Leukocyte Cytotoxicity in a Persistent Virus Infection: Presence of Direct Cytotoxicity but Absence of Antibody-Dependent Cellular Cytotoxicity in Horses Infected with Equine Infectious Anemia Virus

YOSHIKI FUJIMIYA, LANCE E. PERRYMAN,* AND TIMOTHY B. CRAWFORD

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164

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Antibody-dependent cellular cytotoxicity and direct cytotoxicity assays were performed with equine infectious anemia virus-infected target cells, equine leukocytes, and equine anti-equine infectious anemia virus antibody to determine whether these mechanisms play a role in controlling viral replication in equine infectious anemia. Direct cytotoxicity was observed by using peripheral blood mononuclear cells from 7 of 10 infected horses. Antibody-dependent cellular cytotoxicity was not observed. The antibody-dependent cellular cytotoxicity reaction in horses was then studied by using sheep erythrocytes and trinitrophenylated sheep erythrocytes as target cells. Lysis of these target cells was mediated by neutrophils, monocytes, and lymphocytes. The reaction was activated by antibody of the immunoglobulin G class but not by immunoglobulin G(T). Furthermore, immunoglobulin G(T) efficiently inhibited immunoglobulin G in this function.

Equine infectious anemia (EIA), a persistent viral infection of horses, is caused by a nononcogenic retrovirus (2, 9, 10, 40, 41, 58; T. B. Crawford, W. P. Cheevers, P. Kllaver-Anderson, and T. C. McGuire, in J. Stevens, G. Todero, and C. Fox [ed.], Eleventh ICN/UCLA Symposium on Molecular and Cellular Biology, in press). The disease is characterized by clinical cycles 3 to 7 days long during which rapid virus replication is associated with fever, depression, and erythrocyte destruction (18, 20; Crawford et al., in press). A vigorous immune response directed toward both virion (4, 12, 23, 25) and cell membrane antigens (36) contributes to the clinical signs and lesions (5, 37, 38, 45). The number, frequency, and severity of cycles vary among horses. Most horses, however, stop cycling within a few months and become asymptomatic viral carriers for life. The control of viral replication and, consequently, clinical disease seems to be immunological in nature (Crawford et al., in press).

We hypothesized that antibody-dependent cellular cytotoxicity (ADCC) and direct cytotoxicity would be operative in horses with EIA. Furthermore, factors which modulate these mechanisms might contribute to differences in clinical patterns among infected horses. In this study we tested the ability of horse leukocytes, in the presence and absence of antibody, to kill EIA virus-infected target cells. We found that peripheral blood mononuclear cells (PBMC) from infected horses selectively killed virus-infected cells in direct cytotoxicity assays. However, attempts to demonstrate ADCC were unsuccessful. This prompted an attempt to more thoroughly define the ADCC phenomenon in horses by using defined antigens and purified reagents. We found that ADCC in horses is mediated by neutrophils, monocytes, and lymphocytes, is activated by antibodies of the immunoglobulin G (IgG) class, and is inhibited by antibodies of the IgG(T) subclass.

MATERIALS AND METHODS

Effector cells. Neutrophils, monocytes, and lymphocytes were isolated from heparinized blood of control and EIA virus-infected horses. After sedimentation of erythrocytes for 30 min at room temperature, the plasma was layered over Ficoll-Hypaque solution and centrifuged at 400 × g as previously described (19). Cells banding at the plasma-Ficoll-Hypaque interface were >98% lymphocytes and monocytes. The cells were collected, washed three times with Hanks balanced salt solution, and resuspended in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) containing 25 mM HEPES (N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid), 1-glutamine, penicillin, streptomycin, and 5% fetal calf serum. The mononu-

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clear cells were further separated into lymphocytes and monocytes as follows. Cells were resuspended in RPMI 1640 with 10% fetal calf serum and incubated on plastic petri plates at 37°C for 2 h. Nonadherent lymphocytes were removed by gentle aspiration. The plates were washed vigorously with RPMI 1640, and any additional nonadherent cells were discarded. Monocytes were then scraped from the plates. Mononuclear cells were also separated by passage over nylon wool columns. The columns were prepared and eluted as previously described, except that Hanks balanced salt solution was substituted for Puck solution G (49).

Neutrophils were obtained by collecting the cells that pelleted through Ficoll-Hypaque and lysing the erythrocytes with 0.83% ammonium chloride. They were washed three times with RPMI 1640 containing 5% fetal calf serum.

Suspensions of isolated monocytes, neutrophils, and lymphocytes were adjusted to a density of 10^7 cells per ml of RPMI 1640 with 5% fetal calf serum. The respective cell preparations contained >98% neutrophils, >95% lymphocytes, and >95% monocytes, as defined by morphological criteria on Wright-stained smears.

Target cells. Equine dermal fibroblasts persistently infected with the fibroblast-adapted strain of EIA virus (30) were produced as previously described (10). Noninfected dermal fibroblasts were maintained under identical conditions. Sheep erythrocytes (SRBC) and trinitrophenylated SRBC (TNP-SRBC) were also used as target cells. Fresh SRBC were washed three times with RPMI 1640 and resuspended at a concentration of 5 x 10^6 cells per ml. TNP-SRBC were prepared as previously described (47).

Antiserum. Horse anti-SRBC, prepared by repeated intravenous injections of washed SRBC, had agglutinating and hemolytic titers of 1:16 and 1:1,000, respectively. Anti-2,4-dinitrophenol (DNBP) antibody was prepared by immunization of horses with DNBP-bovine gamma globulin as previously described (34). Anti-DNBP antibodies were obtained with affinity chromatography on a DNP-lysin-agarose column and subsequently fractionated into IgG and IgG(T) by diethylaminoethyl-cellulose chromatography (34). Sera from horses infected with the Wyoming strain of EIA virus were used as sources of anti-EIA virus antibody.

Measurement of direct cytotoxicity by 51Cr release. The assay was performed in flat-bottomed microtiter plates (Falcon Plastics, Oxnard, Calif.) as follows. Effector cells were dispensed in 0.1-ml volumes in three wells; this was followed by the addition of 0.1 ml of labeled target cells. After incubation at 37°C for 34 h, the plates were centrifuged, and 0.1 ml of supernatant was removed from each well for measurement of radioactivity. Spontaneous 51Cr release was determined by incubating labeled target cells in the presence of RPMI 1640. Total releasable 51Cr was measured after labeled target cells were incubated in 3% Triton X-100. Results were expressed as percent specific release, computed from the following equations:

\[ R = \frac{\text{counts per minute from infected target cells} - \text{counts per minute from noninfected target cells}}{\text{total counts per minute from infected target cells} - \text{total counts per minute from noninfected target cells in RPMI}} \times 100; \]

\[ R_2 = \left( \frac{\text{counts per minute from infected target cells in Triton X-100} - \text{counts per minute from noninfected target cells in RPMI}}{\text{counts per minute from infected target cells in Triton X-100}} \right) \times 100; \]

plus effector cells – counts per minute from noninfected target cells in RPMI)/(total counts per minute from noninfected target cells in Triton X-100 – counts per minute from noninfected target cells in RPMI) x 100; and percent specific release = \( R \) - \( R_2 \). For experiments performed in this study, \( R_2 \) ranged from 0.7 to 17.1%, with a mean and standard deviation of 8.1 ± 4.7%.

Measurement of ADCC by 51Cr release. SRBC were labeled with sodium 51Crchlorate solution (specific activity, 350 mCi/mg; New England Nuclear Corp., Boston, Mass.) by incubating 5 µl of washed, packed SRBC with 0.5 µl of 51Cr for 2 h at 37°C. The SRBC were washed three times and resuspended to 5 x 10^5 cells per ml. Persistently infected fibroblasts were labeled by exposure to medium containing 350 µCi of Na51CrO4 per ml for 90 min. They were then trypsinized, washed, and resuspended to 10^6 cells per ml. Effector cells (10^5/ml) were incubated with varying concentrations of antisera and then added to flat-bottomed microtiter plates in 100-µl portions. The target cells (100 µl) were then added, and the plates were incubated for 4 h (SRBC) or 24 h (fibroblasts) at 37°C. Cells were then sedimented by centrifugation for 5 min, and 100 µl of the supernatant was removed from each well and counted in a Beckman 300 gamma counter. The percentage of specific isotope release from target cells was calculated from the following formula: percent specific release = [(counts per minute of target cells, effector cells, and antisera – counts per minute of target cells and effector cells)/(total counts per minute in 3% Triton X-100 – spontaneous release of target cells alone)] x 100. All tests were performed in triplicate, and data are expressed as the mean of the three observations.

Ability of antibody type to activate lysis of SRBC by ADCC. The ability of IgG and IgG(T) antibodies to participate in ADCC was tested. Varying quantities of IgG(T) were added to 0.1 ml of effector cells in microtiter wells, and TNP-SRBC were then added. After 4 h at 37°C, cytotoxicity was evaluated as described above. The ability of IgG(T) to inhibit IgG-mediated ADCC was tested by using anti-DNBP IgG and IgG(T) in three different ratios of IgG(T) to IgG (7.78, 1, and 0.124; see Table 4). For each experiment, IgG anti-DNBP was first incubated with TNP-SRBC for 5, 15, 30, and 60 min, IgG(T) and effector cells were then added, and cytotoxicity was evaluated after 4 h. In parallel experiments, the same protocol was followed, except that the IgG and IgG(T) were reversed.

RESULTS

Demonstration of direct cytotoxicity with EIA virus-infected target cells. Direct cytotoxicity reactions were demonstrated by using PBMC from three horses infected with EIA virus. The magnitude of cytotoxicity was dependent on the ratio of effector cells to target cells (Fig. 1). Cytotoxicity was reproducibly observed at ratios of effector cells to target cells of 100:1. Cytotoxicity from control horse PBMC was negligible at this ratio. Therefore, a ratio of 100:1 was used for subsequent studies.
The in vitro kinetics of cytotoxicity were examined by using PBMC obtained from three infected horses and one control horse. The release of \(^{51}\text{Cr}\) increased with time in the presence of PBMC from both normal and EIA virus-infected horses (Fig. 2). A 34-h harvest time provided both convenience and an acceptable ratio between release by infected horse PBMC and release by control horse PBMC.

Table 1 summarizes the results obtained when PBMC from 10 infected horses were evaluated in cytotoxicity assays. Positive responses were obtained 14 to 18 days after infection of four horses with \(5 \times 10^2\) 50% tissue culture infective doses of the fibroblast-adapted strain of EIA virus. Three of six horses infected for 1 to 11 years also had cytotoxic PBMC detectable by this assay.

**Absence of ADCC with EIA virus-infected target cells.** The ability of equine leukocytes to mediate ADCC reactions against EIA virus-infected fibroblasts was examined (Tables 2 and 3). Leukocytes from three control and eight infected horses and anti-EIA antibody from eight horses were used. No ADCC could be demonstrated.

**Characterization of ADCC reactions in horses employing defined antigens and purified reagents.** Neutrophils (polymorphonuclear leukocytes [PMN]), PBMC, monocytes, and lymphocytes from control horses and horses infected with EIA virus were compared in ADCC assays by using SRBC targets sensitized with horse anti-SRBC antibody (Fig. 3). All cell preparations were active in ADCC, and the order of effectiveness was PMN > monocytes > lymphocytes. No obvious differences in effector cell activity between EIA virus-infected and control horses were observed.

PBMC from control and infected horses were separated into adherent and nonadherent subpopulations by passage over nylon wool columns. The activity of adherent and nonadherent cells in ADCC assays was similar to that of unfractionated PBMC (data not shown).

The influence of the ratio of effector cells to target cells was evaluated (Fig. 4). Specific release of isotope from target cells increased as the ratio of effector cells to target cells increased. Effector cells from EIA virus-infected and control horses gave virtually identical results, indicating that EIA virus infection does not in itself affect leukocyte function in ADCC assays.

**Inhibition of ADCC by IgG(T).** Data from experiments to examine the ability of IgG and IgG(T) anti-DNP antibodies to activate lysis of TNP-SRBC are shown in Fig. 5 and 6. IgG efficiently activated ADCC by PMN and PBMC from control and infected horses. Minimal cytotoxicity was observed when IgG(T) anti-DNP was used. Use of IgG anti-DNP and IgG(T) anti-DNP in combination inhibited ADCC in proportion to the relative concentration of IgG(T) present. A ratio of IgG(T) to IgG of 1:10 inhibited more than 50% of the reaction, and a 1:1 ratio virtually abolished it.

To determine whether inhibition depended upon the time allowed for IgG to bind to target cells, experiments were performed in which TNP-SRBC target cells were incubated with IgG for varying lengths of time before addition of IgG(T) and PBMC. In parallel experiments, IgG(T) was added before, in combination with, or without IgG. These studies were done with three different concentrations of IgG and IgG(T) (Table 4). IgG(T) anti-DNP inhibited ADCC under all conditions tested. The amount of in-

**Fig. 2.** Kinetics of direct cytotoxicity of EIA virus-infected target cells. Effector cells were obtained from three horses with EIA (horses 23, 281, 381) and one control horse (horse 333). The ratio of effector cells to target cells was 100:1.
TABLE 1. Release of 51Cr from target cells by PBMC from EIA virus-infected horses

<table>
<thead>
<tr>
<th>Horse no.</th>
<th>Time post-infection</th>
<th>% of 51Cr release from:</th>
<th>Percent specific release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EIA virus-infected fibroblast target cells</td>
<td>Noninfected fibroblast target cells</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>330</td>
<td>NA</td>
<td>14.1 ± 0.4</td>
<td>13.8 ± 0.5</td>
</tr>
<tr>
<td>333</td>
<td>NA</td>
<td>4.0 ± 0.3</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>350</td>
<td>NA</td>
<td>8.0 ± 0.4</td>
<td>9.5 ± 0.5</td>
</tr>
<tr>
<td>325</td>
<td>NA</td>
<td>12.7 ± 0.6</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td>329a</td>
<td>NA</td>
<td>9.8 ± 0.5</td>
<td>10.4 ± 0.5</td>
</tr>
<tr>
<td>391a</td>
<td>NA</td>
<td>10.1</td>
<td>6.9</td>
</tr>
<tr>
<td>392a</td>
<td>NA</td>
<td>11.4</td>
<td>12.0</td>
</tr>
</tbody>
</table>

* NA, Not applicable.

The mean ± standard deviation of triplicate observations.

Horses 328, 329, 391, and 392 were tested before and after infection with EIA virus.

Inhibition depended on the ratio of IgG to IgG(T) and on the order of addition of antibody to target cells. IgG(T) significantly inhibited ADCC (more than 50%) when it was present in one-tenth the amount of IgG and was added as much as 30 min after IgG (Table 4, experiment 3).

Based on the marked inhibition of ADCC by IgG(T) when TNP-SRBC were used as target cells, we attempted to determine whether the presence of IgG(T) caused inhibition of ADCC by using EIA virus-infected fibroblasts as target cells. IgG was fractionated from sera of four horses infected with EIA virus. None of the IgG fractions was entirely pure, as all contained at least 10% IgG(T). No cytotoxicity was observed in ADCC assays when these IgG-rich fractions were employed.

DISCUSSION

Cytolytic removal of virus-infected cells by direct cytotoxicity and ADCC is believed to play an important role in controlling viral infections. Direct cytotoxicity has been demonstrated in vitro with target cells infected with rubella (46), measles (27), mumps (1, 11), herpes simplex (53), infectious bovine rhinotracheitis (50), vaccinia (61), and influenza (55) viruses. ADCC has been demonstrated for many of these same viruses (18, 51, 55, 56, 61), as well as with rabies (16), Epstein-Barr (21), Moloney sarcoma (28), and coxsackie B3 (62) viruses. In general, ADCC is more easily demonstrable than direct cytotoxicity. Furthermore, claims for direct cytotoxicity may, in reality, be ADCC assays, activated by antibody secreted during the incubation period of the experiment (18, 50).

Recently, a role for natural killer cells in nonspecific cytotoxicity of virus-infected target cells has been documented. The reaction occurs in the absence of antibody or specifically sensitized lymphocytes and is augmented by interferon secreted by lymphocytes and/or target cells during the course of the assay (54, 59). The question arises as to the possible role of natural killer cells in our experiments, particularly since genetic compatibility between effector cells and target cells was not required for the cytotoxicity observed in this study. Although natural killing may have contributed to the results obtained in the direct cytotoxicity assays, two observations

### TABLE 2. Absence of ADCC with EIA virus-infected fibroblast target cells when serum from different EIA horses was used

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>Percent specific release with the serum from the following EIA horses:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23</td>
</tr>
<tr>
<td>1:10</td>
<td>-7.0</td>
</tr>
<tr>
<td>1:50</td>
<td>0.4</td>
</tr>
<tr>
<td>1:100</td>
<td>5.5</td>
</tr>
</tbody>
</table>

* PBMC from EIA horse 381 were incubated with target cells for 24 h in the presence of serum from four horses with EIA. All sera had precipitating anti-EIA virus antibody activity as determined by gel diffusion.

### TABLE 3. Absence of ADCC with EIA virus-infected fibroblast target cells when effector cells from normal and infected horses were used

<table>
<thead>
<tr>
<th>Dilution of EIA serum</th>
<th>Percent specific release with the following effector cell types:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EIA PBMC</td>
</tr>
<tr>
<td>1:20</td>
<td>-1.8</td>
</tr>
<tr>
<td>1:100</td>
<td>-0.6</td>
</tr>
<tr>
<td>1:500</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Serum was obtained from EIA horse 23.

* PBMC and PMN were obtained from EIA horse 23 and a noninfected control horse.
suggest a minor role for this phenomenon. First, immunological specificity was demonstrated with cells from several of the horses tested. PBMC from four noninfected horses (horses 328, 329, 391, and 392) were unable to preferentially release 51Cr from EIA virus-infected fibroblast target cells, compared with noninfected targets. However, 14 to 18 days after these same horses were infected with EIA virus, their PBMC were able to specifically lyse EIA virus-infected target cells. And second, interferon was not demonstrated in horses or cell cultures infected with EIA virus (30). Furthermore, it has been shown in one outbred species that genetic compatibility between effector and target cells is not required for T-lymphocyte-mediated cytotoxicity of virus-infected cells (50). Therefore, the cytotoxicity observed in our studies was most likely mediated by T lymphocytes. Absence of defined markers for T lymphocytes, however, prevented rigorous proof of this mechanism.

Our inability to demonstrate ADCC reactions with EIA virus-infected fibroblast target cells prompted us to further investigate the ADCC phenomenon in horses. The results indicated that nonsensitized equine leukocytes, in the presence of specific antibodies of the IgG class, are capable of mediating ADCC reactions. Neutrophils were most active, exceeding monocytes and lymphocytes in cytotoxic potential. Activity of leukocytes from uninfected horses or horses with asymptomatic EIA was similar. This lack of suppression of leukocyte activity during asymptomatic EIA is consistent with previous studies (3, 4). However, others have claimed that transient suppression occurs in the early stages of the disease (25).

Our results also are consistent with studies in other species, in which IgG predominates in ADCC reactions (43, 44, 48, 56, 57, 60), although IgM is effective in some systems (8, 28, 29). In our studies IgG(T), a major equine IgG subclass, not only failed to participate in ADCC, but also inhibited this function by IgG. This inhibition is consistent with the competitive relationship between IgG(T) and IgG in other immunological reactions. In complement fixation, IgG activates equine and guinea pig C1 by the classical pathway, whereas IgG(T) does not. IgG(T) inhibits complement fixation by IgG with synthetic, as well as EIA virion, antigens (35, 39, 42). Because of apparent restriction in its binding domain, IgG(T) also fails to precipitate with large antigens carrying multiple repeating determinants and inhibits the precipitation of antibody with
both synthetic (Crawford et al., in press) and EIA virion antigens (35). Finally, IgG and IgG(T) differ significantly in their ability to interact with Fc receptors on monocytes and neutrophils. IgG-antigen complexes bind to monocytes and neutrophils, whereas IgG(T)-antigen complexes do not, even in the presence of complement (6). This inability to bind to Fc receptors may explain the failure of IgG(T) to activate ADCC. The mechanism of inhibition of ADCC by IgG(T) is presumably by competition for binding sites on target cells.

Repeated attempts to demonstrate ADCC between EIA virus-infected target cells and leukocytes from infected horses were unsuccessful. There are at least four possible explanations for these results. (i) One is the lack of sufficient membrane antigens on infected target cells. Persistent infection with measles virus has been shown to induce less membrane antigen than acute infection (22). However, EIA virus-infected target cells clearly have some membrane antigen. They bind detectable quantities of antibody from horse serum (36), and they are recognized and killed by sensitive lymphocytes. Whether the amount is sufficient to trigger ADCC is still uncertain. (ii) Another possible explanation is the differential potency or effectiveness of effector cells. Several authors have shown that effector cells differ markedly in their ability to lyse different target cells (7, 13-15, 26, 31, 32, 43, 48, 52). For example, Grewal et al. (15)
showed that herpesvirus-infected target cells could be lysed by bovine monocytes and PMN but not by lymphocytes. However, all three bovine effector cell types were able to lyse chicken erythrocyte targets. In our studies with EIA virus-infected target cells, neutrophils and a mixture of lymphocytes and monocytes showed no ADCC. (iii) The sera used in our studies may have lacked antibody with sufficient specificity for target cell membrane antigens. A new antigenic variant of EIA virus appears in the blood of horses with each new clinical cycle (24). It is possible that this antigenic variation is reflected in cell membrane antigens as well. Although sufficient quantities of antibody are bound to allow detection by radioimmunoassay (36), they may be insufficient to trigger ADCC. (iv) A final possible explanation is inhibition by IgG(T). Sufficient specific antibody of the IgG(T) subclass may have been present in all sera tested to inhibit the reaction. To verify this possibility, IgG(T)-free antisera would be required. Since our purest preparations of IgG from sera of horses infected with EIA virus contained approximately 10% IgG(T), this question could not be resolved with available reagents. However, if the efficiency of inhibition by IgG(T) using TNP-erythrocytes is applicable to other antigen systems, the ADCC reaction does not seem likely to be very consequential in vivo. Therefore, if cellular mechanisms are involved in the control of EIA virus replication, it appears that direct cytotoxicity is more important than ADCC.

ACKNOWLEDGMENTS
We thank Travis McGuire for the purified IgG and IgG(T) anti-DNP and Keith Banks for his helpful suggestions and for the anti-SRBC serum used in this study.

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LITERATURE CITED

Table 4. Inhibition of ADCC by IgG(T): dependence on concentration, order, and time of addition of antibody

<table>
<thead>
<tr>
<th>Expt</th>
<th>Amt of IgG(T) anti-DNP added (µg)</th>
<th>Amt of IgG anti-DNP added (µg)</th>
<th>Order of addition*</th>
<th>Percent specific release at:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td>5 min</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>4.5</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>G</td>
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<td></td>
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*TNP-SRBC target cells were incubated with IgG or IgG(T) anti-DNP antibody for 5 to 120 min, after which time effector cells with or without additional anti-DNP antibody were added. G + T, IgG was added before IgG(T); T + G, IgG(T) and IgG were added simultaneously; T → G, IgG(T) was added before IgG. Effector cells were added 5 to 120 min later. Cultures were incubated for 4 h, and percent specific release of 51Cr was determined.