Cell-Mediated Cytotoxicity Toward Measles Virus-Infected Target Cells in Randomly Bred Syrian Hamsters

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Cell-mediated cytotoxicity (CMC) toward measles virus-infected cells was studied by a ^{51}Cr release assay with spleen cells from hamsters inoculated with measles virus (strain Lec) or control antigen and with spleen cells from normal hamsters. Spleen cells from measles virus-inoculated hamsters showed greater CMC toward infected than toward noninfected target cells (designated specific CMC). Specific CMC was maximal 7 days after virus inoculation and was declining by 9 to 10 days. Effector cells were present in a nonadherent cell population. Specific CMC was reduced after treatments of effector cells with antithymocyte serum plus complement. The decrease in cytotoxicity was greater toward infected target cells than toward noninfected target cells. Treatment of infected target cells with antimeasles serum did not increase specific CMC by effector cells from the majority of virus-inoculated hamsters. CMC toward infected target cells by normal spleen cells (natural killer cells) or spleen cells from hamsters inoculated with control antigen was approximately the same as, or more often less than, CMC toward noninfected target cells. Natural killer cells were present in a nonadherent cell population. Treatment of natural killer cells with antithymocyte serum plus complement caused a similar decrease in cytotoxicity toward both infected and noninfected target cells. This study demonstrated virus-specific cellular cytotoxicity of effector spleen cells from measles virus-inoculated hamsters. Although the data were compatible with T cells as the source of effector cells in the virus-specific CMC, definitive identification could not be made. Additional membrane markers for better characterization of hamster lymphocyte subpopulations are required.

Although hamsters have found wide use as animal models for human virus infections (see review in reference 17), there are relatively few published reports on their cellular immune response to viruses (2, 3, 8, 14, 22; S. K. Datta, M. T. Gallagher, and J. J. Trentin, *Fed. Proc.* 38:1363, 1979; J. W. Streilein, *Fed. Proc.* 40:2346, 1981). The finding by Zinkernagel and Doherty of major histocompatibility complex restriction on the cytotoxic activity of virus-specific T-cell effector cells of mice (23) has stimulated interest in the major histocompatibility complex restriction on the T-cell cytotoxic function of hamsters. Recent findings by Nelles and Streilein (14) and Zinkernagel et al. (22) indicate that a number of different inbred strains of hamsters derived from colonies established from the three original hamsters captured in Syria in 1930, as well as strains of partially inbred hamsters derived from wild hamsters captured in 1970 in Syria, do not require strain-specific major histocompatibility complex compatibility between target and effector cells for

killing of virus-infected target cells. Nelles and Streilein (*Fed. Proc.* 40:2346, 1981), in a later study, concluded that cell-mediated cytotoxicity to vaccinia virus in these hamsters was mediated by both antibody-dependent cellular cytotoxicity and by natural killer (NK) cells.

We previously studied cellular and humoral immunity to measles virus in adult random-bred hamsters which had survived antithymocyte serum (ATS) treatment concomitant with measles virus inoculation as neonatal animals (5). Although these hamsters still had a good humoral antibody titer to measles virus as adults, no cell-mediated cytotoxicity for measles virus-infected target cells could be demonstrated. Their spleen cells released an equal percentage of ^{51}Cr from both infected and noninfected target cells. The target cells used in these cytotoxicity assays were from a spontaneously transformed baby hamster kidney cell line (BHK 0-853) (6) derived from animals of our randomly bred colony and infected with measles virus. We postulated that the lack of cellular cytotoxicity might be due to a

^e *n* = 19 hamsters. In this series, spleen cells from 3 of 19 hamsters did not show a specific cytotoxic reaction, i.e., percent specific cytotoxicity was <10%.

panencephalitis strain Lec [1]) at a multiplicity of infection of 1. The cultures were passaged 1 week later and were used as target cells 2 to 3 days after this passage. Fresh target cells were infected for each experiment. At the time of use, 70% or more of the cells had membrane stain by the fluorescent-antibody technique with fluorescein-labeled anti-measles virus immunoglobulin prepared in hamsters. Noninfected BHK 0-853 cells which showed no staining with the fluorescein-labeled antibody were used as control target cells. The target cells were labeled with ^{51}Cr ($>200 \mu\text{Ci}/\text{mg}$; New England Nuclear Corp., Boston, Mass.) at $100 \mu\text{Ci}/10^6$ cells for 1 h at 35°C in an atmosphere of 95% air–5% CO_2 . After being washed three times in PBS-FBS, they were suspended in medium at a concentration of 10^4 cells per 0.1 ml.

(iii) **Method.** Target cells (10^4 infected and noninfected cells) were incubated in round-bottom microtiter wells (Linbro plates, Flow Laboratories, Inc., Inglewood, Calif.) with either 0.1 ml of added medium for spontaneous release (SR) or spleen cells in 0.1 ml of medium for 18 h at 35°C in an atmosphere of 95% air–5% CO_2 . Effector cells were checked at effector cell/target cell (E/T) ratios of 200 through 50, 100 through 25, or 100, as indicated in Results. The microtiter plates were then centrifuged at $700 \times g$ for 10 min, and 0.05 ml of the supernatant fluid was removed for counting in a Beckman gamma scintillation counter. All assays were performed in quadruplicate with spleen cells from individual hamsters. The counts per minute in the 0.05-ml samples were multiplied by 4 for correction to counts per minute in the total volume of the supernatant fluid (0.2 ml). Quadruplicate samples of 10^4 labeled target cells were counted for total incorporation. Calculations were as follows:

% SR =

$$\frac{\text{cpm released from target cells} + \text{medium}}{\text{cpm total incorporation}} \times 100$$

% cytotoxicity =

$$\left(\frac{\text{cpm released from target cells} + \text{spleen cells}}{\text{cpm total incorporation}} \times 100 \right) - \% \text{ SR}$$

Percent specific cytotoxicity equals percent cytotoxicity with infected target cells minus percent cytotoxicity with noninfected target cells. Percent SR by measles virus-infected target cells was greater than by noninfected control target cells (21.7 ± 4.8 versus $13 \pm 2.8\%$).

In vitro treatment of spleen cells with ATS. Samples of ATS were sequentially absorbed with stirring for 1 h at room temperature and overnight at 5°C with equal packed cell volumes of BHK 0-853 cells, hamster erythrocytes, and GD36 lymphoma cells (a hamster B cell lymphoma line [4] received through the courtesy of J. E. Coe). Washed spleen cells (10^7) from hamsters inoculated with measles virus or control antigen were incubated for 1 h at 35°C with intermittent shaking with 0.3 ml of various dilutions of absorbed ATS with and without guinea pig complement (0.3 ml of a 1:8

dilution), with complement only, or with medium only. The cells were then washed two times with PBS-FBS and suspended finally in medium for the cytotoxicity assay. Sera were inactivated at 56°C for 30 min before use.

In vitro treatment of target cells with antiserum to measles virus. Hamsters were immunized at 2-week intervals with three subcutaneous and intramuscular injections of measles virus (strain Lec; infected baby hamster brain suspension) in complete Freund adjuvant (10^5 PFU/inoculum). A fourth injection in incomplete Freund adjuvant was given 6 weeks later. The hamsters were bled and the antiserum collected 8 days later. The 80% plaque neutralization titer of the antiserum was 1:800, and the hemagglutination inhibition titer was 1:1,024.

Washed infected and noninfected target cells were incubated with hamster antiserum to measles virus or with normal hamster serum for 1 h at room temperature with intermittent shaking. The cells were then washed two times with PBS plus 20% FBS. They were suspended in medium for the cytotoxicity assay. The percent SR by the serum-treated and nontreated target cells was determined.

Separation of spleen cells on nylon fiber columns. Preparation of the nylon fiber (Fenwal Laboratories, Division of Travenol Laboratories, Inc., Morton Grove, Ill.) columns and separation of lymphocytes was done as described by Greaves et al. (9). Briefly, 7×10^7 to 8×10^7 spleen cells in Earle saline containing penicillin, streptomycin, and 10% FBS were placed on 300-mg nylon fiber columns. After incubation at 35°C for 30 min, the nonadherent cells were recovered by elution of the column with warm Earle saline containing penicillin, streptomycin, and 10% FBS. The mean percentage of cells recovered from the columns in different experiments was 16% (range, 14 to 20%) of the number of cells placed on the columns. Eighty-five to ninety percent of the eluted cells showed membrane staining by the indirect fluorescent-antibody test with ATS absorbed with hamster erythrocytes, BHK 0-853 cells, and cell line GD36. Four to eight percent of the eluted cells showed membrane staining by the indirect fluorescent-antibody test with rabbit anti-hamster immunoglobulin and fluorescein-labeled goat anti-rabbit immunoglobulin G.

RESULTS

Optimal conditions for assay. To determine optimal conditions for the demonstration of cytotoxicity, the following variations were tried: 2 or 10% FBS added to the medium; 100, 50, and 25 E/T ratios; 3, 5, 7, and 9 to 10 days after virus inoculation for time of explant of spleen cells. No difference in results occurred at the two FBS concentrations. The best results were obtained at an E/T ratio of 100 with cells explanted 7 days after virus inoculation (Fig. 1). The assays were therefore routinely done under these conditions and with 10% FBS in the medium.

Cytotoxicity of spleen cells from inoculated and uninoculated hamsters. Initial studies indicated that spleen cells from animals inoculated with control antigen prepared from noninfected cells

FIG. 2. Cell-mediated cytotoxicity of spleen cells from normal, uninoculated hamsters toward virus-infected and noninfected target cells (control targets) ($n = 61$; E/T = 100).

caused the release of ^{51}Cr from both infected and noninfected target cells. Therefore, a series of experiments was done to evaluate the level of cytotoxicity of spleen cells due to stimulation of hamsters by control antigen versus that induced by measles virus antigen, as well as the cytotoxicity of spleen cells from uninoculated hamsters. The percent cytotoxicity by spleen cells from hamsters inoculated with control antigen was usually greater for noninfected target cells than for infected targets, which resulted in a negative or zero number for percent specific cytotoxicity (Table 1). Rarely, percent cytotoxicity of spleen cells from a hamster had a low positive value (1 to 6%). A positive value for percent specific cytotoxicity, which varied from animal to animal, occurred with spleen cells from hamsters inoculated with virus (Table 1). With 10% specific cytotoxicity as the cutoff for significance, 69% of the hamsters (121 of 176) had a specific response; specific cytotoxicity varied from 10 to 36% among individual hamsters. This information was derived from a compilation of data from

a number of experiments. To evaluate the effect of various treatments of spleen cells on their ability to specifically kill infected target cells, only those hamsters whose spleen cells showed a percent specific cytotoxicity of $\geq 10\%$ under the standard conditions of the test were included.

Spleen cells from most normal, uninoculated hamsters showed some cytotoxicity for both infected and noninfected target cells, with percent ^{51}Cr release usually higher for noninfected cells. Therefore, percent specific release was usually a negative value. The degree of response varied from hamster to hamster, as shown in Fig. 2.

Passage of effector cells through nylon fiber. Nonadherent cells recovered from nylon fiber columns after passage of spleen cells from virus-inoculated hamsters caused the same percent cytotoxicity as nonfractionated spleen cells at an E/T ratio of 100. A slight increase in percent cytotoxicity and percent specific cytotoxicity occurred with the nonadherent cells compared with nonfractionated cells as the E/T ratio dropped to 50 and 25 (Table 2). After passage through nylon fiber columns, cytotoxic cells from spleens of normal hamsters were also present in the nonadherent fraction. As usual, cytotoxicity was greater for the noninfected than for the infected target cells. Cytotoxicity at all E/T ratios was somewhat higher with the nonadherent population than with the nonfractionated population (Table 3).

Reduction of cell cytotoxicity by treatment of effector cells with ATS plus complement. Initial studies on the effect of ATS on effector cell function of virus-inoculated hamsters were performed with ATS preparation 1 at a 1:8 serum dilution. Inhibition of cytotoxicity toward infected and noninfected target cells was similar (93 and 88% inhibition, respectively). Further dilutions were then checked. At higher ATS dilutions, inhibition of cellular cytotoxicity was more pronounced against the infected target cells than against the noninfected target cells

TABLE 2. Cytotoxicity of spleen cells (from virus-inoculated hamsters) recovered after passage through nylon fiber columns^a

Treatment of effector cells	% Cytotoxicity (mean $\pm$ SD) at an E/T ratio of		
	100	50	25
None			
Infected target cells	25.0 $\pm$ 6.2	17.9 $\pm$ 5.9	11.7 $\pm$ 4.0
Noninfected target cells	8.0 $\pm$ 3.5	6.7 $\pm$ 2.8	4.5 $\pm$ 2.0
Nylon fiber passed			
Infected target cells	25.0 $\pm$ 4.5	22.1 $\pm$ 4.3	15.9 $\pm$ 4.0
Noninfected target cells	8.4 $\pm$ 4.2	7.9 $\pm$ 3.9	5.8 $\pm$ 3.0

^a Results with spleen cells from 18 hamsters inoculated with virus 7 days before the test.

TABLE 3. Cytotoxicity of normal hamster spleen cells after passage through nylon fiber columns^a

Treatment of effector cells	% ⁵¹ Cr release (mean ± SD) at an E/T ratio of		
	100	50	25
None			
Infected target cells	2.5 ± 0.5	1.5 ± 0.1	0.5 ± 0.7
Noninfected target cells	8.2 ± 4.7	5.0 ± 0.9	2.8 ± 0.7
Nylon fiber passaged			
Infected target cells	3.6 ± 1.0	2.2 ^b	2.0 ± 1.4
Noninfected target cells	11.6 ± 4.8	6.8 ^b	2.4 ± 1.8

^a Results with spleen cells from five normal uninoculated hamsters.^b Mean results from two of the five hamsters; cell yield was insufficient with the other three hamsters to test at all three E/T ratios.

(Table 4). ATS preparation 2, which had a lower serum cytotoxicity titer for splenic lymphocytes than preparation 1 (1:128 versus 1:1,028), caused similar inhibition, but lower serum dilutions were required. Inhibition of cytotoxicity was greater toward infected than toward noninfected target cells at all dilutions tested. ATS treatment of normal spleen cells also caused reduction in their cytotoxicity, but the percent reduction was similar toward both infected and noninfected targets (Table 5).

Effect of treatment of target cells with antiserum to measles virus. Initial studies with spleen cells from virus-inoculated hamsters were done at an E/T ratio of 100. No enhancement of

cytotoxicity toward the antiserum-treated target cells was seen with spleen cells from 10 hamsters. The second series of experiments was done at E/T ratios of 200, 100, and 50. Specific cytotoxicity of effector cells from 9 of 12 normal hamsters changed from a negative to a positive value (e.g., at an E/T ratio of 200, specific cytotoxicity changed from -1.2 ± 1.3 to 6.3 ± 2.9). Similar results occurred at E/T ratios of 100 and 50. Specific cytotoxicity of effector cells from 3 of 12 virus-inoculated hamsters also was enhanced at the three E/T ratios, whereas the specific cytotoxicity of the cells from the remaining 9 hamsters was neither enhanced nor inhibited at any of the E/T ratios.

TABLE 4. Reduction in cytotoxicity by in vitro treatment of spleen cells from virus-inoculated hamsters with ATS plus complement^a

Treatment of effector cells	% ⁵¹ Cr release (mean ± SD) from:		% Inhibition ^b	
	Infected target cells	Noninfected target cells	Infected target cells	Noninfected target cells
None ^c	31.7 ± 7.3	15.5 ± 3.8		
Plus complement	26.4 ± 6.8	11.1 ± 3.0		
Plus ATS and complement, preparation 1, diluted 1:8	1.8 ± 1.0	1.3 ± 0.9	93.1	88.3
None ^d	23.7 ± 6.9	9.9 ± 2.0		
Plus complement	19.1 ± 4.5	7.4 ± 7.2		
Plus ATS and complement, preparation 1				
1:32	2.2 ± 1.9	2.6 ± 1.0	88.5	64.9
1:128	3.7 ± 0.6	4.9 ± 0.8	80.7	33.8
1:512	13.5 ± 7.7	6.0 ± 1.4	29.4	19.0
None ^e	26.3 ± 8.5	9.8 ± 4.5		
Plus complement	22.0 ± 6.7	8.1 ± 4.1		
Plus ATS and complement, preparation 2				
1:8	2.8 ± 1.7	5.0 ± 3.6	87.3	38.2
1:32	6.0 ± 1.8	7.1 ± 5.3	72.8	12.4
1:128	12.3 ± 7.5	7.7 ± 4.8	39.6	5.0

^a E/T = 100. ATS preparations were absorbed sequentially with equal volumes of BHK 0-853 cells, hamster erythrocytes, and GD36 lymphoma cells for 1 h at room temperature and overnight at 4°C.^b Based on percent ⁵¹Cr release with spleen cells plus complement.^c Spleen cells from nine hamsters.^d Spleen cells from three hamsters.^e Spleen cells from seven hamsters.

DISCUSSION

The percent increased cytotoxicity of spleen cells from measles virus-inoculated hamsters for infected target cells over that for noninfected cells (percent specific cytotoxicity) appears to be virus specific and not due to interferon enhancement of NK cells, as has been reported in studies with mice and human cells (15, 16, 20). Interferon, produced by normal lymphocytes *in vitro* during an 18-h cytotoxicity assay with virus-infected cells, is reported to increase the NK cytotoxicity of lymphocytes for the infected target cells over that naturally present for infected and noninfected target cells. Although the spleen cells from the majority of normal hamsters in our studies showed some cytotoxicity for infected and noninfected target cells, the percent cytotoxicity was usually greater for noninfected target cells (Fig. 2). The lack of increase in cytotoxic activity toward infected target cells by spleen cells unprimed to measles virus was also substantiated by the response of hamsters inoculated with control antigen (Table 1). Cellular cytotoxicity of these animals was not greater for infected target cells than for noninfected target cells. In fact, percent cytotoxicity was usually greater toward noninfected target cells. Thus, the phenomenon of enhancement of NK cell cytotoxicity for infected cells by interferon production *in vitro* apparently did not occur in the present study. The lack of increased cytotoxicity for infected targets by normal spleen cells or spleen cells primed with control antigen could not be attributed to low infection of the target cells, as reported by Santoli et al. (15). Seventy percent or more of the target cells in the present study were infected, as demonstrated by membrane staining for measles virus antigens in each experiment.

Furthermore, since normal spleen cells or spleen cells from hamsters inoculated with control antigen did not cause increased ^{51}Cr release from virus-infected target cells, the observed increased release by spleen cells from virus-primed hamsters was not due to the increased lytic susceptibility of the infected target cells to the manipulative procedures of the assay.

The temporal appearance of increased cytotoxicity of spleen cells from virus-inoculated hamsters also fits with a virus-specific stimulation of cytotoxicity (14, 21, 22) rather than increased NK cytotoxicity due to enhancement by interferon production *in vivo*. NK activity stimulated *in vivo* by virus injection usually is maximal at 3 days after inoculation (8, 10, 19, 21) and declines by 6 to 7 days after inoculation (10, 18, 20), whereas virus-specific cell-mediated cytotoxicity is maximal at 6 to 8 days after inoculation (19, 23). In our study, maximal cytotoxicity

occurred 7 days after inoculation, and it began to decline 9 to 10 days after inoculation (Fig. 1).

Virus-specific cytotoxicity may result from direct cell-mediated cytotoxicity by T cells or from antibody-dependent cell cytotoxicity by K cells. It is possible that the cytotoxicity in this study was antibody-dependent cell cytotoxicity. However, the ability of spleen cells from the majority of virus-inoculated hamsters to kill infected target cells was not increased by treatment of the infected target cells with antiserum to measles virus. If antibody-dependent cell cytotoxicity were involved, enhanced ^{51}Cr release would be expected with antiserum to measles virus-treated target cells due to reaction between Fc receptors on the effector cells with the Fc portion of the antibody molecule attached to the target cell. Our data do not provide a definitive answer in this regard.

The percent cytotoxicity with noninfected target cells by virus-primed spleen cells (Table 1) was of the same order as that by control antigen-primed spleen cells or normal spleen cells (NK cells) (Table 1 and Fig. 2). Thus, the response of spleen cells from virus-primed hamsters appears to consist of two components, an NK component, which may cause from 5 to 6% to as much as 15 to 20% cytotoxicity with noninfected target cells, and a virus-specific T-cell component, which accounts for an additional 10 to 30% of specific cytotoxicity with infected targets. Although both the NK component and the virus-specific component were reduced by *in vitro* treatment of effector cells with ATS plus complement, the percent reduction was greater toward the virus-specific component. Thus, at increasing ATS dilutions, inhibition of cytotoxicity was still pronounced toward virus-infected targets, whereas that toward noninfected targets dropped substantially (Table 4). Virus-specific and NK activities were present in the nonadherent fraction of spleen cells (Tables 2 and 3). Other investigators (8; Datta et al., Fed. Proc.) have also reported the presence of NK activity in the nonadherent fraction of hamster spleen cells. NK cells in the mouse are nonadherent cells which may or may not have surface Fc receptors and may or may not have a low density of θ antigen on their plasma membranes (7, 11, 18), whereas T-cells, active in virus-specific cell-mediated cytotoxicity, are nonadherent cells with a high density of θ antigen on their plasma membranes (23).

Approximately 30% of the hamsters tested did not develop a cytotoxic response to virus-infected target cells that was significantly higher (greater than 10%) than that to noninfected target cells. This is not particularly surprising. There is usually some heterogeneity in the ability of individual animals to respond to antigens,

TABLE 5. Reduction in cytotoxicity by in vitro treatment of spleen cells from normal hamsters with ATS plus complement^a

Treatment of effector cells	% ⁵¹ Cr release (mean ± SD) from:		% Inhibition ^b	
	Infected target cells	Noninfected target cells	Infected target cells	Noninfected target cells
None ^c	8.8 ± 2.4	10.5 ± 0.7		
Plus complement	6.0 ± 1.4	7.3 ± 1.2		
Plus ATS and complement, preparation 1				
1:32	1.6 ± 0.7	2.2 ± 0.8	73.3	69.9
1:128	3.1 ± 0.8	4.4 ± 1.0	48.3	39.7
1:512	4.6 ± 0.5	6.1 ± 1.7	23.3	16.4
None ^d	4.1 ± 2.4	9.3 ± 2.9		
Plus complement	2.3 ± 1.7	6.8 ± 3.3		
Plus ATS and complement, preparation 2				
1:8	0.6 ± 0.9	3.3 ± 1.8	74.0	51.5
1:32	2.3 ± 1.4	5.7 ± 3.3	0.0	16.2
1:128	2.2 ± 1.3	5.5 ± 2.9	0.0	19.2

^a E/T = 100. ATS preparations were absorbed sequentially with equal volumes of BHK 0-853 cells, hamster erythrocytes, and GD36 lymphoma cells for 1 h at room temperature and overnight at 4°C.

^b Based on ⁵¹Cr release with spleen cells plus complement.

^c Spleen cells from four normal hamsters.

^d Spleen cells from seven normal hamsters.

as well as heterogeneity in the height of their immune responses.

If the increased cellular cytotoxicity for measles virus-infected target cells is a T-cell-mediated reaction, as our data suggest, then the finding is similar to that of Zinkernagel et al. with closed colonies of randomly bred mice (24). Lymphocytes of individual randomly bred mice responded to virus-infected target cells established from mice within their own colony, but not to target cells of other unrelated inbred or randomly bred strains. Although our data are more compatible with the view that the virus-specific response in our studies is T-cell rather than K-cell or NK-cell mediated, definitive proof will require isolation and better characterization of specific functional cell subpopulations. Many of the markers used in the identification of mouse and human effector cells have not yet been identified with hamster cells.

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