control experiments showed that, at least in the final transfer, the primed cell response was not decreased by the presence of excess virgin cells – although primed cells suppressed virgin cells. In other experiments where the intermediate hosts were treated with vinblastine, it was shown that primed B cells have a slower turnover rate than virgin B cells. The relevance of these results to the problem of B cell tolerance is discussed.

[I 1369]

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Abbreviations: B cells: Bone marrow-derived lymphocytes **T cells:** Thymus-dependent lymphocytes **TNP:** 2,4,6-Trinitrophenyl **SG:** Sheep globulin **KLH:** Keyhole limpet hemocyanin **LNC:** Lymph node cells **DNP:** 2,4-Dinitrophenyl

1. Introduction

There is some evidence that virgin B cells form a population qualitatively distinct from those B cells primed as a result of contact with antigen. For example, virgin B cells have a fast turnover rate and do not recirculate readily, whereas primed B cells have a slower turnover rate and recirculate from blood to lymph [1]. In addition, primed B cells probably have a