INFECTION AND IMMUNITY, May 1975, p. 1069-1078

Vol. 11, No. 5
Printed in U.S.A.

Effects of Canine Distemper Virus Infection on Lymphoid Function In Vitro and In Vivo

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Received for publication 30 December 1974

In the present study, the immunodepressive effects of canine distemper virus (CDV) infection of dogs on two parameters of lymphocyte function, namely phytomitogen-induced cellular proliferation and skin allograft rejection, were investigated. Infection of susceptible gnotobiotic dogs with virulent R252-CDV resulted in a depression of peripheral blood lymphocyte mitogen response as measured by [^{3}H]thymidine incorporation for up to 10 weeks after inoculation. This effect coincided with the appearance of viral antigen by immunofluorescence in leukocytes but persisted after the virus was no longer detectable. Loss of mitogen reactivity was seen in all infected dogs. However, when these same CDV-infected dogs were challenged with foreign skin allografts, no significant retention of grafts over controls was observed despite the depressed lymphocyte activity. Considering the in vitro and in vivo data it was concluded that, although immunodepressive effects of CDV were demonstrated in vitro, parallel in vivo experiments indicated that less than complete suppression of immune functions occurs during the course of CDV infection.

The interaction of infectious agents with the immune system has received wide attention. Studies in a number of viral systems have shown that one of the prime factors determining host recovery is a vigorous intact immune response. In some systems such as disseminated vaccinia infection, the virus escapes host control as a consequence of previously existing (32) congenital or acquired immunodeficiency. With murine and avian oncogenic ribonucleic acid viruses, a direct effect is seen upon lymphocytes or their precursor stem cells. This is reflected in tumor-bearing animals by decreased mitogen responsiveness, prolonged allograft rejection, and deficient antibody response after antigenic challenge (10, 29).

The suppressive effects of measles virus (MV) on in vitro and in vivo manifestations of delayed-type hypersensitivity are well recognized, although the mechanism is unclear (12, 25, 29 30, 34). Intact delayed-type hypersensitivity is generally considered essential for recovery from MV (6). Fatal infection with conventional MV is seen in patients with genetic or drug-induced immunodeficiency and various lymphoreticular malignancies (2, 12, 22). Reports of immunocompetence in patients with subacute sclerosing panencephalitis, a chronic demyelinating encephalitis associated with an MV-like agent, are conflicting. Some studies indicate that subacute sclerosing panencephalitis is accompanied by subtle immunological abnormalities (14, 17, 26, 28). Other reports find no relationship (2, 27, 33).

Canine distemper infection in dogs shares many features with MV infection in man (3). Previous reports from this laboratory have emphasized morphological changes in lymphoid tissue after infection with distemper virus (18, 24) and, more extensively, lesions of and pathogenetic events leading to chronic demyelinating encephalitis. Since the incidence of fatal encephalitis is less than 100%, study of the immunological capacity of infected and recovered animals may provide insight into mechanisms involved in recovery and reveal methods whereby the course of disease could be altered experimentally.

We have shown that the levels of complement-fixing and neutralizing antibodies present in convalescent serum are reliable indicators of the progress of infection (S. Krakowka, R. Olsen, A. Confer, A. Koestner, and B. McCullough, manuscript in preparation). In the present study, in vitro and in vivo tests for cell-mediated immune function in canine distemper virus (CDV)-infected dogs are sequentially evaluated. A disparity between in vitro and in vivo data was encountered and evaluated in terms of the final outcome of the infection in experimental animals.

MATERIALS AND METHODS

Dogs. A total of 23 colostrum-deprived mixed-breed gnotobiotic dogs from three litters was used in

these experiments. Nineteen dogs (10 infected and 9 control) were used in experiments correlating in vitro lymphocyte blast transformation (LBT) responses and allograft rejection. Four dogs were inoculated with a modified live distemper virus vaccine and observed for any effect on LBT.

All gnotobiotic puppies were surgically derived from pregnant conventional dogs and raised in flexible plastic isolators according to the methods of Griesemer (15, 25). At approximately 5 weeks of age, littermate controls were removed from the experimental dogs and housed in separate gnotobiotic isolation units until termination of the experiments.

Viruses: R252-CDV. The origin, in vivo, and in vitro properties of virulent R252-CDV has been described (A. W. Confer, Masters thesis, Ohio State Univ., Columbus, 1974; 18, 24). The inoculum was stored as a clarified 10% cerebellar suspension in liquid nitrogen. In a typical experiment, two pups were inoculated intraperitoneally with 0.3 ml of freshly thawed inoculum. These animals infected littermates by contact exposure. The onset of disease in contact-exposed animals followed 1 week after that in parenterally inoculated animals. Immunological analysis was adjusted accordingly.

Vaccine virus. D-Vac was purchased from Pitt-man-Moore Corp. and administered to four dogs by subcutaneous injection according to the manufacturer's instructions.

Peripheral lymphocyte blast transformation. Samples of blood for hemograms were collected at weekly intervals. Total and differential leukocyte counts were made (24). Two milliliters of serum was also collected at that time for subsequent analysis of complement-fixing antibodies to CDV (S. Krakowka et al., manuscript in preparation).

Ten milliliters of heparinized blood containing 20 U of preservative-free heparin (Panheprin, Abbott Laboratories, Chicago) per ml was drawn once a week throughout the observation period as a source of lymphocytes for the micro-LBT assay. Four milliliters of blood was mixed with an equal volume of sterile 5% dextran in a syringe and allowed to sediment for 1 h at 37 C. The leukocyte-rich plasma was aspirated and the leukocytes were washed once in Hanks balanced salt solution containing 50 mg of ethylenediaminetetraacetic acid per 100 ml. Contaminating erythrocytes in the cell pellet were lysed by the addition of 9.0 ml of sterile distilled water for 10 s. Isotonicity was reestablished by the addition of 2.9 ml of 3.5% NaCl. The leukocytes were pelleted by centrifugation at 800 rpm for 10 min and resuspended in Eagle minimum essential medium (MEM) containing 20% fetal calf 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) to a final cell concentration of 10^6 leukocytes per ml.

The remaining 6.0 ml of heparinized blood was diluted to 30 ml with Hanks balanced salt solution-ethylenediaminetetraacetic acid and lymphocytes were isolated by centrifugation over a ficoll-hypaque (FH) gradient, specific gravity 1.0805 (21). The final preparation was adjusted to 10^6 lymphocytes per ml with MEM.

Both dextran-sedimented (DS)- and FH-purified lymphocyte preparations were cultured in quadruplicate in flat-bottomed microplates (Falcon Plastics no. 3041, Oxnard, Calif.) at 10^5 cells per well with the plant mitogens, e.g., pokeweed mitogen, at 50 μg of MEM per ml and phytohemagglutinin (PHA-P) at 0.1 μl of stock per ml of MEM for 3 days at 37 C, 10% CO_2.

During the last 18 h 0.5 μCi of titrated thymidine was added to each culture well. Subsequently, cells were collected and washed with a multiple automatic sample harvester (Otto Hiller Co., Madison, Wis.). Radioactivity incorporated into cellular nucleic acid was trapped on glass filter paper and subsequently quantitated in scintillation vials filled with a toluene-base cocktail (Permablend II, Packard Instrument Co., Downers Grove, Ill.) by the channels-ratio method in a Packard Tri-Carb model 3375 Beta scintillation counter.

Detection of viral antigen in leukocytes by immunofluorescence. Convalescent serum from a gnotobiotic dog with a high complement-fixing titer to CDV (>1:256) was fractionated by 50% saturated ammonium sulfate. The resultant globulin was conjugated to fluorescein isothiocyanate (anti-CDV-fluorescein isothiocyanate), after which the unbound dye was removed by passage through a Sephadex G-25 column as described (20). Overlabeled globulin was removed by passage through a diethylaminoethyl-cellulose column equilibrated with 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 8.7. The resultant conjugate was stored in 0.6-ml aliquots at −70 C until use. Specificity of the reagent was confirmed by blocking of immunofluorescence on CDV-infected spleen sections by preincubation with unconjugated anti-CDV serum (19).

Leukocytes from both FH and DS preparations were washed once in saline, smeared on glass cover slips, and air-dried. Cover slips were fixed in acetone at room temperature for 30 min. The smears were stained with anti-CDV-fluorescein isothiocynate for 1 h, washed in saline, and examined.

Split-thickness skin grafts. An unrelated gnotobiotic dog served as the skin donor animal in all grafting experiments. A split-thickness 1-cm^2 skin graft from this donor as well as a control autograft was applied to the left lateral thorax of gnotobiotic CDV-infected dogs and controls (9). The grafts were applied on day 14 after infection. The graft sites were bandaged with sterile vaseline-impregnated gauze and surgical tape for 7 days postoperatively. Thereafter, sites were examined twice daily and the color and flexibility of the allograft were recorded. The criteria used for determining graft rejection were change in color from pink to dark purple and accompanying loss in skin graft pliability. Selected grafts were biopsied for histopathological examination.

RESULTS

Infection with R252-CDV. Ten dogs were infected with R252-CDV. Nine uninfected littermates served as controls. All infected dogs became lymphopenic 1 week after infection. An

example of the duration and magnitude of the lymphopenia in surviving dogs of litter 1 along with littermate controls is shown in Fig. 1.

Four of 10 infected dogs developed signs of acute encephalitis 14 to 28 days postinfection (PI). A rapid progression to death within 48 h of onset was seen in those dogs. The remaining six dogs survived the 10-week observation period without overt signs of disease except for prolonged lymphopenia. No gross or histological evidence of active distemper infection was seen in these dogs at necropsy.

Characterization of lymphocyte culture preparations. FH-prepared lymphocyte cultures from uninfected dogs contained 80 to 95% lymphocytes. Five to 20% neutrophils were present as contaminants. Numerous platelets and erythrocytes were also seen. Approximately 30% of the lymphocytes present in the original sample were recovered by this technique.

In contrast, lymphocytes from CDV-infected dogs made up 40 to 75% of the FH cultures. Paradoxically, the recovery efficiency of lymphocytes from lymphopenic infected dogs average over 60% of starting cells. As in uninfected dogs, erythrocytes and platelets were noted in the smears.

Sedimentation of erythrocytes with 5% dextran resulted in recovery of about 70% of the total starting leukocytes from the uninfected dogs. In CDV-infected dogs, the number of leukocytes recovered by this technique was more variable, ranging from 10 to 90%. In both cases, a significant degree of contamination with nonsedimented erythrocytes and platelets was noted. Differential counts of DS cultures demonstrated that the preparation of lymphocytes in these cultures reflected differential counts in whole blood in both control and infected dogs.

Lymphocyte transformation in uninfected gnotobiotic dogs. The mitogens used in this study regularly and consistently induced deoxyribonucleic acid synthesis as measured by the uptake of [H^3]thymidine in lymphocytes from gnotobiotic dogs. As a rule, despite the lower absolute numbers of lymphocytes in culture, DS cultures yielded higher stimulation values in both stimulation indexes as well as counts per minute than did FH cultures from the same blood sample. The degree of stimulation measured in counts per minute in individual control dogs varied from week to week throughout the experiment.

An additional source of variation was encountered when counts per minute or stimulation index was compared between litters of normal dogs. Lymphocyte cultures from dogs of litter 2

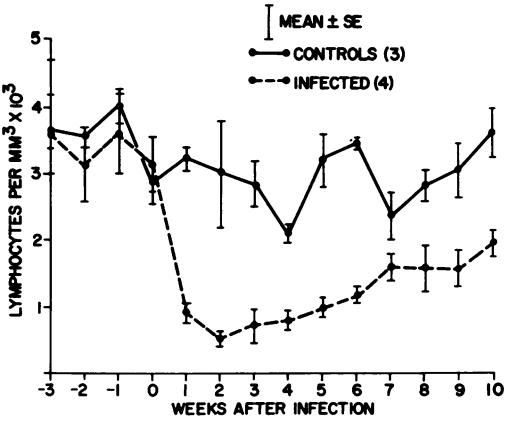

FIG. 1. *Absolute peripheral lymphocyte levels in gnotobiotic dog litter no. 1 during infection with R252-CDV.*

usually gave higher counts per minute than did those of litters 1 and 3. This did not appear to be an age related effect per se as dogs of litter 1 were older and dogs of litter 3 were younger than those of litter 2.

Determination of optimal culture conditions for lymphocyte transformation. Several parameters of the assay were investigated in preliminary experiments to determine optimal conditions for stimulation. The size of the experimental animals as well as the lymphopenia (approximately fivefold) in R252-CDV-infected dogs limited the number of lymphocytes available for weekly sequential study. A cell culture concentration of 10^6 cells/ml (10^5 cells/culture) gave adequate stimulation. Stimulation decreased as the number of cells cultured decreased to a threshold concentration of 0.5×10^6 cells/ml.

Since the degree of lymphocyte transformation is also a function of time in culture, this parameter was investigated. FH-prepared lymphocytes responded maximally at 3 or 4 days in culture. In contrast, DS cultures showed peak stimulation at 3 days with counts per minute falling off rapidly after this time. Based on these results, all subsequent experiments were terminated after 3 days in culture.

Optimal doses of mitogens were determined in preliminary experiments. It was found that a concentration of PHA-P (Difco), reconstituted according to the manufacturer's instructions, at a concentration of 0.1 μl of stock per ml of culture media resulted in maximal stimulation. Likewise, a concentration of 50.0 μg of pokeweed mitogen (GIBCO) per ml of culture media was found optimal.

Peripheral lymphocyte transformation in

R252-CDV-infected gnotobiotic Dogs. Evaluation of results obtained from either FH or DS lymphocyte cultures showed that the results for both mitogens used were comparable throughout the experiment (Tables 1 and 2). Thus, although both isolation procedures were compared throughout the experiments for simplicity, only the data from the DS cultures are given in the results.

Values in counts per minute for mitogen-induced LBT dropped to the level of background counts seen in control cultures maintained without mitogens 1 week after infection with R252-CDV. This event coincided both with CDV-induced lymphopenia and the appearance of viral antigen in peripheral blood leukocytes detected by immunofluorescence. All dogs infected with R252-CDV demonstrated in vitro immunodepression. The results presented in Table 3 indicate that no return of lymphocyte mitogenic activity was observed in three fatally infected dogs. In contrast, a trend toward return to preinoculation values 4 to 6 weeks after infection was seen in dogs which were destined for recovery as illustrated in Fig. 2. Lymphocytes from several of these animals remained incapable of responding to mitogens throughout the 10-week observation period. The LBT assay in the early stages of disease did not distinguish between fatally infected dogs and those surviving infection with R252-CDV.

Explanations for this effect of R252-CDV on peripheral lymphocyte activity were sought. A simple decrease in the numbers of lymphocytes in culture as occurs during the lymphopenia could explain these results. However, the FH lymphocyte cultures which were adjusted to 10^6 lymphocytes per ml showed a comparable depression of stimulation. Further, depression of mitogen response persisted into the convalescent period, a time in which absolute lymphocyte returned toward preinoculation levels.

In vitro viability of cultured leukocytes was compared in control and R252-CDV-infected cultures by trypan blue dye exclusion. In both cases, 40 to 70% of the original cells examined were viable after 3 days in culture.

It was possible that a different dose of mitogen, either suboptimal or supraoptimal, was required to induce transformation in lymphocytes of R252-CDV-infected dogs. Tenfold higher and lower dosages of both mitogens were

TABLE 1. *Comparison of FH and DS prepared lymphocyte cultures: response to PHA-P[a] after infection with R252-CDV*

Weeks PI	FH-prepared lymphocytes[b]			DS-prepared lymphocytes[c]		
	1[d]	2	3	1	2	3
−1	494 ± 22(10.5)[e]	1972 ± 93(28.6)	536 ± 56(11.2)	598 ± 95(15.3)	7759 ± 232(114.1)	967 ± 54(25.4)
1	77 ± 13(0.8)	49 ± 6(1.0)	49 ± 3(1.4)	68 ± 12(1.1)	52 ± 9(1.4)	56 ± 10(1.0)
5	81 ± 6(1.4)	95 ± 8(1.9)	117 ± 10(3.7)	59 ± 3(1.9)	111 ± 3(2.6)	566 ± 22(10.9)
10	237 ± 11(6.1)	296 ± 29(2.2)	1997 ± 141(25.9)	37 ± 3(1.0)	736 ± 65(6.3)	2297 ± 109(54.7)

[a] Difco, Detroit, Mich.; 0.1 μl of stock/culture.
[b] Adjusted to 10^6 lymphocytes/ml (10^5 lymphocytes/culture).
[c] Adjusted to 10^6 leukocytes/ml (10^5 leukocytes/culture).
[d] R252-CDV-infected dog number.
[e] Data expressed as mean counts per minute ± standard error of quadruplicate cultures. Stimulation index (in parentheses) is calculated by dividing counts per minute in mitogen-stimulated cultures by counts per minute in control cultures.

TABLE 2. *Comparison of FH and DS prepared lymphocyte cultures: response to pokeweed mitogen[a] after infection with R252-CDV.*

Weeks PI	FH-prepared lymphocytes[b]			DS-prepared lymphocytes[c]		
	1[d]	2	3	1	2	3
−1	644 ± 42(13.7)[e]	2516 ± 121(36.5)	834 ± 44(17.4)	698 ± 31(17.9)	5844 ± 51(85.9)	751 ± 30(19.8)
1	56 ± 10(0.6)	80 ± 5(1.7)	68 ± 3(1.9)	90 ± 17(1.5)	40 ± 11(1.1)	137 ± 4(2.4)
5	98 ± 3(1.7)	153 ± 12(3.0)	169 ± 13(5.3)	85 ± 6(2.7)	310 ± 2(7.4)	744 ± 48(14.3)
10	461 ± 21(11.8)	908 ± 35	2283 ± 148(29.6)	37 ± 5(1.0)	912 ± 95(7.9)	1268 ± 133(30.2)

[a] GIBCO, Long Island, N. Y.; 50 μg/culture.
[b] Adjusted to 10^6 lymphocytes/ml (10^5 lymphocytes/culture).
[c] Adjusted to 10^6 leukocytes/ml (10^5 leukocytes/culture).
[d] R252-CDV-infected dog number.
[e] Data expressed as mean counts per minute ± standard error of quadruplicate cultures. Stimulation index (in parentheses) is calculated by dividing counts per minute in mitogen-stimulated cultures by counts per minute in control cultures.

TABLE 3. *Effect of infection with R252-CDV: mitogen-induced LBT of peripheral blood lymphocytes[a] in fatally infected[b] gnotobiotic dogs*

Weeks PI	Phytohemagglutinin-P[c]			Pokeweed mitogen[d]		
	1[e]	2	3	1	2	3
−1	584 ± 22[f](12.7)[g]	2629 ± 219(20.9)	3043 ± 72(13.6)	214 ± 9(4.7)	3862 ± 204(30.7)	7656 ± 553(34.3)
0	2045 ± 147(35.9)	657 ± 42(16.4)	756 ± 66(11.0)	2140 ± 192(37.6)	4489 ± 162(112.2)	3886 ± 158(56.3)
1	37 ± 3(1.0)	199 ± 3(2.9)	177 ± 9(2.2)	43 ± 3(1.2)	168 ± 6(2.4)	145 ± 7(1.8)
2	50 ± 2(1.1)	856 ± 95(5.6)	70 ± 14(2.1)	44 ± 4(1.0)	149 ± 17(1.0)	35 ± 2(1.0)
3	38 ± 2(0.9)	417 ± 21(9.3)	−[h]	31 ± 2(0.7)	199 ± 10(4.4)	−[h]
4	47 ± 4(1.3)	397 ± 63(2.9)	−[h]	37 ± 2(1.0)	56 ± 3(0.4)	−[h]

[a] DS prepared lymphocytes.
[b] The dogs died of PI day 29, 20, and 15, respectively.
[c] Stock (Difco) 0.1 μl/culture.
[d] Fifty micrograms (GIBCO)/culture.
[e] R252-CDV-infected dog number.
[f] Data expressed as mean counts per minute ± standard error of quadruplicate cultures. The counts per minute in cultures without mitogen ranged from 50 to 100.
[g] SI, given in parentheses, was calculated by dividing counts per minute in mitogen-stimulated cultures by counts per minute in control cultures.
[h] Dog no. 3 died 15 days after infection.

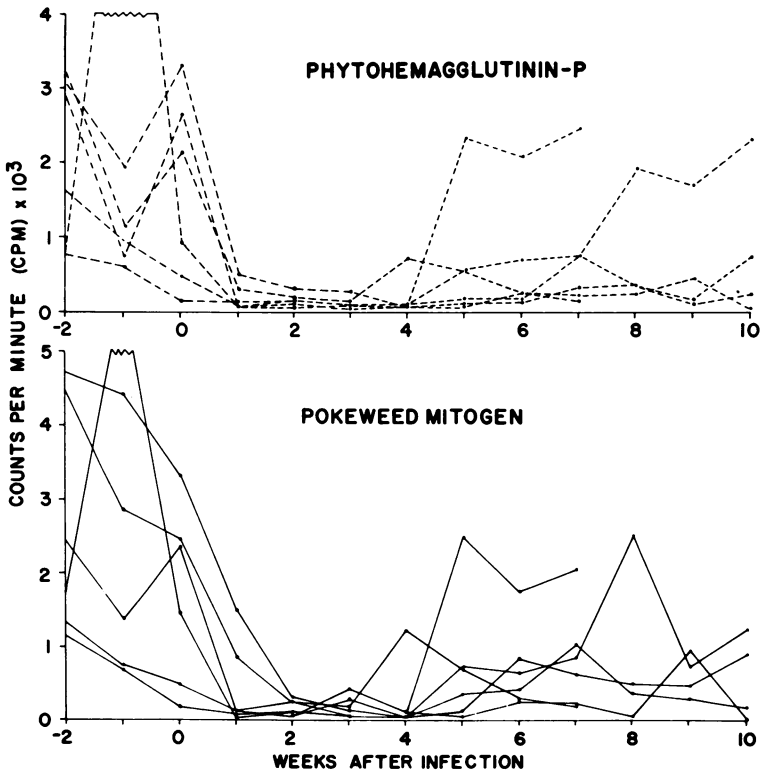

FIG. 2. *Effect of nonfatal infection of six gnotobiotic dogs with R252-CDV on the incorporation of [³H]thymidine into peripheral blood lymphocyte cultures stimulated with PHA-P and pokeweed mitogen.*

tried and did not affect the results (Tables 4 and 5).

A direct viral effect on lymphocytes in cultures was considered a likely possibility since MV, another paramyxovirus, will depress lymphocyte transformation in vitro (25). Immunofluorescent staining of lymphocytes from infected dogs revealed the presence of viral antigen in the cytoplasm of cells of both lymphocytes and monocytes. The proportion of im-

TABLE 4. *Effect of PHA-P concentration on [^3H]thymidine incorporation[a] by lymphocytes[b] from normal and R252-CDV-infected gnotobiotic dogs*

Dogs	Unstimulated control cultures	Concn of PHA-P/ml		
		2.5 μl	0.5 μl	0.1 μl
Uninfected control (2)	39 ± 2	81 ± 5 (2.1)	709 ± 33 (18.2)	1646 ± 207 (42.2)
	62 ± 4	45 ± 2 (0.7)	121 ± 7 (2.0)	566 ± (9.1)
R252-CDV infected (3)	107 ± 33	53 ± 9 (0.5)	60 ± 7 (0.6)	63 ± 9 (0.6)
	30 ± 4	77 ± 18 (2.6)	68 ± 8 (2.3)	81 ± 26 (2.7)
	37 ± 2	50 ± 5 (1.4)	46 ± 5 (1.2)	47 ± 6 (1.3)

[a] Data expressed as the mean counts per minute of quadruplicate samples ± standard error. The SI is given in parentheses.

[b] Leukocytes are adjusted to 10^6 cells/ml and cultured in microculture plates in quadruplicate (10^5 cells/well) for 3 days at 10% CO_2, 37 C.

TABLE 5. *Effect of pokeweed mitogen (PWM) concentration on [^3H]thymidine incorporation[a] by lymphocytes[b] from normal and R252-CDV-infected gnotobiotic dogs*

Dogs	Unstimulated control cultures	Concn of PWM/ml		
		250 μg	50 μg	10 μg
Uninfected control (2)	39 ± 2	1494 ± 92 (38.3)	865 ± 66 (22.2)	442 ± 8 (11.3)
	62 ± 4	916 ± 46 (14.8)	715 ± 65 (11.5)	224 ± 14 (3.6)
R252-CDV infected	107 ± 33	111 ± 14 (1.0)	87 ± 8 (0.8)	78 ± 6 (0.7)
	30 ± 4	85 ± 1 (2.8)	88 ± 8 (2.9)	77 ± 2 (2.6)
	37 ± 2	75 ± 6 (2.0)	54 ± 6 (1.5)	52 ± 3 (1.4)

[a] Data expressed as the mean counts per minute of quadruplicate samples ± standard error. The SI is given in parentheses.

[b] Leukocytes are adjusted to 10^6 cells/ml and cultured in microculture plates in quadruplicate (10^5 cells/well) for 3 days at 10% CO_2, 37 C.

munofluorescent-positive cells was high in dogs dying of acute encephalitis and variable in others. The pattern of fluorescence was diffuse in some cells and restricted to perinuclear inclusions in others. Concurrent light microscopic examination of smears stained with Wright-Giemsa stain failed to detect the inclusions. Sequential immunofluorescent examination of peripheral blood leukocytes did, however, differentiate between fatally infected and recovered animals. Leukocytes from fatally infected dogs contained viral antigen from 1 week postinfection (PI) until death. In contrast, fluorescent-positive cells were seen for only 1 to 3 weeks PI in those animals destined to survive the infection.

Peripheral lymphocyte transformation in gnotobiotic dogs vaccinated with modified live virus vaccine. Inoculation of a modified-live virus measles vaccine in man suppresses manifestations of cell-mediated immunity. Lymphocytes from four normal gnotobiotic dogs were tested for mitogen-induced blast transformation before and after vaccination with a modified-live distemper virus vaccine. The results are summarized in Table 6. No depression of lymphocyte response was noted. Further, neither febrile response nor lymphopenia were observed. Viral antigen could not be demonstrated in cultured leukocytes. Sera from all dogs contained CDV antibody within 28 days after vaccination.

Correlation of LBT and skin allograft rejection. Correlations were sought between in vitro immunodepression as measured by LBT and an in vivo consequence of immunodepression, namely retention of foreign allografts. Grafts were applied to infected dogs 2 weeks PI. The results of these experiments along with parallel in vitro LBT data are given in Table 7.

Despite the high incidence of surgical and postoperative failures in infected (four out of ten) and control (three out of nine) animals in these experiments, the results are unequivocal. No significant retention of allografts was observed in R252-CDV-infected gnotobiotic dogs. Only one dog demonstrated prolonged graft survival as compared to controls (infected dog 1, Table 7). This animal died from CDV-associated encephalitis 16.5 days after grafting.

Allografts placed on control uninfected as well as infected dogs exhibited changes in color and

loss of pliability 11 to 14 days after application. Biopsies of the grafts at this time revealed histological features typical of graft rejection (8, 35) including epithelial and dermal necrosis, cellular infiltration of mononuclear inflammatory cells and neutrophils, and vascular thrombosis and necrosis. Although allografts from CDV-infected animals showed similar overall histological changes, the cellular inflammatory reaction in both the allograft and underlying dermis was less prominent than in uninfected control allografts.

DISCUSSION

A prominent feature of virulent CDV infection in gnotobiotic dogs is prolonged lymphopenia and systemic lymphoid depletion. Previous studies have emphasized the overall effects of CDV on lymphoid tissues in fatally infected dogs (3, 18, 24, 36). However, virus-lymphocyte interactions in infected animals which remain clinically healthy have received less attention. Although development of serum-neutralizing antibodies are correlated with recovery (3), the pathogenetic events resulting in effective immunity have not been elucidated. Initial replication sites for CDV are macrophages and lymphocytes (3). This suggests that CDV-mediated immunodepression constitutes one mechanism whereby a progressive infection would be initiated. Accordingly, tests of lymphoid function should differentiate fatally infected from immune dogs. The present experiments were designed to test this hypothesis in vitro and in vivo.

The in vitro response of peripheral blood lymphocytes to plant mitogens is a nonantigen-specific polyclonal transformation to immature blast cells. Although immunospecificity is lacking, the LBT assay is widely studied because the intracellular events involved in lymphocyte responses to either antigens or mitogens are

TABLE 6. *The effect of vaccination with a modified-live CDV vaccine on immunological responsiveness of four gnotobiotic dogs*

Weeks after vaccination	Absolute lymphopenia	Depression of mitogen-induced peripheal blood LBT	Viral antigen in peripheral blood lymphocytes by immunofluorescence	Complement-fixing antibody response to R252-CDV; sero-conversion after vaccination
0	No	No	No	No
1	No	No	No	No
2	No	No	No	No
3	No	No	No	Yes (2/4)[b]
4	No	No	NT[a]	Yes (4/4)[b]
5	No	NT	NT	Yes (4/4)

[a] NT, Not tested.
[b] Number of dogs containing anti-CDV antibodies per number tested.

TABLE 7. *Comparison of skin allograft survival time, mitogen-induced LBT and antibody response to R252-CDV in control and R252-CDV-infected gnotobiotic dogs*

Dogs	Allograft rejection time (Days)	Depression of mitogen-induced LBT	Complement-fixing antibody response to R252-CDV; sero-conversion after infection
uninfected (6)[a]	11.75 ± 0.5[b]	No	No
R252-CDV infected			
1	16.5[c]	Yes	No
2	14.0	Yes	Yes
3	11.5	Yes	Yes
4	12.0	Yes	Yes
5	11.5	Yes	Yes
6	11.0	Yes[d]	Yes

[a] Two controls from litter one and four control animals from litter 3.
[b] Mean survival time ± standard error.
[c] This dog died of acute encephalitis 16.5 days after grafting. At that time, the allograft was still viable by both gross and histological criteria.
[d] Mitogen response in this dog was significantly depressed for 2 weeks only. Lymphocyte reactivity returned to normal levels during the grafting experiment.

identical (16). The response to mitogenic stimulation in preinoculation and control samples from gnotobiotic dogs was similar to the expected response in conventional dogs. Five uninfected controls were evaluated weekly along with the CDV-infected animals. Normal dog's lymphocytes demonstrated a cyclic periodicity in the amount of [^{3}H]thymidine incorporated. Dionigi et al. observed a similar phenomenon in PHA-stimulated peripheral blood lymphocyte cultures from normal individuals sampled biweekly for several months (11).

Despite any contributing effects of cyclic phenomena, infection with CDV had a profound and prolonged suppressive effect on canine LBT. The effect was seen at the onset of lymphopenia but persisted after peripheral lymphocyte levels returned to the normal range. Several different in vitro and in vivo possibilities were considered as possible mechanisms of lymphocyte suppression. Infection with virulent CDV is necessary since inoculation of dogs with a modified-live virus vaccine failed to affect LBT. Parameters such as decrease in number of lymphocytes in culture during infection, viability differences between infected and control cells, and changes in optimal mitogen dose during infection do not provide a satisfactory explanation for the suppression observed.

An in vivo effect on mitogen responsiveness analogous to antigenic competition has been described by Gershon (13). He found that spleen cells taken from mice immunized 1 to 2 days previously were unresponsive to PHA and that this effect lasted for 1 week after immunization. In the present circumstance an active response to viral antigens might explain part of the observed mitogen insensitivity. Another possibility is that a selective loss of mitogen-sensitive lymphocytes occurs in dogs as a consequence of infection with CDV. Although no direct evidence for this effect was sought, this explanation is consistent with the lymphocyte-depleting effects of CDV on central lymphoid organs such as the thymus (24).

The simplest hypothesis in agreement with available morphological data is that mitogen unresponsiveness is a consequence of a direct viral effect on either lymphocytes or monocytes. With direct immunofluorescence viral antigen was found in leukocytes of all infected dogs 1 to 2 weeks PI. Fatally infected animals remained viremic until death. Virus was not found after 3 weeks PI in the leukocytes of those dogs which survived the infection, yet in some cases, mitogen unresponsiveness was observed for at least 10 weeks PI. Although the reason for this later discrepancy is unclear, it is possible that the method was not sensitive enough to detect small amounts of intracellular virus persisting after the appearance of humoral antibodies.

Loss of mitogen reactivity was seen in all CDV-infected dogs regardless of the outcome of infection. Early in the course of disease, animals which eventually died of generalized infection could not be distinguished from immune dogs by LBT. Mortality was more closely correlated with the overt presence of viral antigen in peripheral leukocytes longer than 3 weeks PI and the absence of neutralizing or complement-fixing antiviral antibodies in serum (S. Krakowka, et al., manuscript in preparation) than by the depressed LBT.

To further clarify the immunodepressive effects of CDV, an in vivo measure of cell-mediated immunity in infected animals was sought. Intradermal skin tests with antigens to which dogs had been previously sensitized by immunization were evaluated in pilot studies. Great difficulty was encountered in production of tuberculin hypersensitivity in normal dogs by repeated immunizations with complete Freund adjuvant. Skin reactions using both Koch old tuberculin and purified protein derivative were minimal in one-third of the dogs and undetectable in the remaining animals (S. Krakowka, unpublished data). Further, positive tests were not reproducible. Demonstration of delayed-type hypersensitivity to another antigen, keyhole limpet hemocyanin, was obscured by the simultaneous presence of intense antibody-mediated immediate-type reactions in the skin and subcutis of immunized animals. Consequently, skin allograft rejection was adapted as the in vivo assay.

Rejection of skin allografts is a reflection of histocompatibility differences between donor and recipient. Thymic-dependent lymphocytes are responsible for detecting these antigenic differences and for initiating the rejection reactions. Further, the ability of lymphocytes to respond to mitogenic stimulation is positively correlated with the capacity to reject foreign allografts (7). In their experience with renal transplants, Rivard et al. found that mongrel dogs with active distemper retain renal allografts which otherwise would have been rejected (31). The results of our skin grafting experiments did not support this observation. Only one CDV-infected dog retained a skin graft significantly longer than controls. In this instance, a coincidental good histocompatibility match is as likely an explanation as any suppressive effects of CDV infection.

Three possibilities were entertained as explanations for the apparent discrepancy between in

vitro and in vivo data. The first was related to the experimental design, in that dogs were routinely grafted 14 days after infection. It is possible that statistically prolonged graft survival could have been attained in more animals if they had been grafted earlier in the course of disease. As to the second alternative, differences between the tests may exist in their sensitivity to immunodepressive influences. The LBT assay may be very sensitive to subtle interference of immunological function, whereas allograft rejection is not. Rejection of foreign transplants in man occurs in spite of procedures known to affect in vitro lymphocyte function such as immunodepressive drugs and antithymocyte globulin. In passive transfer experiments, Lubaroff found that as few as 500,000 lymph node cells given to isogenic X-irradiated rats restored immunocompetence in recipients (23). In reconstituted rats, mean skin graft survival times were the same regardless of the numbers of cells transferred once the threshold dose had been attained. By extrapolation to the present work, prolonged graft survival in CDV-infected dogs would occur only if the number of immunocompetent lymphocytes is reduced below an undefined threshold level. Under ordinary circumstances, infection with CDV does not cause the degree of depletion necessary to result in prolonged graft survival.

A third explanation was also considered. Skin graft rejection correlated with the development of effective antiviral immunity; LBT, on the other hand, did not. It is possible that suppression of lymphocyte activity in culture represents an in vitro artifact which inaccurately reflects in vivo events. Poorly understood variables such as composition of culture media, cell concentration effects, and the obligatory requirements for viable phagocytic cells affect the quality of lymphocyte mitogenic stimulation. This along with the reluctance to accept in vitro behavior of peripheral lymphocytes as reflections of central lymphoid integrity (13), combine to limit interpretation of results obtained with this assay.

In conclusion, we have investigated the effects of CDV on the functional parameters of the immune system. Although interference with lymphocyte function could be demonstrated in vitro, parallel in vivo data indicated that no significant interference with immune functions occurs in those dogs which survive CDV infection.

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