INFECTION AND IMMUNITY, Apr. 1978, p. 1-5
0019-9567/78/0020-0001$02.00/0

Vol. 20, No. 1
Printed in U.S.A.

Cell-Mediated Immunity Assayed by ^{51}Cr Release Test in Mice Infected with Mouse Adenovirus

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Received for publication 19 September 1977

Immune spleen cells (ISC) from mice immunized with a sublethal dose of mouse adenovirus (M-Ad) were shown by the ^{51}Cr release test to be cytotoxic to target mouse embryonic cells or lymphoid cells infected with M-Ad. The number of ISC required for release of statistically significant amounts of ^{51}Cr from target cells varied from one sample to another, ranging from 5 to ≥30 ISC per target cell. When 24-h-infected mouse embryonic cells were used as targets, the release of ^{51}Cr became evident in 6 h after the addition of ISC to the cells, gradually increased with time, and then leveled off. Cytolytic activity of M-Ad ISC is specific for M-Ad, since ISC do not lyse mouse embryonic cells infected with human adenovirus type 12 and vice versa. Kinetic study of the development of cell-mediated immunity to M-Ad assayed by ^{51}Cr release showed that cytolytic activity of ISC in infected mice became detectable 4 days postinfection, reached its peak level about 7 to 10 days postinfection, and fell to undetectable levels about 10 days thereafter. This is consistent with the data obtained by inhibition of intracellular viral antigen synthesis or by the macrophage migration inhibition test in our previous reports.

Our previous papers have shown that mouse adenovirus (M-Ad) immune mouse spleen cells (ISC) inhibit capsid antigen synthesis in M-Ad-infected cells when added within 12 h postinfection (p.i.), ISC kill the infected cells when examined by the dye exclusion test (10), a new surface (S) antigen(s) appears on the infected cells at the early stage of infection (15), and T-cell sensitization against the S antigen(s) can be demonstrated by the macrophage migration inhibition test (13, 14).

These findings favor our hypothesis that a virus-induced S antigen(s) may be recognized as a new antigen, which may serve as a target site for cell-mediated immunity (CMI), through which, in turn, sensitized lymphocytes destroy virus-infected cells, resulting in interruption of intracellular virus multiplication (21, 22).

To further confirm CMI in M-Ad-infected mice, lymphocyte cytotoxicity as another in vitro correlate of CMI was tested against M-Ad-infected cells by ^{51}Cr release from labeled target cells with ISC, and the results are described in this paper.

MATERIALS AND METHODS

Mouse strain. Male 2- to 3-month-old C3H/He mice were used in this study. They have been maintained in our laboratory by brother-sister matings, as described previously (10).

Cells. Two types of cells were used as targets for the cell-mediated cytotoxicity test. (i) Mouse embryonic (ME) cell cultures were prepared from mouse embryos of about 17- to 20-day gestation. They were used as target cells between passages 2 and 10. (ii) For lymphoid cells, surgically removed mouse spleens were minced by using scissors, washed with minimal essential medium (MEM), and suspended in MEM containing 10% fetal calf serum. The viability of the cells was checked by the trypan blue exclusion test (10), and samples containing more than 10% dead cells were discarded.

Cells were cultured in Eagle MEM supplemented with 10% fetal calf serum and 60 μg of kanamycin per ml.

Viruses. M-Ad, strain FL, was propagated in ME cells, and its stock suspension was prepared as described previously (10). Its infectivity titer was approximately 10^8 plaque-forming units (PFU)/ml. Human adenovirus type 12 (Ad12), strain Huie, was propagated in HeLa cells as described previously (15). Its stock virus titer was estimated to be approximately 10^7 mean tissue culture infective doses/ml.

Preparation of ISC. Mice were immunized with 5×10^6 PFU of M-Ad intraperitoneally. Seven to 10 days later their spleens were removed, and their cells were dispersed, washed, and resuspended as ISC suspensions as described previously (10). The viability of the ISC was checked by the trypan blue exclusion test (10). To eliminate erythrocytes, ISC preparations were treated with 0.83% NH_4Cl solution at room temperature for 10 min. There was no reduction in viable cells of the non-erythrocyte population by this procedure. Normal spleen cells were prepared from unimmunized mice in the same way and used as a control.

Cytotoxicity test. The cytotoxicity test was carried out by ^{51}Cr release from target cells using a slight modification of the method of Gardner et al. (7). ME cells, 2×10^5, were plated on flat-bottomed wells (Linbro Chemical Corp., New Haven, Conn.). Overnight cultures were infected with M-Ad at an input multiplicity of infection of about 50 PFU/cell. After adsorption at 37°C for 2 h, cell monolayers were washed twice with MEM supplemented with 10% fetal calf serum and labeled with 1 μCi of ^{51}Cr (sodium chromate of 200-mCi/mg specific activity) in 0.1 ml of phosphate-buffered saline per well at 37°C for 30 min. The labeled cells were washed with MEM at 37°C, incubated for about 24 h at 37°C in 5% CO_2 in air, and used as target cells. As a control, uninfected cells were labeled with ^{51}Cr in the same way as described for infected cells. One milliliter of appropriately diluted ISC suspension was added to each well and incubated at 37°C for an additional 18 h in 5% CO_2 in air. After incubation, a definite amount of supernatant of each well was removed very carefully and centrifuged at 2,000 rpm for 10 min. The radioactivity of centrifuged supernatants was determined by a gamma scintillation counter. ^{51}Cr release was expressed as the percentage of specific lysis using the equation: percent specific cytolysis = [counts per minute (experimental) − counts per minute (spontaneous)]/maximum counts per minute × 100. Maximum counts per minute was a count of ^{51}Cr released in the supernatant after freeze-thawing labeled cells three times in a well in 1 ml of distilled water. Four wells were used for each experimental group.

For the lymphoid cell target, a short-term ^{51}Cr release assay was used. Lymphoid cells, 10^7, which were infected with M-Ad at a multiplicity of infection of about 50 PFU/cell and incubated for about 24 h, were labeled with 10 μCi of ^{51}Cr for 30 min at 37°C with frequent agitation. After cells were washed to remove free radioactivity, 2×10^5 target cells were transferred to each test tube and mixed with ISC at an appropriate target-to-effector cell ratio. After incubation at 37°C for 6 h, the cells were spun down at 2,000 rpm for 10 min, and the radioactivity of their supernatants was determined.

The average values of spontaneous release from infected and uninfected cells as targets were about the same, i.e., about 33% from ME cells and 25% from lymphoid cells, respectively. Since these values were high, percent specific lysis was calculated by the above-mentioned equation.

RESULTS

^{51}Cr release from target cells at various times after virus infection. Spleen cells were infected with M-Ad at an input multiplicity of infection of 50 PFU/cell. After a 2-h adsorption, the cells were washed with MEM and incubated at 37°C in 5% CO_2 in air. At 3, 6, 12, 18, and 24 h after initiation of adsorption, samples were removed, labeled with ^{51}Cr, washed with MEM, and mixed with ISC at a ratio of 60 ISC per target cell. After the mixtures were kept at 37°C for 6 h, the radioactivity of the supernatants was determined (Fig. 1).

There was no significant ^{51}Cr release when the ISC were added to cells sampled at 3 h after the initiation of adsorption. However, when ISC were added to cells sampled at 6 h after the initiation of adsorption, slight but statistically significant ^{51}Cr release was observed. The longer the time after infection, the greater the amount of ^{51}Cr released. There was no difference in the extent of specific ^{51}Cr release between 18- and 24-h-infected cells.

Time course of ^{51}Cr release from target cells after addition of ISC. As described in Materials and Methods, ISC were added to 24-h virus-infected ME cells at a ratio of 30 per target cell and incubated at 37°C. At appropriate time intervals thereafter, samples of the supernatant were removed and their radioactivity was determined (Fig. 2).

Significant specific release was noticed at 6 h after the addition of ISC to target cells, and it

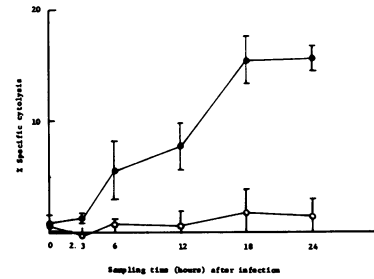

FIG. 1. *Kinetics of development of susceptibility of infected cells to cytolysis by ISC. Mouse spleen cells were infected with M-Ad at a multiplicity of infection of 50 PFU/cell and incubated at 37°C in 5% CO_2 in air. At appropriate time intervals p.i., samples were removed, labeled with ^{51}Cr, mixed with ISC at a target/effector ratio of 1:60, incubated for an additional 6 h, and centrifuged at 2,000 rpm for 10 min; the radioactivity of the supernatants was determined. Symbols: (——) adsorption period; (●) ISC; (○) normal spleen cells.*

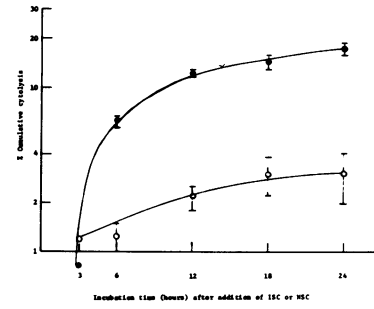

FIG. 2. *Time course of ^{51}Cr release from target cells after addition of ISC. M-Ad infected and ^{51}Cr-labeled ME cells were mixed with ISC (●) or normal spleen cells (○) at a target/effector ratio of 1:30 and incubated at 37°C.*

gradually increased up to 18 h, at which time it reached about 15 to 20%, whereas less than 3% of ^{51}Cr release was observed when normal spleen cells were used instead of ISC (Fig. 2).

Number of ISC required for significant ^{51}Cr release from target cells. ISC were appropriately diluted and added to ^{51}Cr-labeled, M-Ad-infected ME cells or spleen cells. Cytolytic activity of ISC varied from one sample to another, but usually the cytolysis was observed when the ratio of effector-to-target cell was >30. The results with ISC of strong activity and those with ISC of weak activity are shown in Tables 1 and 2.

When strongly active ISC were used against infected ME cells, a statistically significant extent of ^{51}Cr release was observed even at an effector-to-target cell ratio of 5, whereas with weakly active ISC it was observed at a ratio of 30 or greater. When infected lymphoid cells were used as targets in a short-term ^{51}Cr release test, statistically significant cytolysis was observed at a ratio of 10 or greater (Table 3).

Specificity of cytolytic activity of ISC for M-Ad. The specificity of cytolytic activity of ISC was investigated by using ME cells infected with M-Ad or human Ad12 as targets. With the ISC prepared from M-Ad-immune mice, a significant amount of ^{51}Cr was released from M-Ad-infected ME cells, whereas this was not the case with Ad12-infected ME cells (Table 4). Conversely, when Ad12-immune spleen cells were used as effectors, a statistically significant amount of ^{51}Cr was released from Ad12-infected ME cells but not from M-Ad-infected ME cells (Table 4).

TABLE 1. *Number of ISC required for cytolysis of infected ME cells*[a]

Target cells	Target cell/ISC	% Specific ^{51}Cr release by:	
		ISC	NSC
Infected ME cells	1:1	6.5 ± 1.9	ND[b]
	1:5	8.8 ± 1.1[c]	−0.2 ± 0.7
	1:10	12.8 ± 3.8[d]	ND
	1:30	13.8 ± 5.0[e,f]	2.6 ± 1.3
	1:60	16.2 ± 5.9[e]	−1.9 ± 0.6
	1:90	20.4 ± 3.2[c,d]	1.3 ± 1.6
Uninfected ME cells	1:10	0.5 ± 1.3	ND
	1:30	3.2 ± 1.9	ND
	1:90	1.7 ± 1.8	ND

[a] Infected and ^{51}Cr-labeled target ME cells were prepared as described in Materials and Methods, received ISC at the indicated target/effector ratios, and were incubated at 37°C for 18 h. NSC, Normal spleen cells.
[b] ND, Not done.
[c] Significantly higher than lysis by NSC on infected targets ($P < 0.001$).
[d] Significantly higher than lysis on uninfected cells for the same target/effector ratio ($P < 0.001$).
[e] Significantly higher than lysis by NSC on infected targets ($P < 0.005$).
[f] Significantly higher than lysis on uninfected cells for the same target/effector ratio ($P < 0.01$).

TABLE 2. *Number of ISC required for cytolysis of infected ME cells*[a]

Target cells	Target cell/ISC	% Specific ^{51}Cr release by:	
		ISC	NSC
Infected ME cells	1:1	0.9 ± 0.7	ND[b]
	1:10	0.8 ± 0.5	ND
	1:30	4.3 ± 0.5[c]	0.8 ± 0.4
	1:60	6.9 ± 1.1	ND
	1:120	8.5 ± 0.7[c]	1.4 ± 0.5
Uninfected ME cells		ND	ND

[a] See footnote *a*, Table 1. NSC, Normal spleen cells.
[b] ND, Not done.
[c] Significantly higher than lysis by NSC on infected targets ($P < 0.001$).

TABLE 3. *Number of ISC required for cytolysis of infected spleen cells*[a]

Target cells	Target cell/ISC	% Specific ^{51}Cr release by:	
		ISC	NSC
Infected spleen cells	1:1	1.5 ± 2.5	ND[b]
	1:5	1.3 ± 1.1	0.3 ± 1.9
	1:10	5.2 ± 1.0[c]	ND
	1:30	8.9 ± 2.9[d,e]	−1.5 ± 1.1
	1:60	11.6 ± 2.4	ND
	1:120	19.5 ± 5.6[c,e]	−2.6 ± 1.3
Uninfected spleen cells	1:1	0.7 ± 1.3	ND
	1:10	1.0 ± 1.7	ND
	1:30	1.9 ± 0.9	0.9 ± 1.8
	1:120	1.8 ± 1.3	1.5 ± 1.9

[a] Infected and ^{51}Cr-labeled spleen cells as targets were prepared as described in Materials and Methods, received ISC at the indicated target/effector ratios, and were incubated at 37°C for 6 h.
[b] ND, Not done.
[c] Significantly higher than lysis on uninfected spleen cells for the same target/effector ratio ($P < 0.001$).
[d] Significantly higher than lysis on uninfected spleen cells for the same target/effector ratio ($P < 0.005$).
[e] Significantly higher than lysis by NSC on infected targets ($P < 0.001$).

Kinetic study of the development of CMI assayed by ^{51}Cr release. Mice were immunized with 5×10^6 PFU of M-Ad and sacrificed at appropriate time intervals thereafter. Their spleen cells were removed and assayed for cytolytic activity by ^{51}Cr release from infected ME cells as targets. Spleen cells from three mice were pooled and added to target cells at a ratio of 60 ISC per target cell. After incubation at 37°C for 18 h, released radioactivity was determined.

The results are shown in Fig. 3. A significant amount of ^{51}Cr was released when ISC were prepared from mice 4 days p.i. The cytolytic activity of ISC reached its peak level about 7 to

TABLE 4. *Specificity of ISC for virus*[a]

ISC from	% Specific ^{51}Cr release from ME cells		
	Uninfected	M-Ad infected	Ad12 infected
M-Ad immune	1.1 ± 1.5	11.1 ± 1.9[b]	1.0 ± 1.3
Ad12 immune	0.7 ± 1.7	0.6 ± 1.3	5.9 ± 1.5[c]

[a] Mice were immunized with 5×10^6 PFU of M-Ad or with 10^7 mean tissue culture infective doses of Ad12. Seven days later, their spleen cells were used as ISC. ME cells were infected with M-Ad or Ad12 at an input multiplicity of infection of about 50 PFU/cell or 10 mean tissue culture infective doses/cell, respectively, labeled with ^{51}Cr, incubated at 37°C for 24 h, and then used as targets as described in Materials and Methods. The effector-to-target cell ratio was 60:1.

[b] Significantly higher than lysis on uninfected cells ($P < 0.001$).

[c] Significantly higher than lysis on uninfected cells ($P < 0.025$).

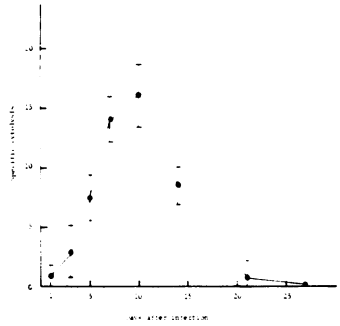

FIG. 3. *Cell-mediated cytotoxic response in M-Ad-infected mice. Mice were immunized with a sublethal dose of M-Ad and sacrificed at appropriate time intervals thereafter. Spleen cells from three mice were pooled and added to infected and labeled ME target cells at a ratio of 60 ISC per target cell. After incubation at 37°C for an additional 18 h, released radioactivity was determined.*

10 days p.i. and fell to undetectable levels about 10 days thereafter.

DISCUSSION

The results described above can be summarized as follows. ISC from mice immunized with M-Ad are capable of inducing specific cytolysis of M-Ad-infected cells, regardless of the mouse cell type used as a target, i.e., ME or spleen cells. Since the extent of release of ^{51}Cr depends on the type of target cells to some extent, a long-term (16 to 18 h) ^{51}Cr release assay was used for ME cells and a short-term (6 h) assay was used for lymphoid cells.

The number of ISC required for release of a statistically significant amount of ^{51}Cr from target cells varied from one sample to another, ranging from 5 per target cell for strongly active ISC (Table 1) to 30 or more for weakly active ISC (Table 2). The amount of ^{51}Cr released from target cells increased roughly in parallel with increasing numbers of ISC, but at high effector-to-target ratios it showed a trend to level off (Table 1).

The kinetic study showed that statistically significant cytolysis became detectable about 6 h after the addition of ISC to infected ME cells (Fig. 2) and about 2 to 3 h after addition of ISC to infected lymphoid cells (data not shown). These results are consistent with the findings on cytotoxic lymphocytes generated in the mixed-lymphocyte reaction (11) or those reported in other virus infections (7, 8).

The susceptibility of M-Ad-infected cells to cytolysis by ISC became detectable by 6 to 12 h p.i. and reached its peak level by 18 to 24 h p.i. By 6 to 12 h p.i. there is S antigen synthesis and no capsid antigen synthesis (10, 13–15). The time course of the development of antigenicity responsible for cytolysis by ISC is similar to that responsible for macrophage migration inhibition (13, 14), although the cytolytic cells are likely to be a different subset than the migration inhibition factor-producing cells, and it also runs parallel to the kinetics of the development of S antigenicity in M-Ad-infected cells (14, 15). Hence, it seems reasonable to assume that the antigen(s) responsible for the cytolysis by ISC is S antigen(s). This favors our hypothesis that the S antigen(s) appearing on the surface of virus-infected cells is responsible for induction of CMI (21, 22), but it remains to be determined whether or not the S antigen(s) demonstrated by antibodies is identical to the antigen(s) responsible for induction of CMI against M-Ad-infected cells, in which effector cells have proved to be T cells (13, 14). The effector cells required for the lysis of M-Ad-infected cells by ISC are also T cells (manuscript in preparation).

The cytolytic activity of ISC is regarded to be virus specific, since ISC cytolytic to M-Ad-infected cells were not cytolytic to Ad12-infected ME cells and vice versa (Table 4).

The kinetic study of the CMI response assayed by ^{51}Cr release in sublethally M-Ad-infected mice revealed a rapid rise and fall pattern, similar to those observed by inhibition of capsid antigen synthesis (10) or the macrophage migration inhibition test (13, 14). From these findings, it is very likely that one and the same phenomenon (CMI) was assayed by different methods, and these data also support the explanation that the inhibition by ISC of intracellular capsid antigen synthesis is due to killing of target cells by

ISC (10). In addition, this development pattern of CMI response seems to be a general phenomenon regardless of types of antigens, for example, alloantigens (4), tumor-specific transplantation antigens (2), and virus-induced antigens in cells infected with ectromelia (3, 8), vaccinia (16, 17), herpes simplex (17, 18), Sendai (6), mumps (20), influenza (5, 12), rubella (19), Sindbis (9), and/or Venezuelan equine encephalomyelitis (1) viruses.

Finally, to our knowledge this is the first report to show that ISC are able to lyse in vitro target cells that are infected with the nonenveloped deoxyribonucleic acid virus.

ACKNOWLEDGMENT

This work was supported by grants from the ministry of Education, Science and Culture, Japan.

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