Natural and Immune Cytolysis of Canine Distemper Virus-Infected Target Cells

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Natural and immune cytolysis of canine distemper virus (CDV)-infected target cells *in vitro* is described. Lymphocytes expressing natural cytotoxicity were found in specific-pathogen-free beagle dogs and in beagle-coonhound crosses before vaccination with CDV and indefinitely after vaccination, when the ephemeral immune lymphocyte-mediated cytotoxicity (ILMC) had declined. In contrast to the natural lymphocyte-mediated cytotoxicity, the ILMC was genetically restricted, could not be blocked by CDV-specific antibody, and was effective against measles virus-infected as well as CDV-infected target cells. Lymphocyte populations were depleted of Fc receptor and surface immunoglobulin-bearing cells by rosetting techniques and tested in comparison. An antibody-dependent cell-mediated cytotoxicity was demonstrated against CDV-infected target cells that were preincubated with CDV antibody when Fc receptor-bearing lymphocytes were not removed. The ILMC was measurable for approximately 10 days, beginning at 6 days post-vaccination. In contrast, CDV antibody measured by virus neutralization and humoral cytotoxicity was detectable by 6 days postvaccination and persisted at peak levels for at least 5 months.

The importance of the immune response in recovery from viral infection has been demonstrated both clinically and experimentally (reviewed in reference 52). The mechanisms by which the immune response promotes recovery from viral infection, including lysis of infected cells, virolysis, and virus neutralization, depend on the mode of virus replication and viral morphology (42). Viruses that code for proteins capable of integrating into the cell membrane render cells susceptible to cytolysis (22). Cytolysis can be effected by natural killer (NK) cells (24), cytotoxic T lymphocytes (CTL) (15), antibody-dependent cell-mediated cytotoxicity (ADCC) (44), or humoral cytolysis (31). The principal benefits of cytolysis to the host include control of cell-associated virus infections (31), destruction of infected cells before infectious virus is transmitted (1, 54), and exposure of intracellular virions to neutralization.

Canine distemper virus (CDV), a morbillivirus closely related to rinderpest and measles viruses, replicates by budding from the cell membrane after integration of viral glycoproteins (5, 6, 28). Consequently, CDV-infected cells are excellent targets for cytolysis. The invariable association of a strong immune response with recovery from canine distemper suggests that host immunocompetence might alter disease pathogenesis.

Besides a humoral immune response (4, 32), cellular reactions may be important (27, 33, 34).

We have demonstrated humoral cytotoxicity by immune serum and complement, ADCC, natural lymphocyte-mediated cytotoxicity (NLMC), and genetically restricted T-cell (immune lymphocyte)-mediated cytotoxicity (ILMC) against target cells infected with CDV.

MATERIALS AND METHODS

Virus preparation and animal inoculation. The Rockborn strain of CDV (CDV-Ro) in the 38th cell passage in primary dog kidney cells and lyophilized dog kidney cell-adapted measles virus (MV) vaccine for dogs (Bio-Ceutic, St. Joseph, Mo.), containing 10^4 IU per dose, were used for vaccination of dogs and for infection of target cells.

Eight 12-week-old specific-pathogen-free (SPF) beagles (series 1 and series 3) and three 12-week-old beagle-coonhound crosses (series D) were inoculated subcutaneously with $3 \times 10^{4.5}$ 50% tissue culture infective doses of CDV-Ro in Eagle minimal essential medium (MEM). One beagle-coonhound cross (D-12) was inoculated intramuscularly with three doses of MV vaccine followed 3 weeks later by intranasal exposure to $2 \times 10^{4.5}$ 50% tissue culture infective doses of the Snyder-Hill strain of CDV (CDV-SH).

Antiserum preparation. Hyperimmune serum against CDV was prepared by immunizing dogs with Formalin-inactivated CDV, challenging with CDV-SH intranasally 6 weeks later, and harvesting the serum 14 days postexposure (4). A fluorescein conjugate of this serum was made by standard procedures. For

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lymphocyte-mediated cytotoxicity (LMC) blocking experiments, this serum was precipitated with 33% (vol/vol) saturated $(\text{NH}_4)_2\text{SO}_4$ followed by further purification on diethylaminoethylcellulose (anti-CDV immunoglobulin). Canine parainfluenza-simian virus 5 (SV5)-specific immunoglobulin (anti-SV5 immunoglobulin) was prepared in an identical manner. Sheep anti-dog (Fab')₂ (ShADFab') and swine anti-sheep gamma globulin (SwAShG) were prepared by intradermal inoculation of a pepsin digest of canine immunoglobulin G and sheep gamma globulin [33% (vol/vol) saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate] in Freund complete adjuvant. The animals were reinoculated with the appropriate antigen in saline 4 weeks after the initial inoculation, and serum was collected 2 weeks later. Fluorescein-conjugated rabbit anti-sheep gamma globulin (RASHg) and swine anti-human gamma globulin (SwAHuG) were purchased from Nordic Diagnostics (London, England). Cerebrospinal fluid from a subacute sclerosing panencephalitis patient with an MV-neutralizing antibody titer of 1:2,000 was kindly provided by V. ter Meulen (Institut für Virologie und Immunbiologie, Universität Würzburg, West Germany).

LMC assay. Target cells were prepared from the testicles of the experimental dogs by trypsinization. Medium consisted of equal amounts of McCoy 5A medium and Leibovitz L-15 medium (GIBCO Laboratories, Grand Island, N.Y.) with antibiotics (penicillin, 500 U/ml, and streptomycin, 1 mg/ml [Pfizer Inc., New York, N.Y.]; and amphotericin B, 2.5 $\mu\text{g}/\text{ml}$ [E. R. Squibb & Sons, Princeton, N.J.]) and 10% fetal calf serum (FCS) (MLA-10). Secondary cultures were inoculated with CDV-Ro or MV and detached with ethylenediaminetetraacetic acid-trypsin 4 days later when cytopathic effect initially became apparent. Infected cells were frozen in small portions in medium with 20% FCS and 7% dimethyl sulfoxide and stored in liquid nitrogen. Target cells were thawed, washed, placed in MLA-10, and allowed to attach overnight to flat-bottom 96-well microplates (Linbro, Hamden, Conn.). Cell concentration was adjusted to have approximately 5×10^3 attached target cells per well. SV5-infected target cells were prepared by infecting attached dog testicle cells in microplates and incubating for 3 days before use. From 40 to 90% of the CDV-Ro- or SV5-infected target cells and 40 to 55% of the MV-infected cells had viral surface antigen detectable by fluorescent antibody.

Target cells were labeled by adding 2.5 μCi of $\text{Na}^{51}\text{CrO}_4^-$ (New England Nuclear Corp., Boston, Mass.) to each well in a volume of 0.025 ml of MEM supplemented with 2 mM glutamine (Microbiological Associates, Bethesda, Md.), antibiotics, and 10% FCS (MEMGA-10), and then incubating the microplates for 90 min at 37°C (5% CO_2 in air). Labeled target cells were rinsed twice with Eagle MEM supplemented with lactalbumin (GIBCO), antibiotics, and 2% FCS (EgLA-2) and returned to 37°C for 60 to 120 min. Subsequently the target cells were washed a third time before effector cells were added.

Lymphocytes were prepared from 10 to 20 ml of defibrinated venous blood. Phagocytic cells were removed by adding 0.2 g of carbonyl iron powder, grade 420 (GAF, New York, N.Y.) per 10 ml of blood and

incubating the mixture at 37°C for 45 min. The blood was then centrifuged at $500 \times g$ for 10 min, and the supernatant serum was removed and saved for serology. The top 2 ml of packed cells, which contained most of the lymphocytes, was removed and diluted in 3 ml of Hanks balanced salt solution. The suspension was layered onto 2 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) and then centrifuged ($400 \times g$ for 25 min) (8). The lymphocyte band at the HBSS-Ficoll-Paque interface was washed twice and counted in a hemacytometer after the few remaining erythrocytes had been lysed with 0.83% NH_4Cl . Cells so prepared either were used directly in the assay or were further separated.

Fc receptor (FcR)-bearing cells were removed by rosetting erythrocyte-antibody (EA) complexes with the lymphocytes and separating the rosetting from nonrosetting cells on Ficoll-Paque (53). Briefly, 0.3 ml of an 8% suspension of sheep erythrocytes (SRBC) coated with rabbit anti-SRBC (Difco Laboratories, Detroit, Mich.) (EA) was mixed with 10^7 lymphocytes and pelleted ($350 \times g$, 5 min). The cell pellet was incubated at 37°C for 30 min, gently resuspended, and centrifuged on Ficoll-Paque for 15 min at $400 \times g$. Lymphocytes depleted of FcR-bearing cells were collected at the Ficoll-medium interface and suspended in MEMGA-10 at the appropriate concentration.

Surface immunoglobulin (SIg)-bearing cells were depleted according to the rosetting procedure developed by Parish et al. (43). SwAShG coupled to SRBC with CrCl was rosetted with lymphocytes preincubated with ShADFab'. Rosettes were removed on Ficoll-Paque. The percentage of rosette-forming cells was determined in a hemacytometer on samples stained with a crystal violet glutaraldehyde solution (43).

Lymphocyte viability, tested by trypan blue dye exclusion, was always greater than 95%. After carbonyl iron-Ficoll-Paque treatment, more than 99% of the cells were mononuclear, and less than 1% were peroxidase positive by the procedure of Kaplow (30). Of these cells, approximately 15% formed Fc rosettes and 20% formed SIg rosettes.

SIg rosetting reduced the number of SIg-positive cells from approximately 15% to less than 1% detectable by indirect fluorescence (53), using ShADFab' followed by fluorescein-conjugated RASHg. After being fixed in absolute methanol for 4 min and covered with phosphate-buffered glycerol, cells were examined on a Zeiss fluorescence microscope (Brinkmann Instruments Inc., Westbury, N.Y.), using an oil dark-field condenser.

Lymphocyte preparations were added to microplate wells at effector-target cell (E:T) ratios of 100:1 to 10:1 (10^5 to 10^4 lymphocytes per well) in 0.2 ml of MEMGA-10. The incubation period was from 6 h to 12 h at 39°C, 5% CO_2 in air. ^{51}Cr release was stopped by placing the microplates at 4°C for 15 to 30 min. Supernatants were completely removed, and the remaining cells were dissolved with 2% (wt/vol) sodium dodecyl sulfate. Both supernatants and dissolved cells were placed in polypropylene sample tubes (Sarstedt, New Brunswick, N.J.), and the radioactivity was determined in total 10-min counts in a Beckman Gamma 4000 counting system (Beckman Instruments, Inc.,

Irvine, Calif.).

Measurement of LMC. The total ^{51}Cr released (TCR) during the assay period was computed as follows: $\text{TCR} = [\text{supernatant} + (\text{SDS-dissolved cells} + \text{supernatant})] \times 100$. The percentage of spontaneous release (SR) was the TCR in the absence of effector lymphocytes. Cytotoxicity caused by the presence of lymphocytes was computed as follows: $\text{LMC} = \text{TCR} - \text{SR}$. If the LMC was caused by nonimmune lymphocytes, it was termed natural lymphocyte-mediated cytotoxicity (NLMC). After exposure to CDV, the percentage of ILMC was computed as follows: $\text{ILMC} = \text{LMC} - \text{NLMC}$.

Humoral cytotoxicity. Humoral cytotoxicity was performed in 96-well round-bottom microplates, using ^{51}Cr -labeled CDV-infected primary dog kidney cells as target cells and guinea pig serum as a source of complement. To each well was added 0.05 ml of a 1:3 canine serum dilution. Subsequently, 5.0×10^4 target cells suspended in 0.05 ml of medium and 0.05 ml of a 1:4 dilution of guinea pig serum were added. The serum, target cells, and complement were allowed to react for 2 h at 37°C in an atmosphere of 5% CO_2 in air. The percentage of specific cytotoxicity (SC) was computed as follows:

$$\text{SC} = \frac{\text{total } ^{51}\text{Cr released} - \text{spontaneous } ^{51}\text{Cr released}}{\text{total } ^{51}\text{Cr added/well} - \text{spontaneous } ^{51}\text{Cr released}} \times 100$$

Neutralizing antibody titers were determined as reported by Appel and Robson (7).

RESULTS

NLMC. Lymphocytes from dogs that were not exposed to CDV and that had no neutralizing antibody were cytotoxic for CDV-Ro-infected dog testicle cells but not for uninfected or MV-infected cells. This NLMC was detectable at the same level against both allogeneic and autologous target cells.

Variation in the level of NLMC caused by lymphocytes from different dogs. Differences in the level of NLMC (Fig. 1) caused by lymphocytes from different dogs were particularly evident in the coonhound-beagle crosses. NLMC ranged from as low as 0% to as high as 20% after a 6-h incubation at an E:T ratio of 100:1. Lymphocytes from dogs D2, D4, and D6 caused significantly ($P < 0.001$) lower levels of cytotoxicity than did lymphocytes from dogs D3, D9, D10, D11, D12, and D13 (Fig. 1).

Recognition of CDV antigen. As stated above, NLMC was detected only against CDV-infected target cells and therefore appeared to require recognition of CDV antigen. To support the conclusion that NLMC was CDV specific, CDV antibody (anti-CDV immunoglobulin) was used to coat viral antigen on the target cell surface and thereby block NLMC (Table 1). A problem in this experiment was ADCC activity

FIG. 1. Variation of NLMC caused by lymphocytes from SPF dogs against CDV-infected target cells. Lymphocytes from dogs listed on the x axis were assayed twice for NLMC on target cells from dogs D2, D4, D6, D7, D8, D10, D12, D13 (E:T ratio = 100:1, 6-h incubation). The bars represent the sample deviation.

in the presence of CDV antibody. To reduce ADCC against target cells pretreated with anti-CDV immunoglobulin, FcR-bearing cells were depleted by EA rosetting as described. This procedure did not reduce NLMC. However, when target cells were pretreated with 5 or 10 mg of anti-CDV immunoglobulin per ml, NLMC by lymphocytes that were depleted of FcR-bearing cells was almost completely inhibited ($P < 0.001$). In contrast, reduction of cytotoxicity by lymphocytes not depleted of FcR-bearing cells was less marked or reversed. This increase in cytotoxicity by lymphocytes not depleted of EA rosette-forming cells was believed to be ADCC, caused by FcR-bearing lymphocytes in the presence of anti-CDV immunoglobulin. Consequently, the antibody-mediated inhibition of NLMC was masked by ADCC. We did not attempt to separate FcR-bearing cells for a direct demonstration of ADCC.

Pretreatment of target cells with anti-SV5 immunoglobulin or various dilutions of SPF canine serum did not influence NLMC (Table 1).

Specific adsorption of NLMC activity onto CDV-infected cells. To corroborate the specificity of NLMC for CDV antigen, an adsorption experiment was performed (Fig. 2). Lymphocytes were adsorbed onto uninfected and CDV-infected cells at concentrations of 1×10^7 and 0.5×10^7 cells per ml. Subsequently, nonadsorbed cells were gently rinsed off the

TABLE 1. *Effect of CDV and SV5 antibody and SPF canine serum on LMC caused by nonimmune lymphocytes before and after removal of FcR-bearing lymphocytes by EA rosetting*

Lymphocytes from series:	Target cell pretreatment ^a	Concn (mg/ml)	LMC		ADCC ^b
			Before EA rosetting	After EA rosetting	
3 ^c	Control		12.9 (1.4) ^d	12.8 (0.35)	0.1 (1.0)
	Anti-CDV immunoglobulin	0.1	14.9 (4.1)	9.5 (1.9)	5.4 (2.3) ^e
		1.0	6.5 (1.1)	2.1 (2.1)	4.4 (1.0) ^e
		5.0	10.7 (0.64)	1.0 (0.78)	9.7 (0.14) ^e
		10.0	6.0 (1.1)	-1.8 (1.8)	7.8 (0.64) ^e
D ^f	Control		19.0 (4.7)	19.4 (3.3)	-0.4 (1.3)
	Anti-CDV immunoglobulin	0.1	22.2 (5.7)	21.8 (2.2)	0.4 (4.0)
		1.0	15.2 (2.8)	15.4 (0.85)	-0.2 (2.0)
		5.0	12.0 (0.8)	2.8 (2.3)	9.2 (3.1) ^e
		10.0	14.4 (2.3)	3.0 (1.3)	11.4 (3.0) ^e
	Anti-SV5 immunoglobulin	5.0	19.2 (2.9)	21.1 (4.8)	-1.9 (3.2)
	SPF serum	Undiluted	19.5 (3.5)	21.1 (2.6)	-1.6 (-1.9)
		1:19 dilution	19.1 (5.3)	21.8 (2.2)	-2.7 (2.0)

^a To each microplate well containing ⁵¹Cr-labeled CDV-infected target cells was added 0.05 ml of the indicated concentration of antibody or dilution of SPF canine serum. After incubating for 15 min at room temperature, lymphocytes were added.

^b ADCC = LMC before EA rosetting - LMC after EA rosetting.

^c E:T ratio = 50:1, 8-h incubation period, *n* = 4.

^d Numbers in parentheses indicate sample deviation.

^e *P* < 0.001.

^f E:T ratio = 100:1, 8-h incubation period, *n* = 4.

adsorbing monolayers and assayed on ⁵¹Cr-labeled CDV-infected target cells for cytotoxic activity. NLMC was significantly reduced (*P* < 0.001) after adsorption onto CDV-infected cells but not after adsorption on uninfected cells. As would be expected, adsorption was more complete at the lower concentration, i.e., 0.5×10^7 cells per ml.

To determine whether this adsorption was CDV specific, CDV-infected monolayers were pretreated with 5 mg of anti-CDV immunoglobulin per ml. This pretreatment inhibited (*P* < 0.001) adsorption of NLMC, albeit incompletely (Fig. 2). This experiment supported the hypothesis that lymphocytes, which caused NLMC, bound to serologically detectable CDV antigen.

ILMC. Six days after vaccination with CDV-Ro, an ephemeral increase in LMC appeared that was maximal between 7 to 9 days after vaccination and usually decreased to insignificant levels (i.e., >10%) by day 14 (Fig. 3). In contrast, neutralizing and cytotoxic antibody, also detectable by day 6, persisted at undiminished titers for at least 5 months. This pattern had two exceptions. ILMC by dog 1-3 lymphocytes against allogeneic 1-5 target cells was not measurable until 7 days after vaccination, whereas ILMC by dog 1-2 lymphocytes against autologous target cells persisted for at least 5

months. All 11 vaccinates displayed significant (*P* < 0.001) ILMC of between 30 and 50%. EA and SIg rosetting of lymphocytes to remove K and B cells did not diminish ILMC (Table 2).

Differentiation between NLMC and ILMC on the basis of kinetics and specificity. (i) Kinetics. NLMC increased linearly over a 12-h incubation period and caused only low level of cytotoxicity. Conversely, ILMC was maximal by 6 h (Fig. 4).

(ii) Specificity: recognition of nonserologically detectable CDV and MV antigens. Data presented above demonstrated that NLMC could be blocked by CDV antibody. In contrast, when anti-CDV immunoglobulin was used to block LMC after vaccination, the percentage of inhibition was contingent upon the level of ILMC (Table 3). When the level of ILMC was high, a smaller part of the total cytotoxicity was caused by NLMC. However, as ILMC decreased, NLMC contributed a higher percentage of the total LMC. Consequently, as the level of ILMC decreased, the percentage of inhibition caused by anti-CDV immunoglobulin increased. When LMC decreased to prevaccination levels, anti-CDV immunoglobulin completely inhibited cytotoxicity. This suggested that NLMC persisted after vaccination and that ILMC did not require recognition of serolog-

FIG. 2. Adsorption of NLMC activity by CDV-infected cell monolayers and inhibition of adsorption with CDV antibody. The adsorption of NLMC activity onto CDV-infected (AI) and uninfected (ANI) allogeneic dog testicle monolayers was performed in 24-well microplates. Monolayers were preincubated for 15 min with 0.2 ml of medium with (AIA) or without (AI) anti-CDV immunoglobulin (5 mg/ml). Subsequently the monolayers were overlaid with 0.4-ml suspensions of nonimmune lymphocytes (four dogs in D series) containing 1×10^7 (1.0) or 0.5×10^7 (0.5) cells/ml and incubated for 2 h at 39°C in an atmosphere of 5% CO₂ in air, to allow adsorption. Nonadsorbed cells were gently resuspended, washed, and tested for NLMC on CDV-infected ⁵¹Cr-labeled target cells at an E:T ratio of 100:1 after an 8-h incubation period.

ically detectable CDV antigen.

Although serologically detectable CDV antigens were not recognized by immune cytotoxic lymphocytes, ILMC was virus specific. Immune lymphocytes did not cause cytotoxicity of SV5-infected or uninfected target cells (Table 4). Conversely, CDV-Ro-immune lymphocytes did lyse MV-infected target cells (Fig. 5). The appearance and duration of MV- and CDV-specific ILMC were similar, although the latter consistently reached higher levels.

Recognition of target cell surface antigens. As stated previously, NLMC was detected equally well against autologous and allogeneic cells. Conversely, ILMC was, in certain instances, detectable only against autologous target cells and allogeneic target cells possessing the appropriate dog leukocyte antigens (DLA).

Reciprocal genetic restriction between dogs D4, D9, and D12 versus D10 and D13 ($P <$

0.005) (Fig. 6) corresponded to data on serologically detectable DLA (Table 5). Dogs D9, D12, and D13 shared n, c, e, and o determinants. However, only D4, D9, and D12 lymphocytes possessed b and k, whereas D10 and D13 lymphocytes were lysed by antisera specific for g and l. Dog 1-2 lymphocytes continued to show strong ILMC against infected autologous target cells after activity against dog 1-5 target cells disappeared (Fig. 7). Dog 1-2 ILMC against autologous target cells persisted anomalously for at least 5 months. Conversely, dog 1-3 lymphocytes did not lyse target cells from dog 1-2. This also corresponds to data in Table 5, showing a lack of shared DLA on lymphocytes from dogs 1-2 and 1-3. Lysis of MV-infected target cells was also genetically restricted.

DISCUSSION

Several types of CDV-specific LMC were demonstrated, including NLMC, ADCC, and genetically restricted ILMC. Differentiating between these types of cell-mediated cytotoxicity was facilitated by control of experimental conditions, including preparation of relatively pure lymphocyte populations, cryopreservation of allogeneic and autologous target cells, and the use of SPF dogs. The latter is particularly important because many studies of cell-mediated cytotoxicity in humans involved individuals of unknown immunological status and lacked adequate control. The study of cell-mediated cytotoxicity in dogs has the advantages of adequate negative control, known time of exposure to the virus, and use of an outbred species to corroborate data obtained from experiments with inbred laboratory animals.

As was determined by blocking and adsorption experiments, cytotoxicity mediated by nonimmune lymphocytes was specific for serologically detectable CDV antigens. Lysis of noninfected fibroblasts observed with human effector cells (46, 51) was not detected, nor was there cytotoxicity of MV-infected target cells. This specificity for viral antigens is analogous to murine NK cell-mediated cytotoxicity of target cells infected with type C virus (25).

Recognition of serologically detectable viral antigens has also been reported for human lymphocytes, perhaps reflecting an NLMC reaction. Inhibition of human antiviral cell-mediated cytotoxicity by immune serum has been reported for mumps (3, 48), measles (19, 38), and rubella (49) viruses. The requirements of high E:T ratios and long incubation periods to demonstrate low levels of NLMC are consistent with many published reports on virus-specific cell-mediated cytotoxicity in humans (3, 35, 38, 48, 49).

The specificity of human NK cells has been

FIG. 3. Primary immune response to CDV vaccination. Humoral cytolytic (—) and virus neutralization (---) antibody titers and ILMC against 1–5 target cells (---) represent data from series 1 dogs ($n = 4$). ILMC against CDV-infected target cells from dog 1–2 (— · — · —) do not include data from dogs 1–2 and 1–3 (i.e., $n = 2$) because of genetic restriction. ILMC was tested at an E:T ratio of 50:1 after a 6-h incubation. An unpaired t test showed that 10% ILMC was significant at $P < 0.05$ and 20% or greater ILMC was significant at $P < 0.001$.

found, in some instances, to be directed by naturally occurring serum antibody on FcR-bearing cells (2, 50, 51). In our study, lymphocyte populations depleted of EA rosette-forming cells were not depleted of their ability to cause NLMC. The latter was not augmented but was inhibited by anti-CDV immunoglobulin. Similarly, Ewan and Lachmann (19) reported that measles-specific cytotoxicity, which in some instances was blocked by immune serum, was not removed by EA rosetting. Lymphocytes from seronegative humans have been shown to be cytotoxic for mumps virus-infected target cells, thus corroborating our finding that virus-specific NLMC is antibody independent (3, 12). However, Harfast et al. (23) found that the mumps-specific cytotoxic lymphocytes had Fc receptors. This might indicate that mumps LMC and CDV-specific NLMC are mediated by different lymphocyte subpopulations. Alternatively, EA rosetting used in this study might not have removed cells possessing low-affinity FcR which were responsible for NLMC. In support of the latter possi-

bility, Zander et al. (53) reported that 23% of canine peripheral blood mononuclear cells formed EA rosettes; in contrast, we only detected 15% rosette-forming cells.

NLMC persisted after vaccination and after the ILMC had disappeared. In contrast, Ho and Babiuk (26) did not observe NLMC against CDV-infected Vero cells by lymphocysts from 6- to 10-week-old mongrel dogs 1 month after vaccination. The absence of NLMC could have been due to the target cell or genotype and age of the dogs. Age and strain have been found to affect NK cell activity in rodents (24, 25). We observed significant differences in the level of NLMC caused by lymphocytes from coonhound-beagle crosses. Similarly, lymphocytes from mumps-seronegative humans were shown to cause variable levels of cytotoxicity (3, 12).

NLMC is of interest for several reasons. First, it may explain the lack of allogeneic and xenogeneic restriction reported in some human and canine cytotoxic assays; second, the level of NLMC could play a significant role in the path-

TABLE 2. Effect of depletion of FcR- and SIg-bearing lymphocytes on ILMC

Day post-vaccination	Target cells from dog:	Lymphocytes from dog:	ILMC		
			Nonrosetted	EA rosetted	SIg rosetted
7	1-2 ^a	1-2	57.1	59.1	
		1-4	54.9	54.3	
		1-6	56.2	56.3	
	1-5 ^a	1-2	44.4	45.6	
		1-3	43.4	47.2	
		1-4	39.4	40.5	
8	3-7 ^b	1-6	44.0	46.1	
		3-7	29.9	32.9	
		3-8	36.5	38.5	
		3-9	34.4	37.6	
		3-10	39.4	41.0	
	3-8 ^b	3-7	28.6	30.4	
		3-8	23.5	26.3	
		3-9	18.2	23.3	
		3-16	27.3	29.1	
7	D-4 ^a	D-4	43.7		46.5
		D-9	50.6		48.4
11	1-2 ^b	1-2	41.8		45.0
		1-4	30.7		34.7
		1-6	13.7		12.3
	1-5 ^b	1-2, 3, 4, 6	11.9		11.1
			(3.8) ^c		(4.6)

^a E:T ratio = 100:1, 6-h incubation period.^b E:T ratio = 50:1, 6-h incubation period.^c Numbers in parentheses indicate sample deviation.

FIG. 4. Comparison of the kinetics of ⁵¹Cr release caused by immune and nonimmune lymphocytes. NLMC (—) by lymphocytes from dogs 1-2, 1-3, 1-4, and 1-6 was tested against CDV-infected target cells from dogs 1-2 and 1-5 at an E:T ratio of 50:1. ILMC by lymphocytes from dogs 1-2, 1-4, and 1-6 was tested against target cells from dog 1-2 (---); ILMC by lymphocytes from dog 1-2, 1-3, 1-4, and 1-6 was tested against target cells from dog 1-5 (....). ILMC was tested 6 days post-vaccination. Bars represent the sample deviation.

TABLE 3. Effect of CDV antibody on LMC before and after vaccination^a

Dogs	Target cell	Day post-vaccination	ILMC		% Inhibition
			Untreated	Anti-CDV immunoglobulin ^b	
1-2, 3, 4, 6	1-2, 1-5	0	10.4 (2.3) ^c	ND ^d	
1-2		10	61.0	58.1	4.0
		14	60.1	58.7	2.3
		60	51.8	48.2	6.9
1-4, 1-6	1-2	10	45.6 (2.0)	41.8 (5.6)	8.3
		14	25.8 (4.3)	15.4 (5.3)	40.3
		60	9.2 (0.55)	0.5 (0.42)	94.6 ^e
1-2, 3, 4, 6	1-5	10	28.1 (5.7)	18.9 (6.9)	32.7
		14	10.8 (1.2)	1.3 (0.14)	88.0 ^e
		60	11.6 (4.6)	0.0 (0.26)	100.0 ^e
3-7, 8, 9, 10	3-7	0	10.9 (1.6)	0.8 (0.56)	92.7 ^e
		8	47.2 (3.4)	49.0 (1.2)	-3.8
		15	12.3 (4.1)	-1.3 (3.2)	110.6 ^e
	3-8	0	7.9 (0.35)	-0.8 (0.95)	111.4 ^e
		6	35.9 (2.7)	37.7 (3.0)	-5.0
		15	7.9 (3.0)	1.1 (1.1)	86.6 ^e

^a All tests were performed with lymphocytes depleted of FcR-bearing cells by EA rosetting. E:T ratio = 50:1, 6-h incubation.^b 0.5 ml/well (5 mg/ml) 15-min incubation at room temperature.^c Number in parentheses indicate sample deviation.^d Not done.^e $P < 0.001$.

TABLE 4. Effect of immune lymphocytes on SV5-infected and uninfected target cells

Lymphocytes from dogs:	Target cells from dogs:	ILMC against:		
		CDV infected	SV5 infected	Uninfected
1-2, 4, 6 ^a	1-2	44.0 (2.0) ^b	7.2 (1.2)	0.3 (0.7)
1-2, 3, 4, 6 ^a	1-5	25.5 (4.5)	0.2 (3.5)	0.6 (1.2)
3-7, 8, 9, 10 ^c	3-7	37.2 (3.4)	3.9 (2.8)	8.1 (0.8)
3-7, 8, 9, 10 ^c	3-8	26.6 (2.8)	4.4 (2.6)	0.2 (1.1)

^a E:T ratio = 100:1, 6-h incubation, 10 days postexposure.^b Numbers in parentheses indicate sample deviation.^c E:T ratio = 50:1, 6-h incubation, 9 days postexposure.

ogenesis of CDV and other viral infections as has been suggested for murine NK cells in cancer (24); and third, NLMC might be an indirect measure of the number of peripheral blood lymphocytes with virus receptors.

When FcR-bearing cells were not removed from nonimmune lymphocyte populations, reduction of cytotoxicity in the presence of CDV antibody was less marked or absent. This higher level of LMC relative to EA rosette-depleted

FIG. 5. Lysis of MV- and CDV-infected target cells by CDV-immune lymphocytes. ILMC of MV- ($\square$) and CDV- ($\bullet$) infected target cells by lymphocytes from dogs D4, D9, and D-13 was assayed after a 6-h incubation at an E:T ratio of 100:1. D4 and D9 lymphocytes were tested on D4 target cells, and D13 lymphocytes were tested on autologous target cells. An ILMC of 10% is significant at $P < 0.05$, and 20% or greater is significant at $P < 0.001$.

lymphocyte populations suggests that FcR-bearing lymphocytes recognized nonviral cell surface antigen associated with virus infection, or that these cells caused ADCC. We interpreted the data to indicate the latter.

ADCC against CDV-infected Vero cells has recently been reported by Ho and Babiuk (26). However, in contrast to the above report, the CDV-specific ADCC reported herein required high concentrations of immunoglobulin to cause low levels of cytotoxicity. This also differs from other homologous systems in which high levels of ADCC against vaccinia (46), measles (19, 45), Epstein-Barr (29), and herpes simplex (41) viruses have been demonstrated in the presence of small amounts of antibody.

Conversely, antiviral ADCC has, in some

FIG. 6. Target cell-restricted ILMC. Lymphocytes from dogs D4, D9, D12, and D13 were tested on CDV-infected target cells from the dogs listed on the x axis. The data from dogs D4, D9, and D13 were obtained in two tests done 7 to 11 days postvaccination. D12 was vaccinated intramuscularly with MV and challenged 3 weeks later by intranasal exposure to the CDV-SH. Tests using D12 lymphocytes were performed between 9 and 15 days after exposure to CDV-SH. The absence of sample deviation bars indicates that only one test was performed. D12 lymphocytes were not tested against D8 target cells. The incubation period for all tests was 6 h.

cases, been more effectively demonstrated in heterologous systems. To demonstrate ADCC against lymphocytic choriomeningitis virus-infected mouse teratoma cells, Zinkernagel and Oldstone (56) used human peripheral blood lymphocytes and guinea pig antisera. Kreth and ter Meulen (36) demonstrated human antimeasles ADCC most effectively when target cells were sensitized with rabbit antisera. Additionally, ADCC is often demonstrated in systems that are neither homologous nor antiviral (18, 44).

The ILMC, measured in response to primary inoculation of CDV-Ro, was differentiated from NLMC on the basis of duration, kinetics, and

specificity. In contrast to NLMC, ILMC was transient, showed genetic restriction, differed in the optimal incubation time, and could not be blocked by CDV antibody. The duration and kinetics of ILMC were therefore similar to those of T-cell-mediated cytotoxicity reported in mice (16, 55).

The observation that ILMC was not inhibited by anti-CDV immunoglobulin indicates that

ILMC is specific for lymphocyte-defined antigens. Similar disparities between the antigen recognized by murine CTL and antibody specific for ectromelia virus (21) and lymphocytic choriomeningitis virus (15) have been reported.

The different specificity of CDV ILMC and immune sera was also observed against MV. MV-neutralizing antibody was not detected in the sera of CDV-Ro-vaccinated dogs (data not shown), whereas CTL from these dogs lysed MV-infected target cells. The absence of MV-neutralizing antibody in dogs exposed to CDV has been reported (6, 28), as has cellular cross-reactivity (10) with MV. The induction of cross-reacting CTL without cross-reacting antibody has also been observed in mice exposed to different strains of influenza A virus (9, 17).

The absence of cross-reacting antibody makes ADCC an unlikely mechanism for ILMC of MV-infected cells. The observation that MV-infected target cells were lysed to a lesser extent than CDV-infected cells could have been due, in part, to the level of infection of the former. Moreover, cross-reacting cytotoxic lymphocytes probably represented only a part of the cytotoxic lymphocyte population. The induction of cross-reacting cytotoxic lymphocytes by CDV vaccination supports the importance of immune mechanisms in protecting MV-vaccinated dogs against CDV infection.

The ILMC also showed specificity for DLA of the target cells. Genetic restriction of LMC has been extensively documented in mice (55), and more recently in humans, against haptens (14, 20), influenza virus (39), and measles virus (37). In contrast, most reports of LMC in humans and

TABLE 5. Serologically defined DLA of experimental animals^a

Lymphocytes from dog:		DLA	
D-4	b, k		c, e, n, o
D-9	b, k		c, e, n, o
D-12	b, k		c, e, n, o
D-10		g, l	n
D-13		g, l	c, e, o
D-2	b, k	l	e, n, o
D-3	k	g, l	c, e, n
D-6	b, k	g, l	c, e, n, o
1-2	b, f, k, m		
1-3		g, l	n

^a Data on serologically detectable DLA were provided by Frances C. Cannon (Mary Imogene Bassett Hospital, Cooperstown, N. Y.). DLA were detected by using ⁵¹Cr release by peripheral blood leukocytes in the presence of antibody and complement. Chromium release of greater than 7% was considered to be significant. The sera were prepared by reciprocal exchanges of skin allografts and subcutaneous inoculation of blood leukocytes (40). Although not monospecific, these sera have been useful in predicting survival of skin, heart, and liver allografts in the Cooperstown dog colony (13).

FIG. 7. Target cell-restricted ILMC. Lymphocytes from dogs 1-2 (●) and 1-3 (○) were tested for ILMC against target cells for dogs 1-2 (—) and 1-5 (---) at an E:T ratio of 50:1 after a 6-h incubation period. ILMC of 10% is significant at $P < 0.05$; ILMC of 20% or greater is significant at $P < 0.001$.

recent reports on CDV (33, 34) and vaccinia virus (27) in dogs have failed to demonstrate genetic restriction.

Reciprocal genetic restriction in the D series occurred between dogs that had common DLA. These common DLA might be unimportant as targets for ILMC. Murine virus-specific CTL recognize only gene products of the K and D loci of the H-2 complex (55). Alternatively, these common DLA might be recognized by CTL but not by distemper-immune CTL. Preferential cytolysis of antigen associated with certain H-2 haplotypes has been documented for tumor viruses (11, 47).

The duration, kinetics, and specificity of ILMC made it unlikely that antibody was involved. CDV antibody persisted long after ILMC had become insignificant. ADCC demonstrated before vaccination was much lower than the peak ILMC. Moreover, ILMC appeared to be genetically restricted and not to recognize serologically defined CDV antigen. This is corroborated by data showing that depletion of SIg- and FcR-bearing cells did not diminish ILMC.

In the present study, NLMC, ILMC, ADCC, and humoral cytotoxic responses in dogs after CDV vaccination have been reported. Studies are in progress to determine the significance of cellular versus humoral immune responses in recovery or viral persistence in dogs after exposure to virulent CDV.

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