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Generation of virus-specific cytotoxic T cells *in vitro* I. Induction conditions of primary and secondary Sendai virus-specific cytotoxic T cells*

H-2-restricted cytotoxic T cells specific for Sendai virus were generated *in vitro* in a primary response from normal mouse lymphocytes cultured in the presence of infective as well as inactivated Sendai virus. Antigen-presenting cells of different origin, including T cells, were found to be effective stimulators. Antibodies to Sendai virus were shown to inhibit the activation of specific precursor killer cells when added to cultures before, but not after, the addition of viral antigen. Data obtained by Lyt phenotyping, revealed that precursor killer cells specific for Sendai virus reside in the Lyt-2,3$^+$ T cell population and that Lyt-1,2,3$^+$ T cells are not required for the generation of cytotoxic lymphocytes. Different activation kinetics were demonstrated for primary and secondary antiviral cytotoxic responses, and the analysis of the proliferation and stimulation requirements suggests qualitative differences.

1 Introduction

In present models of T cell activation, copresentation of determinants of the major histocompatibility complex (MHC) and viral antigens in the recognition and effector phase is required [1]. Activation may therefore be dependent on the tropism of viruses for particular cells and the capacity of these cells to present viral antigens. Therefore, the role of the presenting cells as well as the mode of formation of the new antigenic determinant is of special interest.

If only cell membrane-expressed viral antigens can trigger virus-specific cytotoxic T cell (CTL) precursors, the process leading to infection and/or membrane expression of viral antigen acquires a crucial role in T cell activation. Some viruses carry in their envelopes the relevant antigenic determinants which are expressed on the cell surface immediately after virus-cell fusion. In other viral systems, the formation of viral determinants on cell membranes requires penetration, uncoating and the neosynthesis of proteins. Therefore, the sensitization process of T cells by different viruses may follow different activation patterns,

To establish a model for the study of the activation process *in vitro*, we used the Sendai virus (SV) infection of the mouse. Primary T cell activation to SV has recently been reported [2, 3]. Since SV infection is known to be endemic in many mouse colonies [4], emphasis was laid on the question of discrimination between primary and secondary responses *in vitro*. Using different conditions, we here describe quantitative and qualitative differences between primary and secondary CTL responses to SV.

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Abbreviations: β-PL: β-Propiolactone **CTL:** Cytotoxic T lymphocytes **ELISA:** Enzyme-linked immunosorbent assay **HA:** Hemagglutination **HAI:** Hemagglutination inhibition **HAU:** Hemagglutination unit **LU:** Lytic unit **PBS:** Phosphate-buffered saline **SV:** Sendai virus **Con A:** Concanavalin A **C:** Complement **MHC:** Major histocompatibility complex

2 Materials and methods

2.1 Animals

BALB/c and DBA/2 strain mice were purchased from Gl.Bomholtgård, Ry, Denmark and from the Institut für Versuchstierzucht, Hannover, FRG, at 4–8 weeks of age, and some of the mice were tested immediately after arrival. B 10, B 10.D 2, B 10.BR and DBA/2 mice were raised in our own breeding facility.

2.2 Viruses

SV (kindly provided by Dr. M.-G. Gething, ICRF, London) and influenza H virus A/Victoria (H 3 N 2) (kindly provided by Dr. R. Rott, Gießen, FRG) were grown in 10-day-old embryonated chicken eggs. The allantoic fluid was harvested after two days and the viral antigen concentration determined by hemagglutination (HA). Purification of SV was obtained by centrifugation through a sucrose gradient [20–55% w/v in phosphate-buffered saline (PBS)]. The virus-containing band was collected and concentrated by centrifugation at 120 000 × g for 1 h. Inactivation was achieved by incubating the purified virus [2 mg/ml, ca. 4×10^4 hemagglutinating units (HAU) per ml] for 18 h at 4 °C with β-propiolactone (β-PL, Sigma, München, FRG) at a concentration of 0.025%. β-PL was inactivated by incubation for 30 min at 37 °C. Inactivation was tested by egg infectivity titration. Viruses were stored at − 70 °C.

2.3 Immunization

Mice were injected intraperitoneally (i.p.) with 100 HAU of infective virus. Lymphocytes from spleen and lymph nodes were removed 3–10 weeks afterwards and analyzed *in vitro*.

2.4 Media

Cytotoxic lymphocytes were generated in RPMI 1640 supplemented with L-glutamine (2 mM final concentration), streptomycin and penicillin (50 units/ml), 2-mercaptoethanol (2×10^{-5} M) and 10% fetal calf serum.

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2.5 Antisera

Antiserum to SV was raised in DBA/2 mice by 3 weekly i.p. injections of 100 HAU. Serum was obtaind 5 days after the final injection and tested in hemagglutination inhibition (HAI) and an enzyme-linked immnosorbent assay (ELISA). This test is based on antibody binding to viral antigen which is coupled to plastic surfaces. In a second step, the bound antibody binds horseradish peroxydase-coupled viral antigen. Binding of enzyme-coupled antigen is then demonstrated by the addition of substrate.* Antibody titer to SV was 2.5×10^{-3} (HAI) and 3.5×10^{-5} (ELISA). Antiserum to influenza A substrain Victoria (H3N2) was prepared in a similar way. The titer in HAI was 2×10^{-3}.

AKR anti-C3H antiserum was kindly provided by Dr. B. Rubin, Statens Seruminstitut, Copenhagen, Denmark. Anti-Lyt antisera were prepared as described by Shen et al. [5]. Briefly, anti-Lyt-1.1 antisera were prepared by injecting (BALB/c × C57BL/6) F_1 hybrids with thymocytes from B6 Lyt-1.1 congenic mice (Lyt phenotype 1.1, 2.2, 3.2). Anti-Lyt-2.1 antisera were prepared by injecting B6 (H-2^k) mice with thymocytes from (B6 × CE) F_2 mice homozygous for the Lyt-2.1 allele. After one subcutaneous injection of $50 \times 10^6 - 100 \times 10^6$ donor cells and three additional i.p. injections of $50 \times 10^6 - 100 \times 10^6$ donor cells at 14-day intervals, each mouse was tested individually and only those selected for further immunization that produced good specific titers after removal of autoantibodies by absorption on thymocytes from the recipient strains. Selected mice were bled on day 7 and 10 after each inoculation, and the sera from several bleedings were pooled and stored at −70 °C. Prior to use, the anti-Lyt antisera were absorbed once with 100×10^6 thymus and lymph node cells from the recipients and the B6 congenic strains per ml undiluted antisera to remove autoantibodies. In a microcytotoxicity test, the titer of anti-Lyt-1.1 was 1 : 1224 on thymocytes of B6 Lyt-1.1 congenic mice, and the titer of anti-Lyt-2.1 was 1 : 250 on thymocyts of B6 Lyt-2.1 congenic mice after the absorption of autoantibodies. The anti-Lyt-1.1 antiserum was used at a dilution of 1 : 40, the anti-Lyt-2.1 antiserum at a final dilution of 1 : 10.

Cells were suspended at a concentration of 3×10^7 cells/ml and treated with anti-Lyt or anti-Thy-1.2 antiserum (at a final concentration of 1 : 10) in RPMI 5% fetal calf serum and incubated for 30 min at room temperature. Cells were centrifuged and resuspended in freshly thawed selected rabbit serum (dilution 1 : 11) as a source of complement and incubated for an additional period of 30 min at 37 °C. The treatment of cells with anti-Lyt antisera and complement (C) was repeated once. Cells were then washed, counted and resuspended to the desired concentration.

2.6 Glutaraldehyde treatment

Glutaraldehyde treatment of stimulator cells was essentially as described by Lightbody and Kong [6]. Cells were washed twice in PBS, pH 7.4; 10^7 cells were resuspended in 1 ml of 0.025% glutaraldehyde (Art. 4239, Merck, Darmstadt, FRG) diluted in PBS and incubated with occasional shaking for 20 min at 20 °C. The cells were subsequently centrifuged (200 × g) and washed 3 times with PBS.

* H. C. J. Ertl, W. Gerlich and U. H. Koszinowski, manuscript submitted.

2.7 Cell cultures

P815 (H-2^d), AKR-A (H-2^k) and EL4 (H-2^b) tumor cells were grown in medium at a concentration of 2×10^5 cells/ml with medium change every 48 h. Mouse spleen cells or lymph node cells were suspended in medium at a concentration of 4×10^6 cells/ml. Cells were cultured in multi-dish culture trays (FB-24Tc, Linbro Chemicals, New Haven, CT) or in plastic tissue culture flasks of various sizes. If desired, cells were depleted of erythrocytes by lysis in 0.184 M NH_4Cl or by separation on a Ficoll-Hypaque gradient. B cell depletion was achieved by nylon wool column passage [7]. Macrophages and adherent cells were removed by passage of cells through Sephadex G-10 columns. Phagocytic cells in the cultures were destroyed by addition of 100 µg silica/ml (kindly provided by Dr. Lemke, Institut für Genetik, Köln, FRG) [8].

2.8 Stimulator cells

Various stimulator cells (tumor cells, concanavalin A (Con A) blasts, or spleen cells depleted of erythrocytes) were incubated with 10^2 HAU/10^6 cells or 1 µg SV protein/10^6 cells in a volume of 10^7 cells/ml for 1 h at 37 °C. In order to inhibit proliferation of the stimulator cells, the virus-containing medium was also supplemented with mitomycin C at a concentration of 50 µg/ml. Thereafter, cells were washed 3 times with responder cells at various responder-to-stimulator cell ratios.

2.9 ^{51}Cr release assay

Target cells were incubated with either 10 µg β-PL-inactivated SV/10^6 target cells or with 10^2 HAU infective SV for 1 h at 37 °C. Labeling with ^{51}Cr was performed at the same time. After washing the target cells 3 times, they were incubated at 1×10^4 cells/well together with 4 concentrations of attacker cells in round-bottomed microtiter plates (Linbro IS-MRC 96). Spontaneous release was determined by incubating target cells in the presence of medium without addition of effector cells. Maximum release was determined by addition of 2% sodium dodecyl sulfate in PBS. After 4 h incubation at 37 °C in 5% CO_2 atmosphere, supernatants were harvested and ^{51}Cr release counted in a gamma counter. Data are expressed as ^{51}Cr release according to the formula:

$$\% \ ^{51}\text{Cr release} = \frac{^{51}\text{Cr release by immune cells} - \text{spontaneous } ^{51}\text{Cr release}}{\text{Maximal release} - \text{spontaneous release}} \times 100$$

Specific release was obtained by subtraction of release by cells cultured without antigens from release obtained from specifically stimulated cells.

All values are the mean percent specific ^{51}Cr release of triplicate wells. Standard errors of the means were always less than ± 5% and are omitted from the figures for clarity. Lytic units (LU) [9] were calculated from four attacker : target cell ratios with a Hewlett Packard 67 pocket calculator, using a logarithmic curve fit program.

3 Results

3.1 *In vitro* generation of primary SV-specific CTL by infected and virus-coated stimulator cells

It has been shown before that virus-specific T cells from immune animals can be activated by restimulation with anti-

Table 1. Induction of SV-specific CTL *in vitro*[a]

(A) Stimulator cells[b]	R:S ratio	Virus	% Spec. release from infected P815[c] 50 K:T ratio	10
DBA/2 macrophages	10:1	Inf. SV	69	35
		β-PL-SV	65	40
P815	20:1	Inf. SV	83	54
		β-PL-SV	70	38
DBA/2 Con A blasts	10:1	Inf. SV	78	40
		β-PL-SV	65	28
DBA/2 spleen cells	10:1	Inf. SV	50	22
		β-PL-SV	58	10

(B) No stimulator cells	μg virus/ml medium (inactivated, purified virus)		
	10	40	11
	1	54	25
	0.1	68	46
	0.01	60	35
	Nil	14	3

a) Responder cells obtained from a pool of 5 unprimed DBA/2 mice, negative for antibodies to SV. The test was performed after 6 days of culture.
b) Stimulator cells were incubated for 1 h with 100 HAU of infective or inactivated virus followed by 3-fold washing and treatment with mitomycin C.
c) Percent specific ^{51}Cr release from SV-infected targets over a period of 4 h. Four killer-to-target (K:T) ratios were tested (only 2 given in the table). Means of triplicates are given. SE of means did not exceed 5%.

Table 2. MHC and virus specificity of primary anti-SV effector cells

Effector cells[a]		Target cells[b] P815-SV	P815-Infl.	AKR-A	AKR-A Infl.	EL4-SV	EL4-Infl.
B10.D2	SV[c]	80	22	3	5	18	18
B10.D2	INFL.[d]	12	74	6	6	8	11
B10.BR	SV	16	17	36	4	12	12
B10.BR	INFL.	10	14	6	28	6	8
B10.	SV	15	18	9	7	76	20
B10.	INFL.	13	14	0	5	15	64

a) Ratio effector : target cells = 20 : 1; 4-h ^{51}Cr release assay.
b) Target cells were obtained after incubation of 100 HAU A Victoria/10^6 target cells for 4 h. Spontaneous release from target cells: P815-SV: 11%; P815-Infl: 14%; AKR-A-SV: 4%, AKR-A-Infl: 4%; EL4-SV: 6%, EL4-Infl: 8%.
c) Primary cytotoxic response to SV *in vitro*.
d) Influenza-specific effector cells were generated by priming of mice with 100 HAU influenza A Victoria (H3N2) and secondary stimulation *in vitro* 3 weeks later with medium containing 1–5 HAU A Victoria/ml.

gen *in vitro* [10–12]. The lytic activity of the population in which CTL are generated during a secondary response on virus-infected target cells is about 100-fold higher in terms of cytolytic activity than that of immune T cells tested 7 days after infection of animals. For the induction of primary CTL from unprimed animals, a protocol similar to that of secondary *in vitro* responses was used. Responder cells were incubated for 6 days with stimulator cells pretreated with either infective or inactivated SV. It is shown in Table 1 A that effector cells can be generated by using as stimulators various cell sources including macrophages, lymphocytes, Con A-activated blasts and tumor cells.

H-2 restriction and specificity for viral antigens of these cytotoxic lymphocytes generated *in vitro* were tested with spleen cells from strains B 10, B 10.Br and B 10.D 2 and compared to secondary antiviral cytotoxic responses. It is clearly shown in Table 2 that H-2 restriction and specificity for viral antigens seen in secondary cytotoxic responses [13] is also true for primary effector T cells.

Treatment of primary virus-specific CTL with anti-Thy-1.2 antiserum and C eliminated the cytolytic activity (Fig. 1). Therefore, antiviral CTL are clearly defined as T cells.

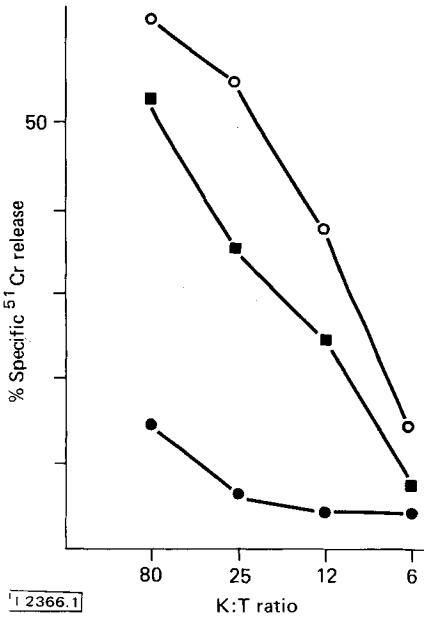

Figure 1. Effect of treatment with anti-Thy-1.2 antiserum and C upon virus-specific CTL. Equal numbers of SV-specific DBA/2 effector cells generated in a primary *in vitro* response were pretreated with C alone (○), with anti-Thy-1.2 (■) or with anti-Thy-1.2 and C (●) as described in Sect. 2. Target cells were P815-SV.

3.2 Generation of effector cells by addition of viral antigens to the *in vitro* culture

The fact that cells from different sources were equally effective as stimulators suggested that the target antigens required for the activation of T cells are not generated by interaction of virus with one particular stimulator cell type only. Therefore, in the next step, infective (data not shown) or inactivated virus (Table 1B) was added directly to the responder cell population. The data demonstrate that virus-specific CTL can be generated from spleen cell populations incubated for 6 days with inactivated virus.

3.3 Effect of antibodies to SV on the induction of a cytotoxic response

From the previous findings, we concluded that the virus added to the responder cell population probably fuses to cells within

this population. This fusion process seems to take place very rapidly since incubation of responder cells for 20 min at 37 °C with 0.1 μg/ml inactivated purified SV, followed by intensive washing, resulted in the generation of CTL (Fig. 2). Since it is known that antiviral sera block virus-cell fusion, it was now of interest to test whether pretreatment of inactivated SV with antibodies would interfere with the induction of CTL. Therefore, antibodies specific for SV and antibodies specific for influenza virus as a specificity control were added to responder cells together with virus. It is illustrated in Fig. 2 that the simultaneous addition of virus plus specific antibodies to the cell culture did not result in the generation of virus-specific killer cells. One could argue that the failure to induce cytotoxic T cells is due to blocking of all relevant antigenic determinants on the virus by the antibody. We therefore tested whether antibodies to SV were able to inhibit the induction of killer cells in responder populations preincubated with virus. The data show that, once the viral antigens are membrane-associated, antibodies seem not to be able to inhibit the process of T cell activation. These results support the view that intimate virus-cell interactions, probably virus-cell fusion, induces the formation of new antigenic determinants. This process of virus-cell interactions can obviously be inhibited by antibodies to SV. The reason for the inability of anti-SV antibodies to interfere with the activation process after preincubation of cells with virus is unclear. It is possible that the determinants recognized by the antibodies are irrelevant for T cell triggering or that the antibody concentration used in the inhibition experiments was too low to block all determinants responsible for the activation of precursor T cells.

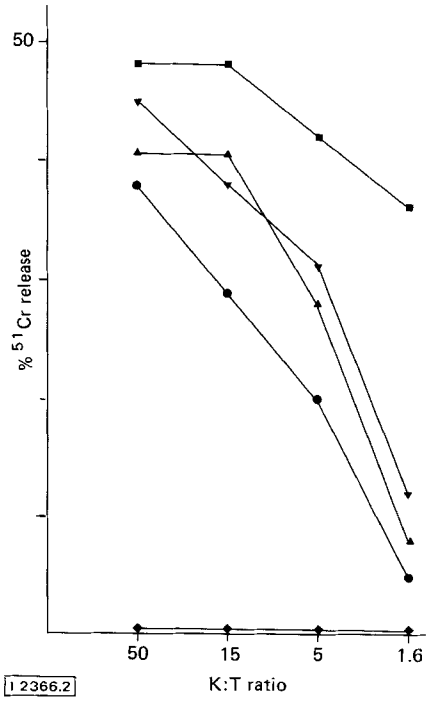

Figure 2. Effect of SV antibodies on CTL induction. (a) Responder cells from a pool of 5 DBA/2 mice were cultivated in the presence of 0.1 μg inactivated SV/ml and tested for specific lysis on P815 cells infected with SV and uninfected control. Release on uninfected targets is subtracted (■). (b) Responder cells incubated with virus for 20 min followed by 3-fold washing and culture in antigen-free medium (●). (c) Culture in presence of antiserum to SV (final dilution 1 : 50) and viral antigen as in (a) (◆). (d) Culture in presence of antiserum to influenza A Victoria virus (H3N2) (final dilution 1 : 50) and viral antigen (▼). (e) Responder cells incubated with viral antigens for 2 h followed by washing as in (b), then addition of antiserum to SV as in (C) (▲).

3.4 *In vitro* primary responses of lymphocytes from different origins

Cells from different lymphoid organs were tested for their ability to generate virus-specific CTL (Fig. 3). Lymph node and spleen cells were found to be similarly active. Lymphocytes from the thymus could not be activated by viral antigen to generate T killer cells. To demonstrate possible inhibitory or amplifying properties of thymus cells, cell mixing experiments were carried out. As can be seen in Fig. 3, there was no detectable help or inhibition by thymus cells regarding generation of T killer cells in spleen and lymph node cell populations under the experimental conditions used.

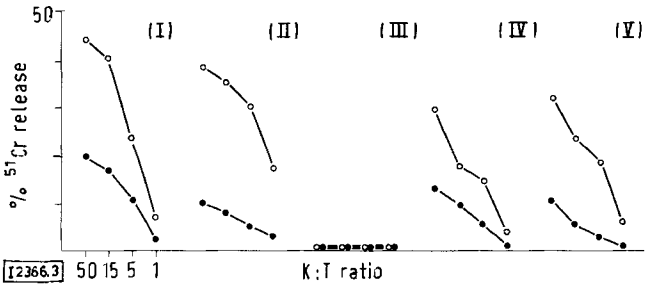

Figure 3. Induction of CTL in cultures of cells from different lymphoid organs. Four × 10^6 cells/ml from DBA/2 mice were cultured in the presence of medium containing 0.1 μg β-PL-inactivated SV/ml for 5 days. Activity is tested in triplicate at different killer-to-target cell ratios on ^{51}Cr labeled P815 SV (○) and uninfected P815 (●) cells in a 4-h assay. (I) Spleen cells; (II) peripheral lymph node cells; (III) thymus cells; (IV) mixture of spleen and thymus cells 1 : 1; (V) mixture of lymph node and thymus cells 1 : 1.

The data described so far show that there is no requirement for a particular stimulator cell in the generation of CTL, but that viral antigens are only sensitizing when they are associated with the cell membrane. Since in these experiments, unselected lymphocyte populations were used, it was now of interest to test whether there are limiting responder cell requirements in the generation of viral specific CTL. We therefore tested T cells selected by nylon wool, or T cells depleted in addition of macrophages by Sephadex G-10 column separation. The macrophage-depleted T cell population was also sensitized *in vitro* to antigens in the presence of silica, which is known to be an agent selectively toxic for macrophages without reducing the viability of lymphocytes *in vitro* [8, 14]. All the populations were able to generate a cytotoxic response to SV (Fig. 4). Although we cannot formally exclude antigen presentation on macrophages, the probability is slight that a phagocytic cell is required as antigen-presenting cell. This suggests that the T cell is not only the responder cell but can also act as stimulator cell in the generation of antiviral T killer cells.

3.5 Lyt phenotype of the primary T killer cell precursors

In order to investigate the type of T cells that participate in the induction phase of the response to SV antigens, the responder cell populations were pretreated with either anti-Lyt-1 or anti-

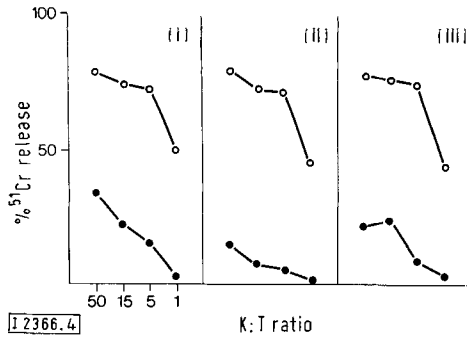

Figure 4. Effect of depletion of adherent and phagocytic cells on the induction *in vitro* of CTL. Culture conditions and cytotoxic test as in Fig. 3. (I) Cells separated by nylon wool column; (II) as in (I) plus additional separation on Sephadex G-10 column; (III) as in (II) plus 100 μg/ml silica to cultures.

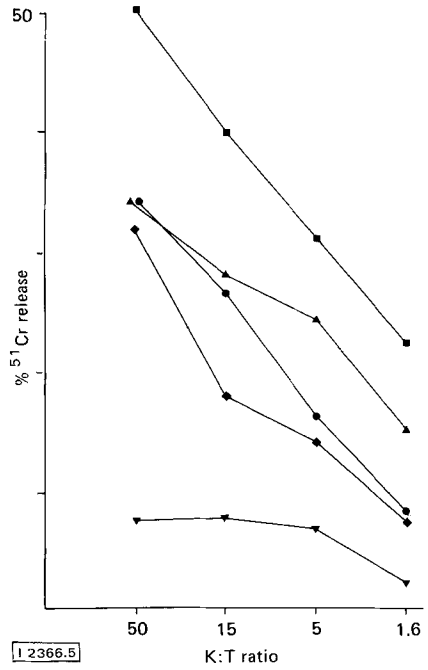

Figure 5. Effect of pretreatment of equal numbers of DBA/2 spleen T cells with Lyt antisera and C prior to culture on the generation of primary virus-specific CTL *in vitro*. No treatment (●); treatment with C (■); treatment with anti-Lyt-2.1 and C (▼); treatment with anti-Lyt-1.1 and C (▲); 50% cells treated with anti-Lyt-2.1 and C plus 50% cells treated with anti-Lyt-1.1 and C (◆).

Lyt-2 antisera and C (Fig. 5). Both populations were incubated with antigen either individually or as a combined fraction containing Lyt-1$^+$ and Lyt-2,3$^+$ but not Lyt-1,2,3$^+$ T cells. It is shown in Fig. 5 that Lyt-2,3$^+$ cytotoxic effector cells are very efficiently generated from Lyt-2,3$^+$ precursor cells even in the absence of Lyt-1$^+$ T cells. This was not always the case since in other experiments, Lyt-2,3$^+$ precursor cells required the presence of Lyt-1$^+$ T cells for an effective generation of CTL. Lyt-1$^+$ T cells were unable to mount a cytotoxic response to SV. Obviously, like in other systems, Lyt-1$^+$ T cells do not contain precursor killer cells, but the remote possibility that the result is due to their possible failure to function as antigen-presenting cells is still open. These experiments show that in contrast to the generation of syngeneic trinitrophenyl (TNP)-specific killer cells [15], Lyt-1,2,3$^+$ T cells are not required for the generation of H-2-restricted and virus-specific CTL.

3.6 Quantitative and qualitative differences between primary and secondary T cell responses to SV

3.6.1 General remarks

It could be argued that the mice used for primary *in vitro* cultures had already been presensitized *in vivo* by infection. We therefore tested routinely the sera of the mice for antibodies to paramyxoviruses. This was done by virus neutralization, HAI and by an ELISA assay in which IgM and IgG antibodies to viral antigens can be found to be 100-fold more sensitive as compared to HAI. Only animals which showed negative results in these tests were used in our experiment. Although antiviral antibodies are usually demonstrable for a very long time after infection, this does not necessarily imply that the absence of a humoral response reflects a virgin state with respect to cellular immunity.

We therefore investigated the kinetics, cytolytic activity, responder cell proliferation and stimulator cell requirements of primarily *in vitro* induced virus-specific CTL and compared the results with those obtained from *in vivo* primed spleen cell populations activated under identical conditions.

3.6.2 Kinetics and cytolytic activity

Cultures were set up with inactivated virus as antigen under identical conditions using as responder cells normal DBA/2 spleen cells or cells from mice primed with either 100 HAU infective or inactivated SV. It was found that in primed spleen cell populations, CTL can be demonstrated as early as 24 h after *in vitro* culture. In unprimed populations, a comparable cytotoxic response was only detectable after 3 days of culture (Table 3). Cells from mice primed with infective virus showed a 10-fold higher activity in terms of LU compared to unprimed animals or animals primed with inactivated virus. In all 3 groups, the peak responses were found on day 5–8. There was

Table 3. Kinetics of killer T cell generation *in vitro*. Cytotoxic activity on P815-SV in LU/10^6 effector cells

Days of *in vitro* culture[a)]	Sensitization of animals: 10^4 HAU SV i.p.	10^4 HAU β-PL-SV i.p.	No sensitization *in vivo*
1	1.0×10^4	6.5×10^3	1.7×10^2
3	6.0×10^5	5.1×10^4	5.0×10^4
6	6.9×10^5	$\underline{5.4 \times 10^4}$	7.2×10^4
8	$\underline{7.4 \times 10^5}$	1.8×10^4	$\underline{7.9 \times 10^4}$
10	2.8×10^5	2.2×10^3	7.4×10^4
13	2.0×10^5	NT	4.6×10^4

a) Culture conditions as in Fig. 2. Cultured cells from primed and unprimed mice were tested on SV-infected and uninfected P815 cells over a period of 13 days. For the sake of brevity, the activities are expressed in LU/10^6 responder cells. 1 LU = ratio attacker-to-target cells resulting in 33% specific lysis. Day of peak activity underlined.

no significant difference between unprimed spleen cells and cells primed with inactivated virus besides an earlier onset of the cytotoxic response in the primed population. In all 3 cultures, cytolytic activity was demonstrable up to 3 weeks (data not shown).

3.6.3 Responder cell proliferation requirements

In the next step, we investigated the requirement of proliferation for primary responder cells and compared it to that of a secondary antiviral response. For this purpose, untreated cells or cells treated with mitomycin C from primed and unprimed animals were cultured in the presence of inactivated SV, and cytotoxic activity was tested at 24-h intervals. The proliferative response was monitored to control the efficiency of mitomycin C treatment (data not shown). Cells from sensitized mice responded to secondary *in vitro* stimulus even after pretreatment with mitomycin C (Fig. 6). However, cells from unsensitized animals did not respond to SV antigens after mitomycin C pretreatment in primary *in vitro* cultures. Thus, proliferation of the responder cell population is an essential requirement for primary but not for secondary cytotoxic immune responses to virus *in vitro*.

3.6.4 Stimulator cell requirements

Since it is known from the induction of alloreactive T cells that secondary but not primary responses can be obtained with fixed stimulator cells or cell fragments [16], we tested whether the antiviral T cell generation *in vitro* is governed by the same principles. Responder cells from primed and unprimed animals were incubated with virus-coated P 815 stimulator cells, either glutaraldehyde-fixed or untreated. It is shown in Fig. 7 that only the primed responder population was able to mount a response to both viable and glutraldehyde-fixed virus-coated stimulator cells. These results strongly suggest that in a primary response to SV *in vitro,* nonfixed cells are required for the appropriate presentation of viral antigen.

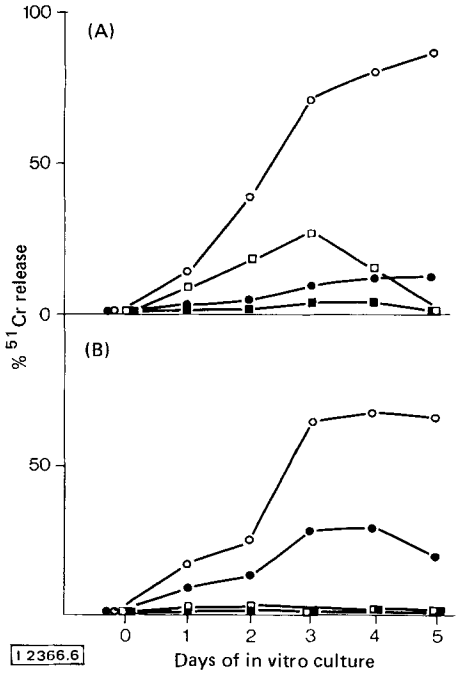

Figure 6. Proliferation requirements for the induction of CTL. Culture conditions and assay as in Fig. 3. (A) Activity of *in vivo* sensitized and *in vitro* restimulated DBA/2 spleen cells on P 815-SV (○) and uninfected target cells (●). Same spleen cell population treated previous to *in vitro* culture with 50 μg mitomycin C/10^6 cells/ml for 45 min. P 815-SV target cells (□), P 815 target cells (■). (B) Activity of unprimed DBA/2 spleen cells after primary sensizitation *in vitro*. Target and responder cell treatment as in (A).

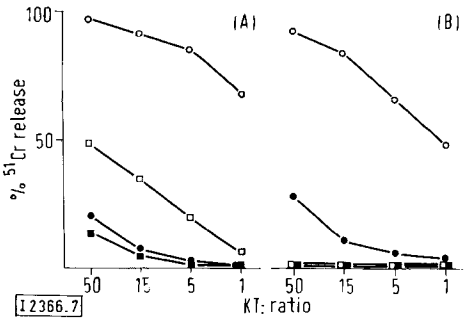

Figure 7. Stimulator cell requirements for the induction of CTL. Culture conditions and assay as in Fig. 3. (A) Activity of *in vivo* sensitized spleen cells after secondary stimulation *in vitro* on uninfected P 815 (●■) or virus-infected P 815 cells (○□). Effector cells generated in the presence of living stimulator cells (○●) or glutraldehyde-fixed stimulator cells (□■). (B) Activity of unprimed DBA/2 spleen cells after primary sensitization *in vitro*. Stimulator cell conditions and targets as in (A).

4 Discussion

In order to analyze the induction phase of antiviral cytotoxic T cells on a cellular level, an *in vitro* system for the generation of primary SV-specific killer cells was established. Being aware of the fact that many mouse colonies are endemically infected with SV [4], the following precautions were taken to exclude previously sensitized mice from the experiments. Animals from breeding stocks, raised under specific pathogen-free conditions, were used immediately after arrival, besides our own mice, and found to be capable of generating primary cytotoxic responses to SV *in vitro*. In addition, mice were tested for antiviral antibodies in sensitive assays (HAU, virus neutralization and ELISA), and only those found to be negative were used in this study. Mice, positive for antibodies to SV, reacted in accord with the criteria we established for a secondary response. Since antiviral antibodies, once generated, could be detected for more than 6 months, a previous contact of the animals with the antigen is very unlikely,although sensitization in a earlier phase cannot formally be excluded. However, the differences found in the induction of cellular immune responses between mice primed to SV and unsensitized animals argue against this possiblity. Furthermore, to circumvent this problem, mice of 8–12 weeks of age were used. A comparison of the primary *vs.* secondary cytotoxic response to SV showed a significant difference in the magnitude of the immune response (secondary immune response 10-fold higher than primary immune response) as well as in the onset of the cytotoxic responses (secondary CTL day 1, primary CTL day 3). In addition, we found that the lymphocyte population from primed and unprimed mice differed also with respect to their induction requirements. Thus, proliferation was not required for the generation of secondary CTL and was essential for the generation of primary CTL to SV. Furthermore, it was shown

that glutaraldehyde-fixed stimulator cells were only able to sensitize virus-specific memory CTL, as found by other investigators [10], but not virus-specific primary CTL. The differences observed are not necessarily qualitative because they could also be explained in quantitative terms. Since, to our knowledge, there is no evidence for clearly defined quantitative differences between unprimed and primed CTL, our criteria for the definition of the virgin state of the precursor T cells are therefore as yet functional.

There are striking similarities between the proliferation and activation characteristics of CTL generated *in vitro* against viral antigens and CTL generated in mixed lymphocyte cultures against alloantigens: (a) peak responses are obtained at about day 6–8, after which CTL activity progressively decreases but is still demonstrable for periods up to three weeks [17]. There is a considerable difference in magnitude between primary and secondary responses [16, 18]. (b) Primary responses require cell proliferation [19, 20] while activation of memory CTL does not require DNA synthesis [21, 22]. (c) The conditions of stimulator cells are critical in both systems during primary induction of CTL *in vitro* [23, 24] but less stringent during secondary stimulation [16, 25]. (d) Similar to the Lyt phenotype of alloreactive T cell precursors [26], cytotoxic precursor cells specific for SV were also shown to reside in the Lyt-2,3$^+$ cell pool.

This conclusion is derived from experiments showing that effector cells could be generated from T cell populations selected for Lyt-2,3$^+$ T cells and in populations containing Lyt-1$^+$ and Lyt-2,3$^+$ T cells which have been depleted from Lyt-1,2,3$^+$ T cells. There was no generation of killer cells in cultures containing Lyt-1$^+$ T cells. From these data, we conclude that Lyt-1,2,3$^+$ cells are not necessarily required during the induction of H-2-restricted and virus-specific CTL. This is in contrast to the results obtained with H-2-restricted and TNP-specific CTL in which the requirement of Lyt-1,2,3$^+$ T cells in the generation of CTL has been shown [15].

It is not yet clear whether the precursors of the H-2-restricted T cell populations specific for viral antigen or TNP, respectively, reside in different T cell pools or whether the Lyt-1,2,3$^+$ T cells act as precursor helper cells during the generation of T cells specific for haptens but not during the generation of virus-specific CTL (M. M. Simon and U. Koszinowski, manuscript in preparation). In some experiments, highly active CTL could be generated from Lyt-2,3$^+$ T cells while in other experiments, Lyt-1$^+$ T cells had an additional amplifying effect on the generation of virus-specific killer cells. Further experiments will have to show to what extent Lyt-1$^+$ helper T cells are involved in the generation of antiviral T cell responses. These findings reveal further similarities between alloreactive and virus-specific H-2-restricted CTL. They also demonstrate that the requirements for T cell subsets in the generation of H-2-restricted CTL are not identical for all antigens.

In contrast to activation against SV, primary induction of CTL against other viruses tested so far has proven difficult. If the SV system turns out to be an exception, one has to be critical as to whether the activation process towards paramyxoviruses is an example for CTL activation to viruses in general. The SV model has some properties in common with other virus models while it differs in other respects. Priming with inactivated virus and secondary challenge with inactivated virus has been possible with SV [2, 13, 27, 28], rabies virus [29] and influenza viruses [30, 31], but not with vaccinia [32, 33] and herpes virus ([34], M. Röllinghoff, personal communication). Paramyxoviruses are known for their fusion capacity while other viruses are less effective in this respect. Investigations on the requirements for target cell formation have shown differences: pox virus and herpes virus seem to require insertion of early virus-induced antigens to the plasma membrane of the target cell, while target cell formation with SV requires neither protein synthesis nor the presence of viral RNA [13]. Early antigens recognized on target cells infected with pox virus are presumably nonstructural proteins while in the SV model, the antigens inducing T cell triggering and target cell formation are spike proteins of the viral envelope [13, 27, 32].

There is little information about the stimulator and responder cell requirements during primary induction of antiviral CTL. Macrophages are reported to be an essential requirement for the secondary *in vitro* induction of ectromelia virus-specific CTL [35]. Furthermore, from data obtained with chimeric mice, which showed that cells from parent into F^1 chimeras could only be induced to generate T killer cells with H-2 specificity for the other parent in an environment containing lymphoid cells expressing the H-2 specificity of that parent, R. Zinkernagel [1] concluded that cells of the lymphoreticular system are in fact required for presentation of the viral antigen. This conclusion is supported by our earlier [11] and present data since we find that all cells used in the induction phase, even T cells can serve as effective stimulator cells, and macrophages are not required in the culture.

Our studies on virus cell interaction suggest that it is mainly the viral activity, presumably fusion, which induces the formation of the new antigenic determinant and that interference with this step will block T cell generation. In this context, it is intriguing to speculate that the role of antigen presentation by lymphoid cells is primarily dependent on the capacity of a given virus to induce the new antigenic determinants on the cell surface which is high for SV and low for pox and other viruses [36]. The high capacity to induce new antigenic determinants is explained by the fact that virus with a low multiplicity of infectivity or even noninfectious virus will create the antigenic determinants immediately after virus-cell contact, while other viruses, less active in fusion, require either higher amounts of viral antigen for immediate antigen expression on membranes or even protein neosynthesis.

Recently, mitogenic activity has been described for some strains of influenza viruses [37]. Generation of SV-specific CTL with cross-reactive activity on allogeneic target cells has been described [38, 39]. We found even generation of alloreactive CTL without cross-reactive properties indicating polyclonal T cell activation by SV (U. Koszinowski, unpublished observations). Furthermore, SV is a potent inducer of viral interferon. Increased production of interferon has recently been observed during mixed lymphocyte culture reactions in which CTL were generated [40], but its role in the induction of CTL remains open.

The combined features of SV, namely (a) T cell activation in the absence of infectivity, (b) fusion capacity, (c) target cell induction by spike proteins, (d) mitogenic activity on T cells and (e) induction of interferon, which are intrinsic for certain viruses (paramyxoviruses) but not others, might be the reason for the possibility to induce primary CTL to SV *in vitro*.

The way by which SV-specific T cell activation occurs is still unclear, but it could be described hypothetically as follows: mitogenic activity stimulates several T cell populations, and unspecific helper factors are induced [41, 42]. Virus fuses with cells within the responder cell population which creates the specific T cell-activating determinants. The fusion capacity of SV might even allow refusion and new presentation when the cell-presenting antigens initially deteriorates, which is indicated by cross-priming and cross-stimulation data in a secondary response ([11] and U. Koszinowski, unpublished observations). This effect may lead to long-term exposure of SV antigens on living cells in an immunogenic form.

Several aspects of this activation process, in particular the immunogenicity of the two surface proteins of SV, the mitogenic effect, the role of fusion and the H-2 requirements during CTL induction are presently under investigation.

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