Brief Definitive Report

THE ROLE OF NATURAL KILLER CELLS AND ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY DURING MURINE CYTOMEGALOVIRUS INFECTION

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Cell-mediated immunity is a significant aspect of the host response to cytomegalovirus (CMV) infection. We have recently demonstrated that BALB/c mice develop a cytotoxic T-lymphocyte (CTL) response to CMV infection (1) and have demonstrated that these cells are distributed through the blood and in infected tissues so that they may contribute to the limitation of virus replication during the viremic stage of infection (2). Other types of effector-cell responses have not been studied, and little is known regarding the significance of natural killer (NK) cells or of antibody-dependent killer (K) cells in virus infections in general. Welsh and Zinkernagel (3) have demonstrated that the cytotoxic lymphocyte responses to lymphocytic choriomeningitis virus (LCM) are biphasic. The first phase of the CTL response is not genetically restricted and apparently not mediated by T cells. The second phase appears to be a typical H-2-restricted CTL response. A similar biphasic response has been demonstrated during Semliki Forest virus infection, but the initial phase in that case appeared to be mediated by macrophages (4). The following studies were performed to determine whether a similar phenomenon could be demonstrated during CMV infection, and to determine the possible significance of this response as a host defense mechanism.

Materials and Methods

Mice and Cell Cultures. Pregnant BALB/c and C3H/HeN mice were obtained from Microbiological Associates, Walkersville, Md. Weanling BALB/c mice were obtained from Charles River Breeding Laboratory, Wilmington, Mass. CBA/n mice were obtained from the Animal Production Unit, National Institutes of Health, Bethesda, Md. Primary mouse embryo cell (MEC) cultures were prepared as previously described (1). RBL5 cells, a continuous lymphocyte line derived from C57BL/6 mice, were provided by Dr. J. Djeu, Department of Health, Education, and Welfare, Food and Drug Administration, Bureau of Biologics, Bethesda, Md.

Antiserum for Antibody-dependent Cell-mediated Cytotoxicity (ADCC) Assays. Anti-C57BL/6 alloantiserum was prepared as follows: spleens, thymuses, and lymph nodes were removed from two adult C57BL/6 mice, the cells were teased into suspension in 4 ml medium, and 0.5 ml was inoculated s.c. into each of eight adult BALB/c mice. The procedure was repeated for six weekly injections, the last of which was administered i.p. 1 wk later, the mice were bled and the serum was pooled.

Viruses. Weanling mice were inoculated with 1×10^4 plaque/forming units (PFU) of in vivo-passaged Smith strain murine cytomegalovirus (MCMV) i.p. (5). MEC cultures were infected with tissue-culture passaged MCMV as previously described (2). Virus titrations and neutralizing-antibody titrations were performed in MEC cultures as previously described (6).

Interferon Assays. Serum specimens from triplicate mice were collected at various times after infection and stored at −20°C. Interferon assays were performed by Biofluids, Inc., Rockville, Md. Serial 10-fold dilutions of serum were tested for interference of vesicular stomatitis virus plaque formation in L929 cells.

NK-cell and ADCC Assays. A microcytotoxicity assay involving an 18-h incubation of lymphocytes with target cells similar to that which we have previously described was used throughout (1). The RBL5 cell line has been previously shown to be susceptible to lysis by NK cells and, when sensitized by allotypic antisera, to lysis by K cells (7). RBL5 target cells were labeled with [^{51}Cr]sodium chromate (1) and resuspended either in diluted heat-inactivated BALB/c anti-C57BL/6 serum for ADCC assays, or in media for NK-cell assays. The cells were incubated for an additional 30 min at 37°C. Additional media was added to yield a final target cell concentration of 1×10^5 target cells/ml, and 0.1-ml vol were dispersed into wells of plastic round-bottomed microtiter plates (Linbro Chemical Co., Hamden Conn.). Pooled spleen cell suspensions of triplicate spleens from uninfected CBA/n mice or from BALB/c mice were prepared in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) with 5% fetal bovine serum at various times after infection. Lymphocyte suspensions were washed twice and then resuspended, usually at 5×10^6 cells/ml, and 0.1-ml vol were added to quadruplicate wells for test lysis. Suspensions of unlabeled RBL5 cells, 0.1 ml/well, were added for spontaneous lysis; 0.1 ml/well of 10% Brij-35 solution (Sigma Chemical Co., St. Louis, Mo.) was added for maximum lysis determination.

MCMV-specific cytotoxic lymphocyte assays were performed in replicates of eight as previously described (1). The percentage of specific immune lysis (% SIL) was calculated from the equation:

$$\% \text{ SIL} = \frac{\text{test lysis} - \text{spontaneous lysis}}{\text{maximum lysis} - \text{spontaneous lysis}} \times 100.$$

Results

The results of serum neutralizing antibody assays and of plaque assays for titration of MCMV infectivity in spleen cell suspensions and salivary gland homogenates are presented in Fig. 1. MCMV titers in spleens reached a maximum within 4 d and then gradually decreased to negative within 3 wk. Salivary gland virus titers continued to

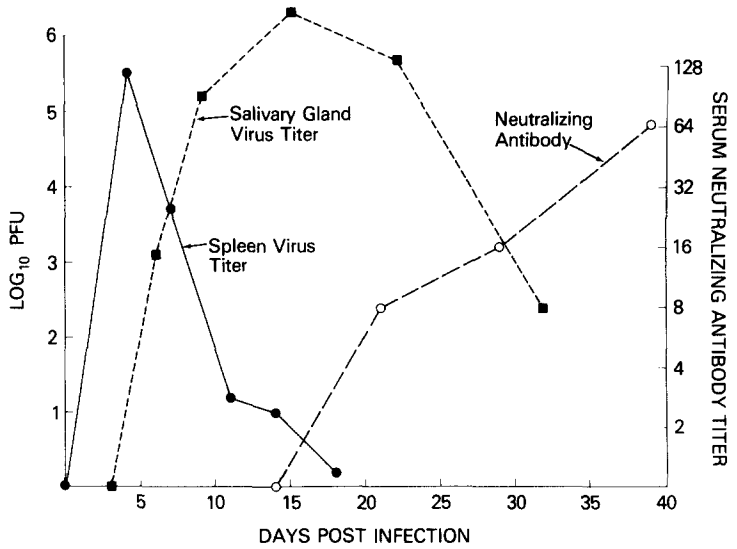

FIG. 1. Reciprocal titers of MCMV in spleen cell suspensions and salivary gland homogenates and of MCMV neutralizing antibody in serum of BALB/c mice at various times after i.p. inoculation of MCMV.

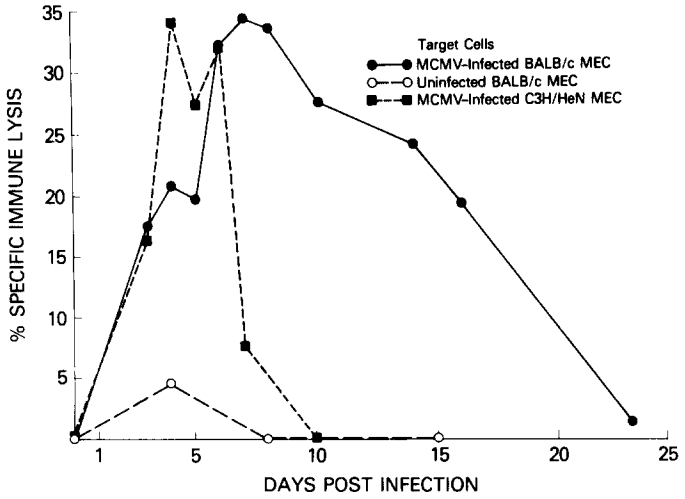

FIG. 2. Cytotoxic activity of BALB/c mouse spleen cells against syngeneic uninfected, and syngeneic and allogeneic MCMV-infected, MEC at various times after i.p. inoculation of MCMV.

rise for 2–3 wk after infection, and then fell more slowly. Neutralizing antibody in serum was first detected 21 d after infection.

No interferon was detected in normal mouse serum. It was first detected on the 2nd day after infection, at which time it had already reached maximum levels of $10^{3.1}$ U/ml. It then declined over the next 4 d to undetectable levels.

The results of cytotoxicity assays using infected and uninfected MEC as target cells are presented in Fig. 2. Specific immune lysis of MCMV-infected BALB/c MEC by syngeneic spleen cells was first detected 4 d after infection, was highest ~8 d after infection, and returned to insignificant levels by 3–4 wk. There was no significant lysis of uninfected BALB/c MEC. Specific immune lysis of MCMV-infected allogeneic C3H/HeN MEC was detected from days 3–6 after infection. The results indicated that the initial virus-specific cytotoxicity was not genetically restricted, whereas the later activity was restricted.

Results of assays of spleen cells for NK- and K-cell activity indicated that the optimum incubation time was 18 h, because the % SIL was greatest at that time, and spontaneous lysis was only 20–30% of maximum lysis. Experiments were performed to determine the optimum effector:target ratio and to determine the maximum useful dilution of the BALB/c anti-C57BL/6 serum used to sensitize target cells for ADCC assays. Results of two of these experiments are presented in Table I. Similar results were obtained using spleen cells from uninfected CBA/n mice or from BALB/c mice 3 d after infection. Cytotoxic cells were not detected in spleens of uninfected BALB/c mice using these assays (results not shown). In subsequent experiments, the antiserum was routinely used at a dilution of 1:100. The optimum effector:target ratio was found to be 50:1; at higher ratios, spontaneous target cell lysis sometimes increased to unacceptable levels. Under these conditions greater % SIL was routinely measured in the ADCC assay than in the NK-cell assay.

The changes in activity in response to infection of the cytotoxic lymphocytes measured by these two assays are shown in Fig. 3. CMV-infected BALB/c mice developed a brisk rise in splenic NK- and K-cell activity beginning 3 d after infection

TABLE I

Assay For NK and K Cells: Determination of Optimum Serum Concentration and Optimum Effector:Target Ratio

Effector:target ratio	% SIL		
	RBL5 cells	RBL5 cells sensitized with anti-C57BL/6 serum diluted:	
		1:100	1:500
BALB/c*			
200:1	9.0	31.9	31.1
50:1	22.2	42.2	58.8
20:1	0.0	12.1	3.2
CBA/n‡			
200:1	0.0	17.8	3.2
50:1	7.4	26.9	17.7
20:1	0.0	12.4	3.2

* BALB/c spleens removed 3 d after i.p. infection with 1×10^4 PFU of MCMV.
‡ CBA/n spleens removed from uninfected 6-wk-old mice.

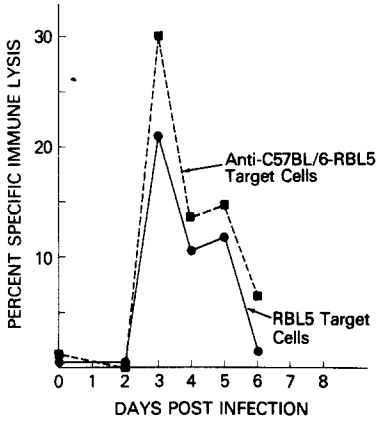

FIG. 3. Cytotoxic activity of spleen lymphocytes of BALB/c mice against RBL5 cells and RBL5 cells sensitized with antibody to C57BL/6 alloantigens at various times after i.p. inoculation of MCMV.

TABLE II

Effect of Pretreatment of Spleen Cell Suspensions on NK- and K-Cell Activity

Treatment*	% SIL‡	
	RBL5 target cells	C57BL/6-RBL5 target cells
None	20.4	30.2
Nylon-wool passaged	17.9	25.6
Anti-theta serum + C'§	14.5	18.7
C'	20.2	24.0
Media control	25.4	28.3

* Spleens removed from BALB/c mice 3 d after i.p. inoculation of 1×10^4 PFU of MCMV.
‡ Results are mean values from two experiments.
§ C', complement.

and persisting through day 5 or 6 after i.p. virus inoculation.

The results of experiments performed to determine the cell type responsible for target cell lysis in the NK- and K-cell assays are presented in Table II. Nylon-wool nonadherent cells were equivalent in cytolytic activity to unfractionated spleen cell

suspensions. Lysis of T lymphocytes in suspensions by treatment with anti-theta serum and complement also failed to significantly affect the activity of the cytotoxic effector cells. These results indicated that the predominant cytotoxic cell type(s) detected in each of these two assays were not macrophages or B or T lymphocytes.

Discussion

Cell-mediated immune lysis of herpes virus-infected cells is potentially important as a host defense mechanism by which infected cells may be destroyed before virus replication has occurred. Although much attention has been given to the frequency of severe and fatal CMV infections in individuals with depressed cellular immunity (8), little is known about specific functions of different types of effector cells as mediators of resistance to these infections. We have previously defined a CTL response to MCMV infection (1). This CTL response is virus-specific, H-2-restricted, and correlates temporally with the viremic phase of infection. During this time, CTL are distributed through the peripheral blood and in cervical lymph nodes (2). These earlier findings suggest that CTL may contribute significantly to the immune control of the acute, viremic phase of infection.

The studies reported here demonstrate that spleen lymphocytes develop the capacity, during days 3–6 after i.p. inoculation of MCMV, to lyse syngeneic and allogeneic MCMV-infected target cells. CTL responses to a number of virus infections have been described (1–4, 9). In each case, these CTL have been found to be H-2 restricted. The initial phase of the cytotoxic lymphocyte response to MCMV infection is, therefore, probably not mediated by CTL, whereas the second phase has been previously shown to be mediated by T lymphocytes (1). A biphasic response with similar characteristics has also been found during murine LCM infection (3), indicating that the pattern of response measured here is not unique to one virus.

Activity of NK and K cells was found to be increased between days 3–6 after infection. In each assay system, the cytotoxic lymphocytes possessed characteristics typical of NK and K cells (10). They were nonadherent, and lacked theta antigen. Because these assays did not involve the use of MCMV-infected target cells, the cytotoxicity measured was clearly not dependent on virus-specific antibody, nor the presence of viral antigens on the surface of the target cells. However, the simultaneous occurrence of this response and the first phase of the virus-specific cytotoxic lymphocyte response suggests that the same effector cell(s) may have been involved.

The mechanism by which NK or K cells could specifically recognize MCMV-infected cells is unknown. We cannot completely exclude the possibility that the cytotoxic effect of these cells was antibody dependent, but we could not detect neutralizing antibody in serum until 2 wk after this cytotoxic-cell response had abated. It is possible that MCMV infection induced the formation of nonviral antigens on infected cells which reacted specifically or by cross-reaction with antigens to which the animal was previously exposed. Regarding this possibility, it has been shown by Boyd et al. (11), that infection of cells with a variety of herpes viruses, including CMV, results in activation of endogenous type C viruses. Antigens of these viruses might be recognized by naturally occurring or preexisting antibody and thus provide specific recognition sites for NK or K cells. Alternatively, virus infection might result in differentiation of a subset of NK cells capable of specific recognition of MCMV antigens in the absence of antibody.

Regardless of the mechanism by which target cell recognition occurs, NK or K cells

probably serve an important role early in the course of MCMV infection. It is likely that these effector cell functions are stimulated by virus-induced interferon, and may then specifically recognize and lyse syngeneic or allogeneic MCMV-infected cells. This broadly reactive response is then replaced within 3–4 d, and as serum interferon levels fall, by the highly specific, genetically restricted CTL response. Thus, NK and K cells may contribute to the role of CTL in controlling the acute, viremic phase of MCMV infection.

Summary

Definition of the functions by which the cellular immune system contributes to control of cytomegalovirus (CMV) infection should permit determination of the specific defects which result in the increased susceptibility to infection of immunosuppressed individuals. Using a murine model, we studied the cytotoxic lymphocyte response to murine CMV infection. This response was found to be biphasic. The initial phase extended from the 3rd to the 6th d after infection, was not genetically restricted, and correlated to a rise in numbers of natural killer (NK) and antibody-dependent killer (K) cells in spleens. The NK- and K-cell responses were preceded, by 24 h, by a rise in serum interferon levels, and occurred before the time when antibody could be measured in serum by neutralization. NK and K cells appear to develop the capacity for specific recognition of CMV-infected cells and the potential to contribute to control of the acute phase of CMV infection.

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