Syncytia Inhibition by Immune Lymphocytes: In Vitro Test for Immunity to Canine Distemper

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Received for publication 4 October 1977

A simple and rapid (24-h) assay for peripheral blood lymphocyte-associated immunity to canine distemper virus (CDV) is described. The test is based upon leukocyte-associated inhibition of CDV-induced syncytia formation in Vero cells. The technique quantitates the response morphologically, thereby eliminating the requirement for release of radiolabeled compounds. Positive results were determined from specific-pathogen-free and gnotobiotic dogs exposed to CDV via hyperimmunization, vaccination with a modified live virus vaccine, and after virulent virus infection. Preinoculation lymphocytes and lymphocytes from non-immune dogs did not inhibit CDV-induced syncytia formation. Maximum responses were observed 7 to 21 days after initial exposure and declined thereafter. The method can be used to further investigate the role of immune lymphocytes in the recovery from CDV infection.

The quality and quantity of the immune response to canine distemper virus (CDV) antigens are major determinants of eventual recovery from infection. Dogs that develop prompt and increasing levels of antibodies recover from infection and are refractory to subsequent virulent virus challenge (2). In contrast, dogs without viral antibodies in serum eventually die of CDV-associated encephalitis (2, 11). Although these correlations permit prediction of the outcome of infection, the actual role of antibodies in terminating infection is unknown. Passive administration of immune serum to dogs with clinical signs of CDV is ineffective (2). With measles virus infection of humans, a systemic and fatal infection is observed in patients with deficits in cell-mediated immune function, despite high levels of antiviral antibodies in serum (15).

Methods for evaluating the role of sensitized lymphocytes in the pathogenesis of CDV-related disease, except for one study (5), have remained elusive. Data from other systems suggest that development of an immune lymphocyte response is more important for immediate recovery than are virus-specific antibodies. In the present study, a simple and reproducible in vitro assay for cellular sensitivity to CDV is described. Positive responses were observed in hyperimmunized, vaccinated, and convalescent groups of dogs. The cytotoxic lymphocyte response was a transient event that developed rapidly after immunogenic exposure and then declined in the convalescent phases of infection.

MATERIALS AND METHODS

Dogs. A total of 22 dogs was used in this study. Four young, adult, specific-pathogen-free (SPF) beagles, derived from gnotobiotic stock, were maintained in Goss Laboratory. Two dogs were hyperimmunized with a commercial modified-live (MLV) CDV vaccine incorporated into complete Freund adjuvant, and two dogs were given one dose of MLV vaccine (Enduracell-d, Norden Laboratories, Inc.) via subcutaneous injection. Six gnotobiotic beagle pups were derived by Caesarean section and delivered into sterile, flexible-plastic isolation units (7). Pups were raised on a milk replacement diet and divided into two groups of three in separate isolation units. Three pups were inoculated intraperitoneally with 0.2 ml of virulent R252-CDV (13) at 14 days of age. The three remaining littermates were used as CDV-negative controls. Blood and serum samples from 12 additional dogs were obtained from an SPF colony maintained in the Department of Veterinary Clinical Sciences, The Ohio State University.

Lymphocyte isolation. Lymphocytes were isolated from fresh heparinized peripheral blood using Ficoll-Hypaque density gradient centrifugation (10). A total of 75 to 95% of the cells collected at the interface were identified as lymphocytes. Contaminating leukocytes consisted of neutrophils, eosinophils, and monocytes. After washing, lymphocytes were adjusted to a concentration of 1×10^6 to 10×10^6 viable cells per ml in Eagle minimal essential medium with Spinner salts containing 20% fetal bovine serum, 1% sodium bicarbonate (8.8% wt/vol), and 1% antibiotics (penicillin, 20,000 U/ml; streptomycin, 10 mg/ml; and nystatin, 5,000 U/ml).

Viruses and cell culture methods. The Onderstepoort strain of CDV (Ond-CDV) adapted to growth in African green monkey kidney (Vero) cells

was used. Virus titrations (50% tissue culture infective dose, TCID₅₀) were performed in microtest plates as described elsewhere (4). Levels of virus-neutralizing antibodies in heat-inactivated serum or plasma were assayed in a microtest system using 100 TCID₅₀/0.025 ml (3) and read 3 days after inoculation of virus antibody mixtures as described by Confer et al. (4).

Virulent R252-CDV (used as inoculum in three CDV-susceptible gnotobiotic dogs) was stored as a 20% clarified suspension of spleen and thymus from dogs infected 10 days previously. In vivo and in vitro properties of this isolate have been described (4, 10-13).

Lymphocyte-mediated cytotoxicity assay. A 0.1-ml amount of Vero cells (0.5×10^6 cells per ml) was inoculated into microtest plate wells (Falcon no. 3040). A 10-TCID₅₀ amount of Ond-CDV (0.025 ml/well) was then added, and the plates were covered and incubated at 37°C in 10% CO₂ for 6 h. The supernatant fluid was then discarded, and the infected monolayers were washed twice with Eagle minimum essential medium with Spinner salts plus 10% fetal bovine serum. Uninfected control dog lymphocytes as well as virus-infected and uninfected cells were included in each test. A 0.1-ml amount of immune and control Ficoll-Hypaque-purified leukocyte suspensions was then added, and the plates were returned to 37°C, 10% CO₂ for 18 h. For staining, the supernatant fluid was removed, and cells were fixed in a solution of 10% Formalin in 0.9% NaCl for 3 h. Plates were subsequently stained with May-Grünwald-Giemsa stain for

45 min, washed, and air dried. Syncytia were counted with a conventional light microscope at 10× magnification. The average number of syncytia per well was determined on 6 to 12 replicates per sample 24 h after infection.

RESULTS

Characteristics of Ond-CDV infection in Vero cells. Detailed light and electron microscopic and immunofluorescence studies of the growth cycle of Ond-CDV in Vero cells have been published (4). Cytopathic effects on Vero cells after inoculation of Ond-CDV consist of formation of multinucleated giant cells (syncytia) visible within 24 h (Fig. 1) and most extensive 3 to 4 days postinfection.

The relationship between viral dose (TCID₅₀) and syncytia formation as discrete foci of infection 24 h postinfection is given in Table 1 (medium control). In these and other experiments, 10 TCID₅₀ yielded 4 to 8 syncytia per well that were easily counted with the microscope.

The effects of lymphocytes from two CDV-hyperimmunized (tested 29 days after initial immunization; see Fig. 3) and two CDV-free control dogs upon the number of syncytia formed within 24 h by using various virus doses (10 to 100 TCID₅₀/well) were tested, and the data are

FIG. 1. Cytopathic effects of CDV infection in Vero cells. Nonlytic syncytia formation. May-Grünwald-Giemsa stain. $\times 250$.

TABLE 1. *Effects of increasing virus dose upon inhibition of CDV-induced syncytia formation by Ficoll-Hypaque-purified lymphocytes^a*

TCID ₅₀ /well	Medium control	Unvaccinated dog no.		Hyperimmunized dog no.	
		1	2	1	2
10	6/6 ^b (4.0) ^c	6/6 (3.3)	6/6 (4.2)	0/6 (0.0)	0/6 (0.0)
20	6/6 (8.0)	6/6 (6.7)	6/6 (7.7)	0/6 (0.0)	0/6 (0.0)
50	6/6 (14.0)	6/6 (11.1)	6/6 (13.5)	0/6 (0.0)	0/6 (0.0)
80	6/6 (17.7)	6/6 (17.8)	6/6 (17.2)	1/12 (0.1)	0/12 (0.0)
100	6/6 (20.5)	6/6 (19.5)	6/6 (20.5)	3/12 (0.25)	6/12 (0.5)

^a Tests were performed on hyperimmunized dogs 1 and 2 29 days after initiation of vaccination protocol; see Fig. 4.

^b Number of wells containing syncytia per number of wells examined.

^c Average number of syncytia per well.

TABLE 2. *Effects of lymphocyte concentration upon lymphocyte-mediated inhibition of viral syncytia formation*

Dog no.	% Reduction in syncytia ^a at concn (lymphocyte/ml) of:				
	1 × 10 ⁶		5 × 10 ⁶	10 × 10 ⁶	
	Expt 1	Expt 2	(Expt 1)	Expt 1	Expt 2
CDV-immune					
1	50	51	64	77	100
2	27	48	59	62	97
3	52	43	82	— ^b	100
4	47	34	65	—	100
5	39	62	85	—	100
Nonimmune control					
1	2	2	9	8	—
2	12	0	—	—	23

^a Percent reduction in syncytia calculated by 100: (average number of syncytia per lymphocyte-treated well/the average number of syncytia per media control well) × 100.

^b —, Not done.

shown in Table 1. Syncytia formation in monolayers treated with immune lymphocytes was completely inhibited at low virus doses when compared to monolayers treated with nonimmune lymphocytes or untreated control wells. The dose response relationship between the number of syncytia per well and the number of TCID₅₀ inoculated was similar between the medium only and normal lymphocyte-treated wells.

The effects of lymphocyte concentration with a constant viral input of 10 TCID₅₀/well was then examined. The data obtained from five immune and two control dogs are shown in Table 2. A dose response effect was observed in that increasing the number of lymphocytes from 1 × 10⁶ to 10 × 10⁶ cells per ml resulted in a decrease in the number of syncytia per well. Because the number of lymphocytes isolated from the dogs was limited, subsequent experi-

ments employed only 1 × 10⁶ and 5 × 10⁶ lymphocytes per ml.

Requirement for viable leukocytes. Immune lymphocyte-mediated inhibition of CDV-induced syncytia should require living cells. Lymphocyte suspensions (1 × 10⁶/ml) from control and immune dogs were subjected to three cycles of freezing in dry ice and alcohol, alternating with thawing in a 37°C water bath. Viability was assessed by trypan blue dye exclusion. The data from two different experiments with five immune and two control dogs are shown in Table 3. Wells treated with viable immune lymphocytes produced 1.5 to 4.0 syncytia per well. After freeze-thawing, the number of syncytia per well equalled that of controls (5.3 to 6.2).

Syncytia inhibition: relationship to immunogenic exposure with CDV. The temporal sequence of development of cell-mediated and humoral immune responses to CDV after vaccination was examined by vaccinating two young SPF dogs with one dose of a commercial MLV-CDV vaccine. These animals, along with nonvaccinated controls, were tested biweekly for 3 weeks for lymphocyte-mediated syncytia inhibition and virus-neutralizing antibodies. Data are shown in Fig. 2. Inhibition of CDV-induced syncytia was first evident 5 days after vaccination. Peak response was seen 7 to 10 days postinfection, with responses decreasing in the lower lymphocyte concentrations tested by 14 days postinfection.

This short-lived response was also evident when data collected from two hyperimmunized dogs were tabulated and compared with the immunization schedule (Fig. 3). A progressive decrease in the syncytia-inhibiting properties of lymphocytes with time, despite continuing immunization, was observed. The final immunization given at 55 days produced a transient increase in inhibition that subsequently disappeared.

Lymphocytes from 12 previously vaccinated SPF dogs of various ages were tested for syncytia

TABLE 3. Requirement for viable cells in lymphocyte-mediated inhibition of viral syncytia formation

Dog no.	% Reduction of syncytia ^a			
	Expt 1		Expt 2	
	Before F/T ^b	After F/T	Before F/T	After F/T
CDV-immune				
1	75	0 ^c	51	10
2	59	2	48	13
3	— ^d	—	43	5
4	—	—	34	7
5	—	—	62	0
Nonimmune control				
1	30	0	2	8
2	0	0	—	—

^a Percent reduction on syncytia calculated by 100: (average number of syncytia per lymphocyte-treated well/the average number of syncytia per media control well) × 100.

^b F/T, Freeze-thaw lymphocyte suspensions (1×10^6 cells per ml) three times by treatment with alternate cycles on dry ice and alcohol followed by a 37°C water bath.

^c Data designated as 0% reduction in syncytia formation showed 0 to 0.9 more syncytia per well than in medium control.

^d —, Not done.

FIG. 2. Cell-mediated and virus-neutralizing antibody responses in two dogs vaccinated with one dose of MLV-CDV at zero time (arrow). LY, Lymphocytes.

inhibition. Data are shown in Table 4. The only dog that gave a positive significant response in the cell-mediated immune assay was one that received a final vaccination 1 month previously. Serum from all dogs contained virus-neutralizing antibodies.

FIG. 3. Relationship between vaccination schedule and cell-mediated immune response to CDV in two hyperimmunized dogs. Heavy arrows: one dose of MLV-CDV in complete Freund adjuvant; light arrows: one dose of MLV-CDV given intravenously.

TABLE 4. Relationship between age, vaccination history and cellular and humoral immune responses to CDV in SPF dogs

Dog no.	Age (mo)	Time (mo) since last vaccination ^a	% Lymphocyte-mediated reduction in syncytia ^b at concn (lymphocytes/ml) of:		Virus-neutralizing antibody titer
			1×10^6	5×10^6	
1	3	1	20	66	128
2	3	1	20	28	128
3	6	2	8	5	256
4	22	4	8	18	40
5	13	7	22	26	≥256
6	12	8	4	31	≥128
7	12	8	0 ^c	8	128
8	16	12	8	0	203
9	21	12	0	9	10
10	19	15	0	0	128
11	29	23	0	— ^d	25
12	42	34	12	0	102

^a Commercial MLV distemper-hepatitis-leptospirovaccine.

^b Percent reduction of syncytia calculated by 100: (average number of syncytia per immune lymphocyte-treated well/average number of syncytia per nonimmune lymphocyte-treated control well) × 100.

^c Data designated as 0 per reduction in syncytia formation showed 0 to 0.8 more syncytia per well than in medium control.

^d —, Not done.

DISCUSSION

The present study has investigated lymphocyte-associated immune response to CDV in SPF and gnotobiotic dogs exposed to live virus via hyperimmunization in Freund adjuvant, one inoculation with a commercial MLV-vaccine or in the convalescent stages following virulent virus infection. A positive correlation was established between recent immunogenic exposure to

CDV and reactivity in the *in vitro* test system used.

The assay as described is easy and rapid to perform. As such, the method has significant technical and economical advantages over other *in vitro* methods used to evaluate cell-mediated immunity to viral antigens, namely antigen-specific blastogenesis (6, 19) and chromium 51 release tests (1, 9, 18). Both systems require expensive equipment and facilities to handle and dispose of radioactive reagents. For chromium 51 release, a target cell system containing viral antigens on cell membranes of most, if not all, cells is required. Thus, either high multiplicities of infection must be used, or stable, persistently infected cell lines must be available for testing.

For *in vitro* blastogenesis tests, relatively large quantities of inactivated but still antigenic virus preparations are necessary. In addition, lymphocytes must be capable of deoxyribonucleic acid synthesis and [³H]thymidine incorporation *in vitro*. With canine distemper, 10^{4.5} to 10^{5.5} TCID₅₀/ml is the maximum amount of virus obtainable from roller-bottle cultures (S. Krakowka, unpublished data). The virus must be inactivated because CDV is lymphotropic *in vivo* and *in vitro* (12, 18). Furthermore, we have shown that CDV infection in gnotobiotic dogs is associated with a profound depression of lymphocyte responses to phytoimitogens (10). This *in vitro* suppressive phenomena coincides with the onset of viremia and persists for at least 10 weeks after infection or 8 weeks after virus can no longer be demonstrated in leukocytes. For this reason, the probability is low that antigen-specific stimulation of deoxyribonucleic acid synthesis can be detected in lymphocyte cultures when the response to phytoimitogens is negligible. This factor prompted us to develop an alternate method for assaying CDV-specific lymphocyte activity.

Possible variables in the test system include viral dose and passage level of Vero cells and lymphocyte concentrations tested. Regarding doses of virus employed, there was a somewhat constant relationship between viral input (TCID₅₀) and number of syncytia observed. A 10-TCID₅₀ amount produced an average of six syncytia per well; 5 TCID₅₀ produced 3 to 4 syncytia per well. Six syncytia per well represented a compromise between sensitivity under test conditions and visual capability required to distinguish syncytia as discrete foci of infection. The passage level of Vero cells affected results in that higher passage levels (>156) produced one-half or fewer syncytia per well than did cells at passage level 150 or below (S. Krakowka, unpublished data). The lymphocyte concentration used in the test also affected results. A dose-

dependent effect was observed in that syncytia-inhibiting ability increased as the lymphocyte concentration was increased from 1 × 10⁶ to 10 × 10⁶ cells per ml.

Perhaps the most important feature of the experiments described here is the relationship between a positive test result and recent immunogenic exposure to CDV. Lymphocytes collected recently after exposure were capable of syncytia inhibition, and this ability decreased with time. Lymphocytes from hyperimmunized dogs completely inhibited syncytia formation early in the immunization schedule (Fig. 3 and Table 1). Lymphocytes from dogs receiving less-vigorous exposure to CDV through vaccination or virulent virus challenge, though positive, did not completely inhibit development of syncytia.

The lack of correlation between positive cell-mediated syncytia inhibition and presence of virus-neutralizing antibodies deserves further comment. The precise immunological requirements for recovery from CDV infection have not been established, although prompt development of neutralizing antibodies in serum is closely correlated with recovery (2, 11). With measles virus infection in humans, congenital deficits in cell-mediated immune function result in fatal generalized measles despite development of anti-measles antibody, whereas measles in children with humoral deficiencies is generally innocuous (15). Measles-specific cell-mediated responses clear the virus from tissues. Humoral antibody (chiefly immunoglobulin G) functions as the chief protective factor against reinfection. Because CDV infection in dogs is similar in many respects to measles virus infection in humans it is likely that a parallel situation exists.

The mechanism that terminates cell-mediated syncytia inhibition has not been determined. Two explanations are possible. Viral-antigen specific lymphocytes may be short-lived and, therefore, only present in peripheral blood for a short period after vaccination. Alternatively, suppressor T cells may be generated in the convalescent stages of infection which in turn would prevent lymphocyte-mediated syncytia inhibition. This inhibitory effect can be overcome by increasing the lymphocyte concentration to 10 × 10⁶ cells per ml. Suppressor T-cell activity may be detected by recombination experiments with cryopreserved lymphocytes collected at various intervals after infection. This approach avoids the requirement for histocompatible animals and is currently under investigation.

Finally, the nature and origin of the lymphoid cells effective in producing syncytia inhibition has yet to be determined. Again, by analogy to measles virus infection in humans, two effector cell types could be involved: virus-specific T

lymphocytes and/or nonimmune cells active in antibody-dependent cellular cytotoxicity (ADCC) called K cells (8). In ADCC, cells possessing an Fc receptor for immunoglobulin G including monocytes and neutrophils kill target cells coated with specific antibody. Specific antibody will inhibit CDV-induced syncytia formation (S. Krakowka, unpublished data). However, the carry-over of virus-specific antibody into lymphocyte preparations as an explanation for syncytia inhibition was discounted because cellular lysates prepared by freezing and thawing did not affect viral cytopathic effects on the monolayer. Three observations tend to decrease the possibility of ADCC phenomena as an explanation for the results obtained in the present study. Passive transfer of antibodies in plasma is unlikely in that the cells were washed, and others have shown that these antibodies are loosely bound to lymphocytes and K cells (8, 14, 16). Secondly, the lack of correlation between presence of neutralizing antibodies and lymphocyte-mediated syncytia inhibition suggest that these responses are two separate immunological events. If ADCC were responsible for our results, syncytia inhibition should have paralleled viral antibody responses. Finally, ADCC is mediated by immunoglobulin G. It is unlikely that immunoglobulin G would appear in the circulation as early as 5 days after vaccination.

In conclusion, a simple, reproducible 24-h test for demonstration of lymphocyte-associated immunity to CDV has been developed and described. Studies in progress on the types of cells involved and their role in *in vivo* recovery from infection should further clarify the immunological mechanisms responsible for recovery from CDV.

ACKNOWLEDGMENTS

We thank David Long and Renee C. Mezza for their excellent technical assistance.

This research was supported by Public Health Service grant no. 2 RO1 AI-09022-08, from the National Institute of Allergy and Infectious Diseases, and by The State of Ohio Canine Research Funds.

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