INFECTION AND IMMUNITY, Sept. 1980, p. 1050-1054
0019-9567/80/09-1050/05$02.00/0

Vol. 29, No. 3

Antivirus Antibody-Dependent Cell-Mediated Cytotoxicity During Murine Cytomegalovirus Infection

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BALB/c mice infected with murine cytomegalovirus were studied to determine whether antibody-dependent cell-mediated cytotoxicity contributes to the immune control of this infection. Antibody-dependent killer cells from uninfected mice were used as effector cells to assay for antibody in sera of infected mice. Secondary immune sera were found to contain both cytomegalovirus-specific and autoreactive antibodies. After primary infection only cytomegalovirus-specific antibodies were found. These were detected by antibody-dependent cell-mediated cytotoxicity within 8 to 10 days after onset of infection, but usually not until day 21, by a neutralizing antibody assay. Antibody titers were about 10-fold higher by antibody-dependent cell-mediated cytotoxicity than by neutralization. The results indicate that cellular immunity to cytomegalovirus infection includes an antibody-dependent cell-mediated cytotoxicity response which is likely to be highly efficient and may contribute significantly to control of both acute and later stages of infection.

A direct inhibitory effect of immune lymphocytes on the replication of herpes simplex virus was first demonstrated in vivo and later by reduction of plaque formation in vitro by Ennis and Wells (1, 2). Since this effect appeared to be cell mediated, and since cellular immunity appears to be extremely significant in control of clinical infection, a number of investigators have directed their efforts towards defining the kinds of lymphocytes which mediate this effect. Shore and co-workers (10, 15, 16) and Ramshaw (14) have demonstrated that killer (K) cells may mediate lysis of herpes simplex virus-infected cells. Early in the infectious cycle, before the formation of complete virions, cells become susceptible to lysis by antibody-dependent cell-mediated cytotoxicity (ADCC). In our laboratory we have studied the types of effector lymphocytes which may mediate lysis of cells infected with another member of the herpesvirus group, murine cytomegalovirus (MCMV). We have demonstrated in previous studies that the acute viremic stage of infection is accompanied by specific cytotoxic T-lymphocyte (12, 13) and natural killer cell (NK-cell) responses (11). While these effector cells may account in part for the pathogenesis of infection (3) it is likely that they also contribute significantly to its control through the inhibitory effect described by Ennis and Wells (1, 2). However, adequate infection control probably involves the interaction of these and other immune functions. Through the studies reported here we have demonstrated that MCMV-infected cells may also be lysed by ADCC and that this effector mechanism may well be critical for control of chronic and latent infection.

MATERIALS AND METHODS

Viruses. Virulent Smith strain of MCMV (8) was maintained by in vivo passage in BALB/c mice and stored in 35% sorbitol solution at −70°C. The virus was adapted to tissue culture by 13 serial passages in primary BALB/c mouse embryo cell (MEC) cultures. Infected cultures were harvested by scraping the cells into the media when all cells were cytopathic; the cells were disrupted by sonication, cell debris was removed by centrifugation at 400 × *g* for 10 min, and the supernatant was stored without preservative at −70°C to be used for a virus pool.

Mice. Weanling male BALB/c, C3H/HeN, C57BL/6N (Charles River Laboratories, Boston, Mass.), and CBA/N (Animal Production Unit, National Institutes of Health) mice were used in these experiments. Lymphocyte suspensions were prepared from spleens and thymuses of uninfected mice for use as effector cells as previously described (12).

Serum specimens. BALB/c mice were infected intraperitoneally as weanlings with 10^5 plaque-forming units of virulent MCMV. Groups of three mice were exsanguinated at various times after primary infection; the sera were collected and pooled and stored at −20°C for later use. Secondary immune sera were obtained from mice infected with the same dose of virus 28 days after primary infection and then bled 2 weeks later. All sera were heated at 56°C for 30 min before use. Some sera were adsorbed to uninfected MEC (10^7 cells per ml of serum) for 1 h at 37°C and then for 18 h at 4°C before use. Sera were routinely diluted in phosphate-buffered saline (Media Production Unit, National Institutes of Health).

Preparation of target cells. Confluent monolayer

cultures of BALB/c MEC were infected 24 h before use with 1 to 2 plaque-forming units per cell of tissue culture-passaged MCMV, harvested by trypsinization, labeled with ^{51}Cr-labeled sodium chromate, and washed twice as previously described (12, 13). These cells and similarly prepared uninfected cells were divided, and parts of each were suspended in dilutions of mouse sera or in phosphate-buffered saline alone. These cells were then incubated for 30 min at 37°C in a water bath and then RPMI 1640 (Media Production Unit, NIH) with 5% fetal bovine serum was added to adjust the final cell concentration to 10^5 cells per ml. Each assay thus included infected and uninfected target cells, both sensitized and unsensitized.

Assays for antibody-dependent K cells. Assays for K cells were performed as previously described (11) with target cells prepared from the RBL5 tumor cell line sensitized with allotypic antisera. The RBL5 cell line is only moderately sensitive to NK cell-mediated lysis; thus, it is useful as a target cell for anti-allotype ADCC (11).

Detection of anti-CMV antibody by ADCC. Serum antibody to cytomegalovirus (CMV) was measured by an 18-h chromium release microcytotoxicity assay similar to that which we have previously described (11–13). Effector cell suspensions were prepared from spleens of weanling CBA/N mice. Spleen cells were teased into suspension, washed twice, and suspended at the appropriate concentration. Thymocyte suspensions were prepared in a similar fashion. To appropriate numbers of wells of round-bottomed microtiter plates (Linbro, Hamden, Conn.) were added 0.1-ml volumes of each type of target cell suspended in RPMI 1640 medium with 5% fetal bovine serum. Replicates of eight wells each, containing each target cell, received 0.1-ml samples of spleen cell suspension for test counts per minute (cpm), of thymocyte suspension for control cpm, and of 10% BRIJ-35 solution (Sigma Chemical Co., St. Louis, Mo.) for maximum cpm determination. Thymocytes were used for determination of control cpm since these cells were found not to mediate ADCC. The assays were incubated for 16 to 18 h at 37°C in a 5% CO_2 in air atmosphere, after which the supernatants were collected using the Titertek Supernatant Collection System (Flow Laboratories, Inc., MacLean, Va.), and the specimens were counted on a gamma counter. Control cpm was generally 20 to 30% of maximum cpm. Experiments in which control cpm was greater than 30% of maximum were repeated. Percent specific immune lysis (%SIL) was calculated by the equation: %SIL = [(test cpm − control cpm)/(maximum cpm − control cpm)] × 100%. Significance of lysis was determined by comparing test cpm to control cpm using the Student t test for independent variables. If significant lysis of infected unsensitized (IU) or of uninfected sensitized (US) target cells occurred, the %SIL for infected sensitized (IS) target cells was corrected to determine ADCC by the equation: ADCC = %SIL(IS) − %SIL(US) − %SIL(IU).

Detection of anti-CMV antibody by virus neutralization. The procedures used for these assays have been previously described (17).

RESULTS

Spleens of four strains of mice were assayed for the presence of K cells in the ADCC assay using sensitized RBL5 cells for target cells. The results of a typical experiment are presented in Table 1. This experiment and all experiments described subsequently were performed at least twice, and results of representative experiments are described. Spleens of 4- to 8-week-old CBA/N and C3H/HeN mice were consistently found to have high levels of K-cell activity, whereas K cells were often not detected in spleen cells of BALB/c and C57BL/6N mice using this assay system. These data indicated that spleens of either CBA/N or C3H/HeN, but not BALB/c or C57BL/6N, mice would be useful sources of effector cells for ADCC assays designed to measure anti-CMV antibody. Therefore, in subsequent experiments spleens of CBA/N mice were used as the source of effector cells.

The results of ADCC assays for anti-CMV antibody with sera obtained after primary and secondary infection of BALB/c mice are compared in Table 2. Sera obtained after secondary infection from two separate groups of mice were found to sensitize both infected and uninfected target cells to K cell-mediated lysis to an equal degree. The nonspecific effect could be removed from these sera by adsorbing them to uninfected MEC before use. In contrast, sera obtained after primary infection were not found to contain autoreactive antibodies, and prior adsorption was not necessary. These data suggested the possibility that secondary MCMV infection had induced the formation of autoantibodies. Since the virus pool used for infecting mice was obtained from salivary glands of BALB/c mice, this autoreactivity may have been induced by tissue antigens contained in the virus inoculum. Although this possibility was not investigated in greater detail, it seems unlikely, since the virus pool, test sera, and MEC used for target cells were all obtained from BALB/c mice. Since preadsorbed secondary immune serum produced

TABLE 1. *Antibody-dependent and NK-cell activity in spleens from mice of different strains*

Source of effector cells	Effector cell/target cell ratio	%SIL of RBL5 target cells sensitized with:		
		Unsensitized	Anti-C57BL/6N serum	
			1:100	1:500
BALB/c	50:1	0.0	0.0	ND[b]
C3H/HeN	50:1	0.0	22.0[a]	ND
C57BL/6N	50:1	0.0	4.0	ND
CBA/N	200:1	0.0	17.8[a]	3.2
	50:1	7.4	26.9[a]	17.7[a]
	20:1	0.0	12.4	3.2

[a] Results expressed as significant difference between test cpm and control cpm by the Student t test.

[b] ND, Not done.

CMV-specific sensitization of target cells, it was included in subsequent experiments as a positive control.

The use of different effector cell/target cell ratios was compared in experiments employing MEC adsorbed secondary immune serum, as described in Table 3. A 50:1 ratio was found to produce lysis of the sensitized, infected target cells equivalent to that obtained at higher ratios without producing significant lysis of control target cells. NK cell-mediated lysis of CMV-infected target cells has been previously described (11). Use of lower ratios resulted in decreased sensitivity of the assay. Subsequent experiments were performed with the 50:1 ratio, thereby eliminating, in most instances, the need for correcting the %SIL value obtained for the sensitized, infected target cells.

Serum specimens obtained after primary infection were routinely tested in serial 10-fold dilutions. The endpoint was considered to be the last dilution that produced significant sensitization of the infected target cells to K cell-mediated lysis. The results of a typical series of comparative titrations by ADCC and neutralization assays of sera obtained at various times after infection are presented in Fig. 1. Antibody could be routinely detected by the ADCC assay 8 to 10 days after inoculation, rose to maximum titers of 1:500 to 1:1,000 by 3 to 4 weeks, and persisted at this level for at least 2 months. The apparent decrease in antibody titer from 1:100 to 1:10, measured by ADCC on day 15, is not a significant decline in titer since it represents a difference of only a single dilution. The neutralizing antibody assay proved to be much less sensitive. In this laboratory we have detected

TABLE 2. *Comparison of specificity of primary and secondary MCMV immune sera*

Sensitized with[a]:	%SIL ± standard error of BALB/c MEC	
	Uninfected	CMV-infected
Unsensitized	2.8 ± 7.7	1.5 ± 5.8
Serum A (2° immune), 1:10		
Unadsorbed	23.0 ± 7.2[b]	22.9 ± 7.5[b]
MEC-adsorbed	0.0 ± 4.2	28.9 ± 3.0[b]
Serum B (1° immune), 1:10		
Unadsorbed	5.5 ± 4.1	24.2 ± 3.1[b]
MEC-adsorbed	6.1 ± 5.2	16.9 ± 2.5[b]
Serum C (1° immune), 1:10		
Unadsorbed	0.0 ± 2.1	18.7 ± 3.2[b]
MEC-adsorbed	0.0 ± 3.9	21.0 ± 3.1[b]

[a] Serum B was obtained 29 days and serum C was obtained 48 days after primary infection.

[b] Significant difference between test cpm and control cpm as determined by the Student *t* test, $P < 0.05$.

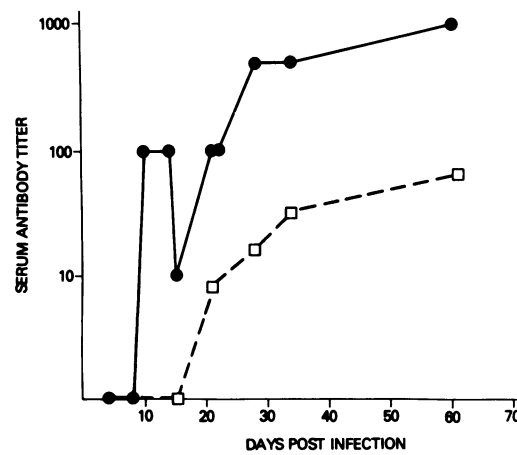

FIG. 1. *Comparison of sequential serum antibody titrations obtained using the ADCC (●) and neutralizing antibody (□) assays.*

TABLE 3. *MCMV-Specific ADCC at different effector cell/target cell ratios*

Effector cell/target cell ratio	%SIL ± standard error							
	Uninfected MEC sensitized with:			CMV-infected MEC sensitized with:			ADCC[a]	
	Unsensitized	Anti-CMV Antibody[b]		Unsensitized	Anti-CMV antibody		Anti-CMV antibody	
		1:10	1:100		1:10	1:100	1:10	1:100
20:1	0.0 ± 1.7	0.9 ± 2.2	0.0 ± 4.2	0.0 ± 4.2	9.1 ± 4.5[c]	12.8 ± 4.8[c]	9.1[c]	12.8[c]
50:1	1.0 ± 3.9	2.6 ± 2.5	9.1 ± 2.2	2.4 ± 3.6	34.8 ± 4.0[c]	33.5 ± 2.6[c]	34.8[c]	33.5[c]
100:1	10.1 ± 2.6	7.5 ± 2.3	4.2 ± 1.6	21.2 ± 3.2[c]	28.7 ± 1.8[c]	22.1 ± 2.3[c]	7.5[c]	0.9

[a] To determine ADCC, lysis of infected, unsensitized target cells and of uninfected, sensitized target cells were subtracted from lysis of infected, sensitized cells only if there was statistically significant lysis of either or both of the two controls. Corrections were not made for %SIL values for lysis of infected unsensitized or uninfected sensitized target cells which were not significantly greater than 0.0 by the Student *t* test.

[b] Anti-CMV antibody is a serum obtained after secondary infection, previously adsorbed to uninfected MEC.

[c] Significant difference between test cpm and control cpm as determined by the Student *t* test, $P < 0.01$. For all other values $P > 0.05$.

antibody by the neutralization assay before day 21 on only one occasion. Serum neutralizing antibody titers were about 10-fold lower than those obtained by ADCC.

DISCUSSION

These experiments were performed to determine the possible role of virus-specific ADCC in recovery of mice from MCMV infection. In previous studies we have demonstrated the sequential appearance of two types of cytotoxic effector cells during the course of this infection. An initial rise in NK-cell activity which extends from day 3 to 6 postinfection (11) is followed by an *H-2*-restricted cytotoxic T-cell response which begins about day 6, peaks between day 7 to 10, and disappears by day 21 (12, 13). The NK cells appear to lyse either MCMV-infected MEC or an uninfected tumor cell line, whereas the cytotoxic T cells lyse only MCMV-infected MEC. Both responses occur during the acute viremic stage of infection, but are no longer measureable during the subsequent chronic stage of salivary gland infection or after complete recovery. However, lymphocytic infiltration of the salivary gland has been found to precede resolution of chronic infection (5), and treatment of mice with antilymphocyte serum can reactivate latent infection (7). Both of these observations suggest an important role for cellular immunity during these later stages of infection. Since lymphocytes of normal mice may possess antibody-dependent K-cell activity, the possibility that these functions were mediated by ADCC was examined.

The anti-CMV ADCC assays were performed with effector cells from uninfected mice so that a positive test result would reflect the presence of antibody in the test serum. The technique was found to be a very sensitive assay for antibody to MCMV. Antibody was detected relatively early in the course of infection, on day 8 to 10, more than a week earlier than generally detected by the conventional neutralization assay. It was also possible to detect antibody in sera 10-fold more dilute than was possible by neutralization. The experiments thus confirmed the occurrence of an ADCC response to MCMV infection and suggested the possibility that delivery of small amounts of antibody to infected tissues would be sufficient to promote specific ADCC in vivo.

The finding of anti-CMV antibody by the ADCC assay in serum obtained during the period of chronic infection suggested the possibility that ADCC may have a significant role in vivo at this time. Such an hypothesis must take into account the previous findings that K-cell activity is increased only early in CMV infection (11) and that K cells generally cannot be detected in spleens of adult mice older than 12 weeks (6). These observations are not necessarily incongruous. Other cell types may function as effector cells for ADCC, such as neutrophils and macrophages, as has been noted in herpes simplex virus infection (4, 9). Additionally, available assay techniques are not sensitive enough to completely exclude the possibility that adult mice possess a small number of circulating K cells. The differences in K-cell activity that have been noted between mice of different ages, between different strains of mice and between different species of animals may, on the other hand, be an indication of the relative importance of ADCC as an infection control mechanism in vivo in these animals.

The relationship of the ADCC response to the cytotoxic T-lymphocyte response we described previously is also of interest. Antibody was first detected by the ADCC assay at the time of peak cytotoxic T-lymphocyte activity, and then titers increased progressively as cytotoxic T-lymphocyte activity waned. This inverse relationship suggests the possibility that the two immune functions are subject to a common control mechanism. More important, however, is the consideration that cytotoxic T-lymphocyte activity is, in effect, replaced by ADCC. Maintenance of a significant level of cytotoxic T-lymphocyte activity would require that the circulating lymphocyte population contain a large number of fully committed effector cells. If cytotoxic T-lymphocyte activity persisted indefinitely after each virus infection, the immune system would eventually become overwhelmingly committed to past infections and deficient in effector cells capable of responding to new antigens. In contrast, the maintenance of an efficient mechanism for cell-mediated immune cytotoxicity through ADCC should require only the presence of circulating antibody and the normal presence of nonspecific circulating K cells. This transition from the cytotoxic T lymphocyte effector mechanism to ADCC may well be absolutely essential since it should permit continued cellular immune control of chronic and latent infection, and resistance to reinfection, without requiring the continued presence of a highly committed lymphocyte pool.

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