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Hygiene-Institut der Universität Göttingen,
Lehrstuhl Medizinische Mikrobiologie, Fed. Republ. Germany

Cell-Mediated Cytotoxicity against Sendai-Virus-infected Cells[1])

H. Ertl and U. Koszinowski

With 4 Figures

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Abstract

After injection of Sendai virus, a parainfluenza virus type 1, mice generate cytotoxic lymphocytes which lyse specifically Sendai-virus-infected target cells in vitro. Their action is not inhibited by specific antibody in vitro. Killer cell activity appears 4 days after infection, reaches a maximum on the 7th day and disappears on the 14th to 16th day. Decrease of cytotoxic cell activity is correlated with an increase of haemagglutinating antibodies. The cytotoxic effector cell could be characterized as a thymus-derived cell, there is no specific activity in antibody-dependent cell-mediated cytolysis (ADCC).

The degree of cytotoxic effector cell activity is only slightly influenced by the dose of injected infective virus.

[1]) This work was supported by the Deutsche Forschungsgemeinschaft.

List of Abbreviations

ADCC	= antibody-dependent cell-mediated cytotoxicity
CFA	= complete Freund's adjuvant
CL	= cytotoxic lymphocytes
CMC	= cell-mediated Cytotoxicity
CRBC	= chicken red blood cell
EID_{50}	= 50% egg infective dose
E-monolayer	= monolayer of CRBC
EA-monolayer	= monolayer of CRBC coated with antibodies to CRBC
i.p.	= intraperitoneal
LCM	= lymphocytic choriomeningitis
LD_{50}	= 50% lethal dose
MEM	= minimal essential medium
N-modified	= N-(3-nitro-4-hydroxy-5-iodophenylacetyl)-β-alanylglycyl-glycyl-modified
NMS	= normal mouse serum
PBS	= phosphate buffered saline
rpm	= revolution per minute
SD-antigen	= Serologically defined antigen
SRBC	= Sheep red blood cell
$TCID_{50}$	= 50% tissue culture infective dose
TNP	= Trinitrophenyl
T cell	= Thymus-derived cell

Using different syngeneic Sendai-virus-infected cells as targets for cell-mediated cytotoxicity, a tumor line was not lysed by cytotoxic lymphocytes in spite of viral surface antigens.

Preliminary experiments were performed to demonstrate the H-2 gene restriction of the cytotoxic interaction. Using macrophages and tumor cells as targets only syngeneic infected target cells were lysed.

Introduction

Infection with some viruses results in the generation of cytotoxic effector cells. In the mouse viruses of 2 groups, 1 arenavirus (LCM-virus) and 2 poxviruses (Vaccinia and Ectromelia virus) have been well studied (1, 2, 3). In these systems the cytotoxic effector cells have been shown to be thymus-derived cells, which selectively lyse target cells expressing the same H-2 serologically defined antigens as the effector cells.

Similar immune reactions are induced *in vivo* by several syngeneic tumor cells (4) or by H-Y differences (5) and *in vitro* by TNP- or N-modified syngeneic stimulator cells (6, 7).

The 2 viral systems show similarities such as H-2 restriction and virus specificity but also some differences. In the LCM-virus-system PFIZENMAIER et al. (8) observed 2–5 days after the infection early killer cells, which lyse uninfected syngeneic cells. Such self-reactive cells were not found in the Vaccinia virus infection.

The cytolysis of Vaccinia-virus-infected cells was specifically inhibited by anti-Vaccinia virus serum (9). Inhibition of cytotoxicity was seen with anti-viral sera in the Sindbis virus infection (10) and with antiserum to TNP in the effector phase against TNP-modified target cells (11). These effects were not found in the LCM-system (PFIZENMAIER, pers. communication).

To study the significance of these differences, the investigations should be extended to other viral infections. Data presented here show generation, turnover and specificity of cytotoxic effector cells after infection of mice with Sendai virus, a para-influenza virus type 1, an RNA virus which maturates by a budding process in the same way as LCM virus.

Materials and Methods

1. Viruses

Sendai virus – strain D-52 (Behringwerke AG, Marburg/Lahn) – was propagated in the allantoic fluid of 10 days-old fertile eggs. The 27th passage was used throughout. The virus was diluted to a titer of 10^7 EID_{50} per ml.

Vaccinia virus – strain WR was propagated on VERO (monkey kidney cells) or on OMK (owl monkey kidney cells) cells and diluted to a titer of $10^{5.5}$ $TCID_{50}$ per ml.

LCM virus – strain WE-3, kindly provided by Dr. LEHMANN-GRUBE, Hamburg, was used in a concentration of 300 LD_{50} per ml.

2. Mice

Inbred mice of strains C3H (H-2^k), DBA/2 (H-2^d), AKR (H-2^k) and C57Bl/6 (H-2^b), purchased from B. L. Bomholdgard, Ry, Denmark, were used at the age of 8–12 weeks.

3. Antisera

Antiserum to Sendai virus was prepared as described elsewhere (12). Anti-Θ-serum was raised in AKR mice according to the method of Reif and Allen (13). The serum was absorbed with C3H bone marrow cells before use.

4. Separation of IgG-bearing cells and Macrophages

Immunoglobulin-bearing cells were eliminated by adherence to nylon wool columns (14). The decrease of IgG-positive cells was monitored by immunofluorescence. Macrophages were eliminated by adsorption of spleen cell suspensions on plastic surfaces.

5. Testing and elimination of ADCC effector cells

Absorption of Fc-bearing lymphocytes was performed on antibody-coated chicken red blood cell (CRBC) monolayers according to the method of Kedar et al. (15). Efficiency of the absorption was tested in ADCC. The same volume of rabbit antiserum to CRBC (diluted 1:1000, titer in ADCC 1:60,000), was added to 5×10^4 ^{51}Cr-labelled CRBC in a volume of 0.05 ml (sodium chromat, Amersham, Buchler, Braunschweig, No CJS1P, spec. activity 100–200 mCi/mg Cr). Absorbed or not absorbed lymphocytes (5×10^6) were incubated with the antibody-coated erythrocytes over a period of 4 hours. For calculation supernatants and pellets were harvested separately and ^{51}Cr release was determined according to the formula:

$$\%\ ^{51}\text{Cr release} = \frac{\text{radioactivity in supernatant}}{\text{radioactivity in supernatant} + \text{pellet}} \times 100$$

Specific lysis was calculated by subtracting ^{51}Cr release in the presence of CRBC + antibody without lymphocytes from ^{51}Cr release in the presence of CRBC + antibody + lymphocytes.

6. Indirect immunofluorescence

Indirect immunofluorescence of viral surface antigens on viable infected cells was performed as described previously (16).

7. Target cells

L-929 cells (H-2^k) were grown as monolayers in Eagles minimal essential medium supplemented with 10% calf serum and 100 μg/ml penicillin and streptomycin. Mastocytoma P-815 (H-2^d), Meth A (H-2^d) and EL-4 (H-2^b) tumour cells, kindly provided by Dr. Schirrmacher, Heidelberg, were serially passaged at 8–10 days intervals in the peritoneal cavity of syngeneic mice. Macrophages were harvested 3 days after i.p. injection of 2 ml thioglycollate and separated from other cells by affinity to glass or plastic surfaces and trypsin resistance.

8. Infection of target cells

A volume of 0.5 ml Sendai virus diluted in 20 ml medium was added to about $1–2 \times 10^7$ L-929 monolayer cells. 1 hour later the medium containing virus was discarded and fresh medium was added. Tumour cells (5×10^6 in 5 ml) were infected for 1 hour with 0.2 ml Sendai virus, centrifuged and resuspended in fresh medium. Macrophages were cultivated for 4 hours in petri dishes. The non-adherent cells were removed and 0.2 ml Sendai virus was added. After 1 hour incubation the virus-containing fluid was replaced by fresh medium.

9. Cytotoxic assay

The assay was performed with some modifications according to the method of BRUNNER et al. (17) and WAGNER (18). The number of 5×10^4 14 hour infected or uninfected ^{51}Cr-labelled target cells were incubated with 5×10^6 lymphocytes of normal mice or mice immunized 7 days previously with Sendai virus in a volume of 1.0 ml in plastic tubes. Spontanous lysis was determined by adding medium instead of lymphocytes, the maximal lysis was determined by adding 1 m HCl to the target cells. After 8 hours of incubation supernatants and cells were harvested seperately.

The % ^{51}Cr release was calculated using the formula:

$$\% \ ^{51}\text{Cr release} = \frac{\text{counts in the supernatant}}{\text{counts in the supernatant} + \text{in the pellet}} \times 100$$

Specific lysis was calculated by subtraction of % ^{51}Cr release in the presence of normal lymphocytes from % ^{51}Cr release in the presence of immune lymphocytes.

Assays were done at least in triplicate, SD values of all data presented here were less than 5%. Significance was calculated using the student t-test.

Results

1. Kinetics of development of cytotoxic lymphocytes in vivo

Eight groups of 4 C3H mice were injected i.p. at 2 day intervals with 1 ml Sendai virus containing 10^7 EID_{50} per ml. 2 days after the last injection the spleen lymphocytes were harvested and tested for

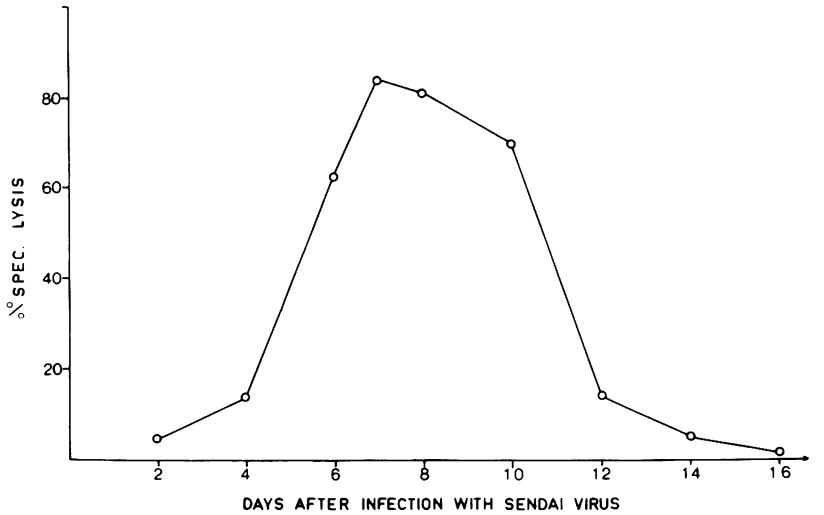

Fig. 1. Specific cytotoxicity against Sendai-virus-infected L-929 cells of spleen lymphocytes harvested from 8 groups of animals infected with Sendai virus (10^7 EID_{50}). % Specific lysis was calculated by subtraction of % Cr release caused by normal spleen cells from % Cr release caused by immune spleen cells. Each point represents the mean of 4 wells.

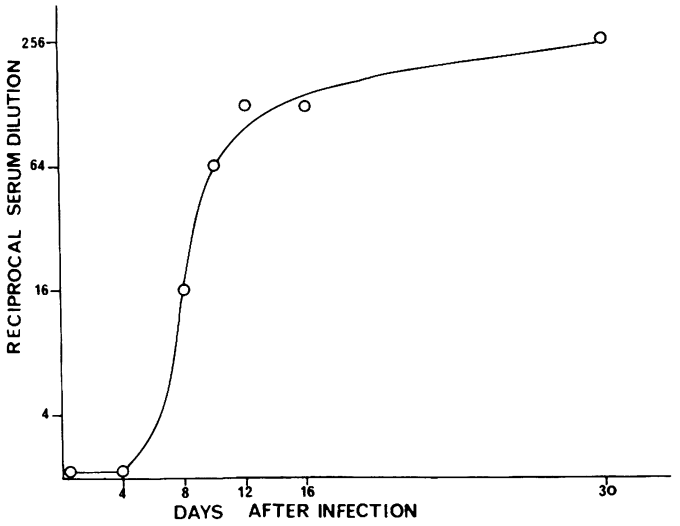

Fig. 2. Activity of anti-Sendai-virus serum from C3H mice infected 4, 8, 12, 16 and 30 days previously, was tested in a haemagglutination inhibition assay.

cytotoxicity on Sendai-virus-infected and normal L-929 target cells at an effector cell to target cell ratio of 100:1. Spleen lymphocytes of normal animals were used as controls. Significant cytolysis was observed on day 4 after infection, reaching a maximum on day 7–8 (Fig. 1), 2 weeks after infection only weak cytotoxicity was detectable.

2. *Kinetics of development of antibody in vivo*

The antibody titer of C3H mice injected 4, 8, 12, 16 and 30 days previously with Sendai virus was tested in the haemagglutination inhibition assay. As shown in Figure 2 antibody activities against Sendai virus were found on day 12–16.

Tab. 1. Effect of anti-Sendai serum on cell-mediated cytotoxicity. Specific lysis of Sendai-virus-infected L-929 cells by immune C3H lymphocytes was tested in the presence of normal mouse serum and anti-Sendai-virus serum raised in C3H mice.

Target cell	Effector cell	Serum	% spec. Lysis
L-929, Sendai-virus-infected	C3H	–	30.2
		normal	20.4
		anti-Sendai-virus	36.2
EL4, Sendai-virus-infected	C57Bl/6	–	22.7
		normal	18.4
		anti-Sendai-virus	29.9

3. Effect of anti-Sendai-virus serum on cell-mediated cytolysis

The incubation of target cells and effector cells in the presence of anti-Sendai-virus serum (titer 1:1024, final dilution 1:4) caused no inhibition of cell-mediated cytolysis in an 18 hours assay. In some experiments even a slight increase of specific lysis could be observed (Tab. 1).

4. Dose of in vivo injected virus and in vitro cytotoxic activity

C3H mice were infected by i.p. injection with Sendai virus diluted serially 10-fold with medium. Seven days later the spleen cells were harvested and the cytotoxic assay was performed. Little relationship with the amount of injected, infective virus could be observed. Lymphocytes of animals which received 10^1 EID_{50} virus showed the same activity as lymphocytes of animals which obtained 10^7 EID_{50} virus (Fig. 3). This indicates that the degree of antiviral CMC is independent of the primary dose. Only lymphocytes of animals which were infected with more than 10^8 EID_{50} virus showed higher cytotoxicity. About 50% of these mice died 4–5 days after infection.

5. Efficiency of target cell lysis in vitro

The activity of day 7 immune C3H lymphocytes on Sendai-virus-infected L-929 cells was tested using different ratios of target cells to lymphocytes. As control, spleen lymphocytes of normal mice were

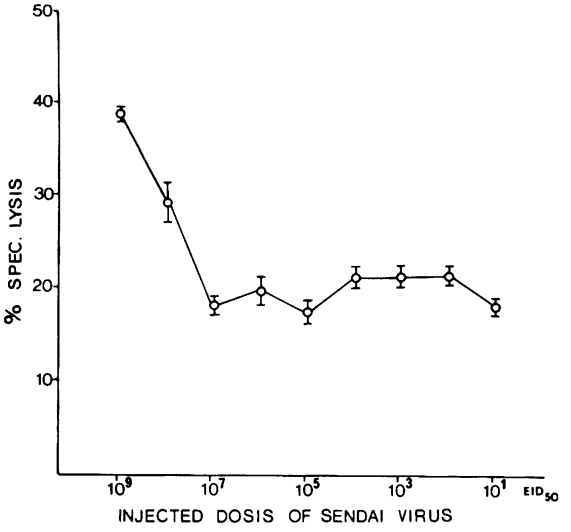

Fig. 3. Specific cytotoxicity against Sendai-virus-infected L-929 cells of spleen lymphocytes from animals infected 7 days previously with Sendai virus diluted serially 10-fold with medium was tested.

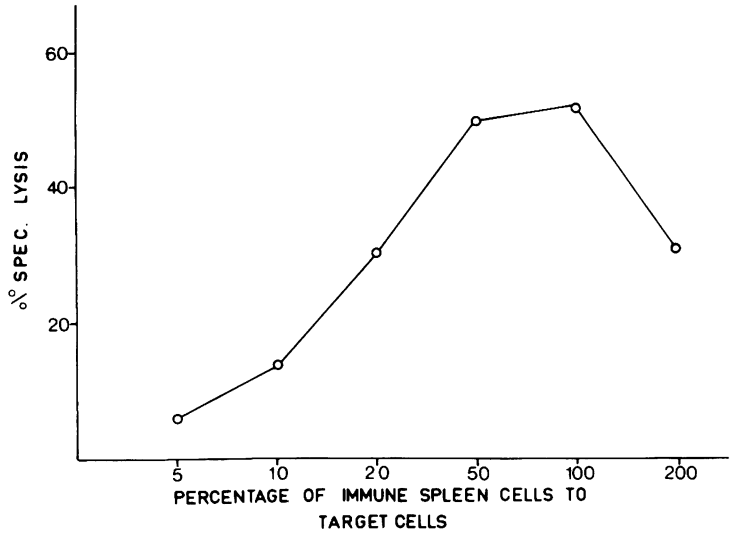

Fig. 4. Effector to target cell ratios and percentage of virus-specific CMC (target cells: Sendai virus infected L-929 cells; effector cells: C3H spleen lymphocytes). % Specific lysis was calculated by subtraction of % Cr release in the presence of normal spleen cells from % Cr release in the presence of immune spleen cells, using the same ratios.

added in the same ratios to the target cells. As seen in Figure 4, maximal cytotoxicity was obtained at ratios between 1:50 and 1:100 target cell to effector cells.

6. Virus specificity of cell-mediated cytolysis

C3H mice were infected with $10^{5.5}$ $TCID_{50}$ Vaccinia virus, 10^7 EID_{50} Sendai virus or 300 LD_{50} LCM virus. Spleen cell activity on Vaccinia-,

Tab. 2. Specific lysis of Vaccinia-, LCM- or Sendai-virus-infected C3H macrophages by vaccinia, LCM or Sendai immune spleen lymphocytes was tested. Mean of 4 groups; differences are statistically significant ($P < 0.001$).

Target cells	% Specific Cytolysis		
	Vaccinia virus immune C3H lymphocytes	Sendai virus immune C3H lymphocytes	LCM virus immune C3H lymphocytes
Normal macrophages	−0.2	0.7	−3.3
Macrophages infected with vaccinia virus	36.9	7.8	5.3
Macrophages infected with Sendai virus	1.1	42.2	1.7
Macrophages infected with LCM virus	−0.4	−1.5	27.4

Sendai-, or LCM-virus-infected macrophages was tested on day 7. As seen in Table 2 only target cells infected with the same virus as used for sensitization were lysed by immune lymphocytes, no cross-reactivity could be observed.

7. Characterization of the cytotoxic effector cell

Anti-Θ-serum: The number of 5×10^6 spleen lymphocytes from C3H mice sensitized 7 days previously to Sendai virus were incubated with 0.5 ml AKR anti-Θ-serum for 30 minutes at 37° C, washed 2 times and then incubated for another period of 30 minutes in 0.5 ml guinea pig complement. The latter had been preabsorbed on L-929 cells. Control cells were incubated with complement or with anti-Θ-serum alone. The results (Tab. 3) show, that the effector cell is sensitive for anti-Θ-serum.

Nylon wool column passage: To eliminate IgG-bearing cells the effector cells were passed through a nylon wool column (14). This procedure reduced the number of Ig-positive cells to 1–2% in the effluent fraction. Removal of B cells did not reduce virus-specific effector cell activity (Tab. 3).

Adsorption on plastic dishes: Macrophages were removed by incubation of effector cell suspensions (10×10^6) in 6 cm plastic dishes for 2 hours at 37°C. Control absorptions were run at 4°C. The results indicate that macrophages are not significantly involved in specific killing of infected target cells (Tab. 3).

Depletion of ADCC effector cells: Some inhibition assays with anti-Sendai-virus serum gave a rise in specific cytolysis. We tested therefore the activity of the effector cells after absorption of Fc-positive cells on EA-monolayers or on control E-monolayers. The efficiency of the separation was controlled by ADCC with CRBC target cells coated with rabbit anti-serum to CRBC (Tab. 4). The results suggest that Fc-

Tab. 3. Characterization of the Sendai-virus-specific cytotoxic effector cell. Mean % ^{51}Cr release from groups of 3 tubes. Lymphocytes were harvested from C3H mice 7 days after i.p. sensitization with 10^7 EID_{50} Sendai virus. Significant reduction ($P < 0.001$) of target cell lysis after pretreatment of lymphocytes with anti-Θ-serum and complement.

Pretreatment of immune lymphocytes	% Specific lysis at an attacker:target cell ratio of	
	100:1	10:1
None	34.2	2.8
Anti-Θ-serum	23.8	0.7
Complement	21.1	1.2
Anti-Θ-serum + complement	1.1	1.1
Nylon wool column	34.1	4.9
Absorption on plastic dishes	37.6	3.4

Tab. 4. Depletion of ADCC effector cells. Spleen cells originate from C3H mice inoculated (i.p.) 7 days previously with 10^7 EID_{50} Sendai virus. ADCC was performed in a 4 hour assay, reduction of cytolysis on EA-monolayers is significant ($P < 0.001$). CMC was performed in an 8 hours assay.

Cells	Treatment	Target cells CRBC ADCC	L-929, CMC
C3H spleen cells sensitized to Sendai virus	Control	62.9	34.2
	E-monolayer adsorption	51.7	29.7
	EA-monolayer adsorption	20.4	32.7
C3H spleen cells	Control	62.4	0
	E-monolayer adsorption	58.4	0
	EA-monolayer adsorption	20.9	0

positive cells are not the effectors in this reaction. Table 5 shows the activity of sensitized effector cells before and after immunoabsorption in the presence of anti-Sendai-virus serum. There is no decrease of cytotoxicity after removal of Fc-positive cells.

8. Lysis of different target cells by cytotoxic lymphocytes

Spleen lymphocytes from sensitized C3H, DBA/2 and C57Bl/6 mice were tested for cytotoxic activity on syngeneic target cells. As target cells infected macrophages, fibroblasts and tumour cells were used. As seen in Table 6 infected macrophages were significantly lysed

Tab. 5. Depletion of ADCC effector cells and cytolysis of target cells in the presence of antibodies. Spleen cells were obtained from C3H mice, infected 7 days previously with 10^7 EID_{50} Sendai virus. Antiserum to Sendai virus was raised in mice, HHT titer was 1:1024, final dilution of serum in the test: 1:10. No significant differences of cytotoxic activity after addition of normal or anti-viral serum to absorbed and non-absorbed spleen cells.

Treatment of effector cells	Serum added in vitro	Specific cytolysis of Sendai-virus-infected L-cells
None	–	34.2
	NMS	39.0
	anti-Sendai-virus antibodies	37.5
Absorption on EA-monolayers	–	32.7
	NMS	38.7
	anti-Sendai-virus antibodies	39.8
Absorption on E-monolayers	–	29.2
	NMS	38.4
	anti-Sendai-virus antibodies	33.9

Tab. 6. Data represent the mean of 4 groups. Standard error of the mean did not exceed 3%.

Target cells	Lymphocytes	% Specific lysis at attacker:target cell ratio 100:1	10:1
H-2^k Macrophages		3.9	−1.1
H-2^k Macrophages, infected	C3H	18.8	3.9
L-929		−1.0	3.1
L-929, infected		37.9	1.8
H-2^d Macrophages	DBA/2	0.6	1.2
H-2^d Macrophages, infected		28.3	2.4
P-815		1.5	0.9
P-815, infected		20.5	1.4
Meth A		−0.1	2.6
Meth A, infected		−1.8	1.1
C57Bl/6 Macrophages	C57Bl/6	6.8	2.7
C57Bl/6 Macrophages, infected		12.6	7.2
EL-4		7.2	6.9
EL-4, infected		36.7	18.0

by syngeneic sensitized lymphocytes. Using permanent cell lines in the H-2^k system L-929 cells were also killed. In the H-2^d system Mastocytoma P-815 cells were lysed in significant amounts by DBA/2 effector cells but syngeneic Meth A tumour cells were not affected. Immune C57Bl/6 lymphocytes killed macrophages and EL-4 tumour cells as well.

To control the percentage of infected cells, immunofluorescence studies with anti-Sendai-virus serum were performed on viable cells. The results clearly indicate, that all cells that were lysed by syngeneic effector cells as well as the tumour cell line resistant to virus-specific lysis, expressed Sendai-virus-specific surface antigens.

Tab. 7. In vitro cytotoxicity of Sendai-virus-sensitized C3H, DBA/2 and C57Bl/6 spleen cells to syngeneic and allogenic normal and Sendai-virus-infected target cells at an attacker:target cell ratio of 100:1 and 10:1.

Target cells	% Specific lysis (8 hours assay) of the effector cells					
	C3H		DBA/2		C57Bl/6	
	100:1	10:1	100:1	10:1	100:1	10:1
C3H-macrophages	3.1	0.9	−3.3	−2.2	−2.1	4.8
C3H-macrophages, infected	**14.8**	3.1	−6.8	−2.1	0.1	2.9
P-815	0	0.1	1.4	1.8	3.8	1.1
P-815, infected	−0.1	0.1	**14.3**	0.7	3.6	4.9
C57Bl/6-macrophages	0.6	0.8	−1.0	5.4	5.4	2.2
C57Bl/6-macrophages, infected	2.5	1.4	−8.4	3.3	**10.6**	5.8
EL-4	0.8	2.2	n. t.		5.8	5.6
EL-4, infected	−0.4	3.5	n. t.		**30.4**	14.3

9. H-2 gene restriction of effector cell activity

Virus-specific sensitized cells from C3H (H-2^k), DBA/2^d) and C57Bl/6 (H-2^b) mice were incubated with various target cells. Normal and infected tumour cell lines were used in this assay as well as normal and infected macrophages. The effector cells killed only syngeneic infected target cells (Tab. 7).

Discussion

The data of the present investigation demonstrate, that mice infected with Sendai virus, generate cytotoxic effector cells which are able to kill Sendai-virus-infected syngeneic target cells.

The kinetics of the generation of cytotoxic lymphocytes *in vivo* were similar to the other experimental viral systems (1, 3). Also *in vitro* conditions of target cell lysis using different effector to target cell ratios were comparable to other virus-specific cellular immune responses, maximal lysis was obtained with a ratio between 50–100 attacker cells to 1 target cell.

The effector cells killed only infected but not non-infected syngeneic control target cells. Pfizenmaier et al. (8) found early self-reactive lymphocytes *in vitro* after experimental LCM virus infection in mice. Sendai and LCM viruses are both RNA-containing viruses which maturate by a budding process on the cell surface. Since we could not find self-reactive cells in the early stages of Sendai virus infection the generation of these cytotoxic cells does not generally characterize the cellular immune response to RNA-containing budding viruses.

Haemagglutinating antibodies were demonstrable 8 days after the infection and reached a plateau after 12–16 days. Thus, these antibodies appeared at the same time as cytotoxic effector cell activity decreased. Possibly there is a correlation between disappearance of killer cells and rise of antibodies (19).

Inhibition of CMC by anti-Sendai-virus serum could not be observed. It is possible that an antibody which shows haemagglutination of Sendai virus does not react with the target of CL. The slight increase of cytotoxic effects in the presence of antibody cannot be attributed to an additional activity of non-thymus-derived, Fc-receptor-bearing lymphocytes. Using different criteria for the characterization of cytotoxic effector cells *in vitro* evidence was provided that the cytotoxic lymphocytes were thymus-derived cells. Adsorption of spleen cells from sensitized mice on EA-monolayers (15) did not decrease the specific activity of virus-activated T cells. The data show conclusively that the spleen cells contain ADCC effector cells against antibody-coated CRBC which can be specifically absorbed,

but there was no ADCC of Sendai-virus-infected cells in the presence of specific antibody.

When we used various target cells, we could observe that in the H-2^k, H-2^d and H-2^b systems syngeneic infected macrophages were generally killed. Infected L-929 cells, mastocytoma P-815 or EL-4 tumour cells were also lysed by syngeneic effector cells, sometimes even better than macrophages. However, Meth A cells were not killed by cytotoxic lymphocytes in spite of viral surface antigens. As it was shown by LESLEY et al. (20), different tumour cells are characterized by a rather low density of H-2 antigens. It is possible that infected tumour cells, which are not killed by sensitized lymphocytes are marked by a low content of H-2 specificities or by a resistance to cell-mediated cytolysis *in vitro*. Using Meth A cells as stimulator cells for *in vitro* induction of allogenic C3H killer cells we could not obtain cytotoxic effector cells reactive against H-2^d target cells. Testing C3H effector cells, sensitized *in vitro* against H-2^d, we found lysis of DBA/2 macrophages or mastocytoma P-815 tumour cells while Meth A cells were not killed (unpublished results).

The results presented in Table 7 demonstrate that cytotoxic cells generated against Sendai-virus-infected target cells require H-2 compatibility between effector and target cells. By using congenic mouse strains virus-specific recognition of modified products of the I region and the M locus can be excluded. In general, the specificity of the cytotoxic lymphocyte generated during Sendai-virus infection in mice seems to follow the same genetic restrictions described in the reaction towards viral- (2, 3, 21) chemical- (6, 7, 12) H-Y- (5) or minor histocompatibility antigen- (22) modified target cells.

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Dr. U. Koszinowski, Hygiene-Institut der Universität Göttingen, Lehrstuhl Medizinische Mikrobiologie, Kreuzbergring 57, D-3400 Göttingen.

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