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Dissertation

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

George Steven Krakowka, D.V.M.

* * * * * *

The Ohio State University
1974

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CHAPTER I

MYELIN SPECIFIC AUTOANTIBODIES ASSOCIATED WITH CENTRAL NERVOUS SYSTEM DEMYELINATION IN CANINE DISTEMPER VIRUS INFECTION

Introduction

The role of the immune response in the etiology of demyelinating diseases such as multiple sclerosis (MS) and canine distemper remains to be clarified. It has been proposed, and evidence presented that demyelination in MS may proceed by immune mechanisms (4,5,6). In this regard, experimental allergic encephalomyelitis (EAE) has been used as a model for MS on morphological and biochemical grounds (24,31). This model is of limited value in that only myelin basic protein sensitized lymphocytes are able to induce the disease. Recent reports indicate that an infectious agent in conjunction with a suspected sensitization to brain components may be involved in the etiology of MS (12,17). Accordingly, the interplay between viruses, host tissues, and immune factors in chronic neurological disorders must be considered in the investigation of demyelinating disease.

Measles virus, or closely related viral variant(s), are strongly implicated as the etiologic agent in subacute sclerosing panencephalitis (SSPE), an uncommon chronic demyelinating disease of man (26). Seroenepidemiologic surveys of MS patients and their families enhances speculation that measles virus is also associated with MS (28). Several in-
vestigators have presented evidence that canine distemper virus (CDV), a paramyxovirus biochemically and antigenically closely related to measles virus, may initiate a brain reactive immune response which could contribute to the demyelinating phase of the disease in dogs (1,19,20,33).

Complement-dependent serum demyelinating factors have been described using the cerebellar explant culture technique in EAE, MS, and Guillain-Barré syndrome, a demyelinating disease of peripheral nerves (5,9,10,15). Recently we reported that some but not all distemper sera were capable of demyelinating explant cultures of canine cerebellum in vitro (20). The myelinotoxic effect was complement-dependent in the more convincing cases.

These observations prompted us to investigate further these myelinotoxic serum factors. The present study was undertaken to determine if antimielin antibodies could be detected and characterized in the sera of dogs with naturally occurring demyelinating distemper encephalomyelitis by serological means. A further objective was to correlate this antibody response with clinicopathological features of experimental CDV-induced demyelination in gnotobiotic dogs.

**Materials and Methods**

**Canine Experimental Sera**

Canine distemper (CD) sera were obtained from 2 sources. The first group of 34 sera were collected from naturally occurring cases of distemper-associated demyelinating encephalitis as confirmed by histologic examination. Representative cases with definite and widespread central nervous system (CNS) lesions of demyelination were selected for detailed
immunologic study.

A second group of CD sera consisted of weekly serial bleeds from 31 gnotobiotic dogs infected with R252-CDV, a neuropathogenic virus isolated from a naturally occurring case of distemper. Detailed clinical and pathologic descriptions of these infected dogs are reported separately (21). Briefly, the infected dogs were separated into 3 groups based on histological and clinical criteria. Group I (11 dogs) became moribund at various intervals after infection and had microscopic lesions of non-inflammatory distemper virus-induced demyelination with accompanying systemic lymphoid depletion.

Group II (5 dogs) survived the 12 week observation period with little or no clinical evidence of neurological involvement. In these dogs, lesions of demyelinating encephalitis characterized by prominent hematogenous inflammatory cell infiltrates were demonstrated in the CNS microscopically. Group III (15 dogs) remained asymptomatic throughout the observation period and had no detectable lesions in the nervous system.

Control sera were obtained from 3 sources. Twenty sera were obtained from a colony of conventional dogs ranging in age from 6 months to 7 years. The dogs had been vaccinated for canine distemper with a modified live virus vaccine according to published recommendations (2). The second control group consisted of serum samples from 33 surgically deprived colostrum-deprived gnotobiotic dogs raised according to the techniques of Griesemer and Gibson (14). Serial bleeds from 9 uninfected gnotobiotic dogs constituted the normal controls for the experiments with R252-CDV.
Preparation of Myelin Antigen and Reference Antisera

Central nervous system myelin was prepared from canine spinal cord using discontinuous sucrose density ultracentrifugation according to the method of Autillo et al. (3). An aliquot of the preparation was examined by electron microscopy.

High titered hyperimmune antiserum was produced in rabbits and in gnotobiotic dogs by bimonthly subcutaneous and/or intramuscular injections of 10.0 mg myelin antigen suspended in 1.0 ml saline and emulsified in an equal volume of complete Freund's adjuvant. Complement-fixation (CF) titers of 1: ≥ 256 were obtained after 4 injections.

Serological Methods

Titration of Antigen and Reference Antibody

Lyophilized myelin antigen was resuspended to 5.0 mg/ml in veronal buffer with 0.5% sodium azide and stored at 4 C. An aliquot of this stock suspension was block titrated against selected bleeds of CF reference antisera in microtiter plates (Cooke Engineering, Alexandria, Va.). The CF test consisted of incubating serial 2-fold dilutions of antigens (0.025 ml) with an equal volume of 2-fold dilutions of heat inactivated sera and 3 hemolytic units of complement (C'H₅₀) for 12 hours at 4 C.

Amboceptor-coated sheep red blood cells (0.025 ml of 2.5% suspension in veronal buffer) were then added and the plates were incubated an additional 30 minutes at 37 C. One complement-fixing antigen-antibody unit was designated as those final dilutions of serum and antigen that permitted less than 50% of the lysis in indicator cells. Routinely, reference antisera gave titers of 1: ≥ 256 when used with antigen excess (3 units). Using 3 units of antibody, complement-fixation occur-
Complement-fixation/Complement-fixation Inhibition Test

The complement-fixation (CF) and complement-fixation inhibition (CFI) test as adapted to the microtiter system by Olsen and Yohn (23) was employed to evaluate myelin-binding antibodies in test sera.

Sera were evaluated for CF antibody using 3 units of antigen. Titers were expressed as the last dilution of serum resulting in less than 50% lysis of the indicator cells.

For the CFI test, 2 units of antigen were incubated with serial 2-fold dilutions of serum for 1 hour at 37 C. This was followed by a 12 hour incubation period at 4 C with 2 added units of hyperimmune anti-myelin antibody. CFI activity was indicated by the absence of reference antibody-antigen associated complement activation and detected visually when greater than 50% lysis of indicator cells was seen.

Test sera were examined for CF and CFI activity simultaneously with controls for anticomplimentary activity of test serum, antigen and nonspecific interaction of reference antiserum with test serum.

Absorption Experiments

Serological specificity was investigated by absorption experiments using lyophilized canine myelin, spleen, and liver powders, rabbit liver powder, and heterologous rat myelin. Five mg of saline moistened organ powder were incubated with 2.0 ml test serum at 37 C for 1 hour and then for an additional 12 hours at 4 C. Insoluble material was removed by low speed centrifugation. When necessary, lyophilized guinea pig complement was added to remove anticomplementary activity. The treated sera
were heat inactivated and filtered with 0.45 um and 0.22 um pore size membrane filters (Millipore Corp., Bedford, Mass.) respectively before testing. Absorbed and unabsorbed sera were also tested for CF antibody to suspensions of liver, spleen and rat myelin in veronal buffer.

Whole Serum Fractionation

Ion exchange chromatography of CD sera was performed on columns of DEAE-cellulose (Sigma Chemical Co., St. Louis, Mo.) equilibrated with 0.01M phosphate buffer, pH 8.0 (7). The IgG containing fraction was eluted with 0.05M NaCl in phosphate buffer. The remaining serum proteins including IgM were eluted with 0.6M NaCl in phosphate buffer. Samples with 0.05% glycine added were concentrated back to starting volume with a Diaflo apparatus (Amicon, Lexington, Mass.) using a membrane exclusion size of 10,000 molecular weight. Residual anticomplementary activity was removed by absorption with lyophilized guinea pig complement.

Gel filtration of selected sera was accomplished on a Sephadex G-200 column (2.5 x 93 cm) equilibrated with 0.4M NaCl, 0.01M phosphate buffer, pH 7.2 adjusted to a flow rate of 50 ml/hour (32). Eluates were monitored at 280 nm and collected in 5.0 ml volumes. Protein peaks were concentrated back to original volume and tested for CF/CF1 antibody.

Reduction of CD Sera with Mercaptoethanol

The effect of a reducing agent on CF positive sera was evaluated by pre-treating the sera with an equal volume of 0.2M 2-mercaptoethanol for 1 hour at 37 C before testing (18).
**Immunofluorescence Microscopy**

The indirect immunofluorescent method was used to visually confirm the binding of immunoglobulins to myelin. Rabbit anticanine globulin with strong anti-IgM activity for use as the fluorescein-conjugated secondary reagent was prepared in the following manner: A dog was injected intravenously with sheep red blood cells and exsanguinated 10 days later. This serum had a mercaptoethanol-sensitive sheep red blood cell hemagglutinating (H.A.) titer of 1:512. After euglobulin precipitation, the redissolved protein solution was applied to a Sephadex G-200 column and the exclusion peaks (HA 1:16) from 2 runs were pooled and rechromatographed on the same column. Immunoelectrophoretic analysis of the leading shoulder of the first peak showed a single precipitin line consistent with the described position and electrophoretic mobility of canine IgM (25). Rabbits were immunized with 5.0 mg of this material in complete Freund's adjuvant. Immunoelectrophoretic analysis of these antisera against whole canine serum showed a single heavy precipitin line in the IgM region and several precipitin lines in the IgG region. The anti-globulin reagent was conjugated to fluorescein isothiocyanate (FITC) by the dialysis technique of Clark and Shephard (8) except that unbound dye was removed by passing the conjugate through a column of Sephadex G-25.

Blocks of canine spinal cord were frozen in liquid nitrogen. Four micron sections were cut on a cryostat at -20 C, mounted on ovalbumin-coated coverslips, and air dried. After washing, the primary reagent (CD sera or control sera pre-diluted 1:10 in 3.0% isotonic rhodamine-conjugated bovine serum albumin counterstain) was applied and the coverslips incubated 1 hour at room temperature. Following the second washing, a pre-
viously determined optimal dilution of fluorescein-conjugated antiglobulin was added and all the coverslips were incubated 37 C for 1 hour. Each test was accompanied by control coverslips treated with conjugate alone, coverslips treated with known positive primary (antimyelin reference serum) and a known negative serum (heterologous normal rabbit serum).

The specificity of the test was established by showing: i) the conjugate alone did not stain myelin, ii) normal dog serum did not stain myelin, and iii) unconjugated rabbit anti-canine globulin serum, but not pooled normal rabbit serum, successfully blocked myelin fluorescence when applied to spinal cord sections previously treated with myelin binding CDV sera.

RESULTS

Electron Microscopy of Myelin Antigen

Examination of isolated myelin revealed a preparation consisting almost entirely of multi-lamminated membranes and fragments characteristic of myelin (figure 1). Occasional synaptosomal structures were seen. No intact organelles were identified.

Reference Antisera and Antigen

The stock suspension of myelin was stable for several months when stored at 4 C. Further, no significant differences in antigenicity in CF/CFI was noted with similar preparations from 5 different dogs.

Complement-fixing antibody to myelin was detected in all animals 4 weeks after immunization and reached maximum titers of 1:≥ 256 by 6 weeks. No significant differences in the titers were noted when a myelin preparation of rat origin was substituted. Several animals also reacted in low
titers (1:4 to 1:16) with a reconstituted liver powder preparation, but this activity could be removed by absorption with liver powder. The above absorption decreased antimyelin titers from 1:≥ 256 to 1:64. In reference antisera used to measure CFI antibody 2 units of antigen and reference antiserum were contained in dilutions well beyond cross reactivity with liver. Reference antisera reacted strongly with myelin membranes when examined by indirect immunofluorescence (figure 2).

**Myelin Antibodies in Sera from Cases of Naturally Occurring Canine Distemper**

Serum from distemper infected dogs contained antibodies that are capable of reacting with CNS myelin in vitro (table 1). Twenty-one of 34 CD sera had CF titers ranging from 1:16 to 1:≥ 256, with a group mean titer of 1:62.5. CFI activity was detected in 12 of 34 CD sera and varied from 1:4 to 1:16 with a mean titer of 1:8. Ninety-seven percent of CD sera tested reacted to myelin in either CF or CFI. None of the sera examined showed simultaneous CF and CFI activity. In contrast, only 30% of sera from conventional normal dogs and 39% of gnotobiotic sera contained myelin antibodies. Moreover, mean CF titers in the control groups (1:8 and 1:4.2 respectively) were significantly lower (P< 0.005) when compared to the distemper group. CFI antibody was detected in only 2 of 20 conventional dogs and in none of the gnotobiotic sera.

Representative CD sera were chosen for further immunologic characterization on the basis of completeness of clinicopathologic records and amount of serum available for use. These cases represented the well established clinical spectrum of findings in neurological canine distem-
CD Serum Absorption

The nonneural tissue distribution of myelin membrane antigen(s) under investigation was evaluated by absorption experiments and additional direct complement-fixation tests (table 2). Myelin of rat origin reacted with all CF positive sera. In contrast, veronal buffer suspensions of canine liver and spleen, equivalent on a per weight basis to 6 antigenic units of myelin, reacted with only 2 of 7 sera. Absorption of CD sera with heterologous or homologous myelin decreased CF antibody titers 2 to 7-fold whereas absorption with homologous liver or spleen did not reduce antimyelin activity significantly (table 2).

CD Serum Fractionation

Further investigation of CD sera by anion exchange chromatography and gel filtration identified specific antibody activity in 2 immunoglobulin classes (table 3). CF antibody was eliminated by treatment with 2-mercaptoethanol and appeared only in the 19S peak after passage through a Sephadex G-200 column. CFI antibodies were found only in the 7S peak after gel filtration. 7S CFI activity was uncovered in several complement-fixing CD sera after separation of IgM and IgG immunoglobulin classes. Protein losses due chiefly to visible aggregation accounted for the marginal recovery of antibody activity in the eluates.

Indirect Immunofluorescence with CD Sera

The results obtained with indirect immunofluorescence correlated well with serological data (table 4). Three of 5 CD sera with CF titers above 1:16 gave myelin positive fluorescence. One of 4 CFI CD sera
(titer $\leq :16$) reacted with myelin in this test system. Cross and longi­tudinal sections of myelin reacted with specific antibody in an intense and homogenous manner (figure 2). Dull green background nuclear and cytoplasmic staining was easily distinguished from positively stained myelin. CD sera also reacted positively with air dried smears of isolated myelin; control sera were negative. A variety of commonly employed tissue fixatives (cold acetone, methanol, picric acid formaldehyde, and formalin) eliminated myelin specific fluorescence.

**Sera CF/CF1 Tests for Myelin Antibodies in Experimentally Produced Canine Distemper**

Weekly serum samples from 31 gnotobiotic dogs infected with R252-CDV along with 9 uninfected gnotobiotic controls were tested by CF/CF1. CF antibody predominated in all dogs tested; CF1 antibodies occasionally were detected late in the observation period in infected dogs only.

Throughout the 5 week survival period, mean antimyelin antibody titers in dogs of group I did not differ significantly ($P > 0.1$) from control levels (figure 3). Dogs of group II produced statistically significant ($P < 0.01$) antibody responses by post-infection day (PID) 21. Rising antibody titers at the time of sacrifice is also indicated ($P < 0.01$).

The serological response to myelin in group III dogs was surprising in view of the absence of neural lesions in these dogs. Highly significant ($P < 0.005$) antibody levels were evident on PID 21. Titers gradually declined toward control levels. This peak response is significantly greater ($P < 0.01$) than the peak response noted in group II.
DISCUSSION

Experiments reported here describe antibodies that bind to myelin in sera of dogs with CDV-induced demyelinating encephalomyelitis. Low levels of antibodies (<1:8) were also detected in control sera. In this regard, the findings of Edgington and Dalessio (11) in man are relevant. They reported low levels of IgG myelin binding antibodies (range 1:<4) in 88% of normal individuals. Since these "autoantibodies" appeared at the time of active myelination in the brain, they speculated that antibody appears in response to myelination. This conclusion is supported in the present study by noting that appearance of antibody coincides roughly with the time of CNS myelination in the dog.

Edgington also found that sera of all multiple sclerosis (MS) patients examined contained IgM antimyelin antibody in relatively high titer, analogous to the findings reported here. Other investigators have reported complement-fixing antibodies of the IgM class in EAE (3), MS (8) and Guillain-Barré syndrome (7). All 3 diseases are characterized by demyelination of a suspected immunologic nature.

Absorption of CD sera with several tissues including myelin indicates that the membrane antigen while present in the greatest amounts on myelin, may be a component of other plasma membranes. Preliminary quantitative absorption experiments (unpublished) indicate that this antigen, or one similar to it, is found in lymphoid tissues, especially thymus. In certain strains of mice, a shared brain/thymocyte antigen, designated theta antigen, is well documented (30).

The evidence that IgM is responsible for the complement-fixation observed is 3-fold: (i) CF activity is lost after mercaptoethanol
treatment, (ii) CF was seen only in the 19S peak of Sephadex G-200, and (iii) in the immunofluorescence experiments, only a FITC-conjugate with strong anti-canine IgM specificity was effective in demonstrating myelin binding antibodies in CD sera.

CFI antibody uncovered after fractionation appears to be IgG based upon its electrophoretic mobility in immunoelectrophoresis and behavior on DEAE cellulose. IgM mediated complement-fixation can obscure CFI antibody in whole serum (table 3). Thus, the type of antibody measured by the CF/CFI test depends upon which antibody class contains the most specific activity at the time of sampling.

Indirect immunofluorescence provided morphological conformation of the myelin binding capacity of these antibodies. This technique was relatively insensitive when compared to the serological test. Also, positive myelin staining was obtained only when a secondary reagent with strong anti-IgM activity was employed. Experiments using a secondary reagent with only anti-IgG specificity failed to demonstrate antimyelin antibody. Thus the choice of reagent was critical. Finally, preservation of tissue sections in a variety of fixations eliminated myelin specific immunofluorescence.

Study of experimentally infected gnotobiotic dogs provided additional pertinent information. Antibody response to myelin in the subacutely infected dogs of group I did not differ significantly from controls. An exact counterpart of this group is not represented in the naturally occurring cases of distemper as all dogs studied here had inflammatory cell infiltrates in areas of demyelination and presented a chronic clinical course of illness.
In contrast, dogs with inflammatory infiltrates associated with demyelination (group II) showed marked differences from controls. These dogs probably represent infected dogs who appear to recover from the acute respiratory and enteric forms of distemper and yet eventually die with demyelinating encephalitis at a later date (16). It appears that these dogs remain persistently infected, even though clinically asymptomatic. In this group, viral invasion and persistence in the CNS may provide the stimulus necessary to incite an anti-brain response.

No satisfactory explanation can be given for the serologic response observed in the dogs of group III. The paradox of a vigorous antmyelin response in the absence of demyelination is difficult to reconcile with information available at this time. Short term viral replication in the brain, terminated by the onset of active anti-viral immunity, may expose myelin to invading immunocompetent cells with resultant antibody production. Alternatively, the previously noted lymphoid tissue-myelin cross antigenicity may provide the necessary antigenic stimulus for the response in the absence of viral persistence in the brain.

The possibility that the antigen involved is a normal constituent of all plasma membranes and degree of specificity observed with myelin represents a fortuitous concentration of antigen on this membrane cannot be excluded at this time. Further sequential pathogenesis studies are necessary to resolve these points.

Data from these dogs indicates another possible effect of CDV on susceptible dogs, that of immunosuppression. Immunosuppressive effects have been well documented for measles and rinderpest viruses, which are other members of the paramyxovirus group (27,29). It appears that based
on this magnitude of humoral antibody response, infected dogs of group I (figure 3) are the most severely compromised, while those of group III show the least evidence of depressed humoral immune responsiveness. Group II dogs occupy the intermediate position between these extremes. This impression is further strengthened by noting that cellular infiltrates in the brain usually associated with immunocompetence (i.e., lymphocytes and plasma cells) are not a feature of disease in group I, but are a prominent histological finding in the demyelinating lesions of group II. The role of specifically sensitized lymphocytes (i.e., cell-mediated immunity) in distemper associated demyelination has not been investigated. Morphological changes in lymphoid tissues induced by R252-CDV, are described elsewhere (22). Experiments designed to investigate viral effects on both cellular and humoral immunocompetence are in progress with particular emphasis on how this may relate to chronic infectious neurological disease.

The precise role of these antibodies in the etiology of demyelinating disease is still unknown. The demonstration of CNS-bound immunoglobulin in demyelinating lesions of CDV infected dogs (19), provides tentative support for an active role in the disease process. We presently believe that these antibodies are contributing factors in the pathogenesis of demyelination associated with CDV and serve to amplify the basic lesion of noninflammatory virus-induced demyelination in the brain.
SUMMARY

Sera from dogs with spontaneously occurring and experimentally produced canine distemper virus associated demyelinating encephalitis were examined for antibodies to CNS myelin by the complement fixation (CF) and indirect immunofluorescent methods. Complement-fixing IgM antibodies and non-complement-fixing IgG antibodies were found in 97% of the spontaneous cases. In comparison, only 28% of control sera contained these antibodies; further, mean antibody titers in the control groups were significantly lower (P<0.005) when compared to the distemper group. Complement-fixing antimyelin antibodies were also demonstrated in gnotobiotic dogs with experimentally induced distemper virus associated demyelination. The antibody response could be correlated with clinico-pathological features of the disease produced. Results of this study indicate that demyelination in canine distemper may proceed by immune mechanisms.
Table 1. Distribution of Myelin Antibody Titers in Naturally Infected Canine Distemper (CD) Dogs and Control dogs.

<table>
<thead>
<tr>
<th>Source of Test Serum</th>
<th>Total Number Test Serum</th>
<th>Complement-Fixation (CF) Titer</th>
<th>Complement-Fixation Inhibition (CFI) Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;1:2 1:2 1:4 1:8 1:16-64 ≥1:128</td>
<td>1:4 1:8 1:16</td>
</tr>
<tr>
<td>CD Infected Dogs</td>
<td>34</td>
<td>13^a 0 0 0 16 5</td>
<td>4 5 3</td>
</tr>
<tr>
<td>Normal Vaccinated Conventional Dogs</td>
<td>20</td>
<td>16 0 2 1 1^b 0</td>
<td>2 0 0</td>
</tr>
<tr>
<td>Normal Gnotobiotic Dogs</td>
<td>33</td>
<td>20 5 5 3 0 0</td>
<td>0 0 0</td>
</tr>
</tbody>
</table>

^a 12 of 13 sera had CFI titers of 1:4 to 1:16 as indicated in right hand column.

^b Titer 1:16.
Table 2. Organ Specificity of Myelin Antibodies in Canine Distemper (CD) Sera; Evaluation by Tissue Absorption

<table>
<thead>
<tr>
<th>CD Serum Number</th>
<th>Myelin&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Myelin&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Liver&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Spleen&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7</td>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5,6,7</td>
<td>4 (3)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3 (3)</td>
<td>0 (3)</td>
<td>0 (3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> 1 CF antibody unit defined as 1 log<sub>2</sub> dilution of CD sera.

<sup>b</sup> Absorbed with 2.5 mg tissue powder/1.0 ml CD serum.

<sup>c</sup> Dog origin.

<sup>d</sup> Rat origin.

<sup>e</sup> 2 of 7 sera reacted with canine liver and spleen powders in veronal buffer, equivalent to 6 units of myelin antigen on a weight basis, with titers of 1:8 and 1:32 (liver) and 1:8 and 1:4 (spleen) respectively.

<sup>f</sup> No. of sera tested.
Table 3. Fractionation of Myelin Antibodies in Canine Distemper (CD) Sera by Gel Filtration and Anion Exchange Chromatography.

<table>
<thead>
<tr>
<th>CD Serum Number</th>
<th>Unfractionated Antibody Titer</th>
<th>Antibody Titer after Sephadex G-200 Gel Filtration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Antibody Titer after DEAE Chromatography&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peak I (19S)</td>
<td>Peak II (7S)</td>
</tr>
<tr>
<td></td>
<td>CF</td>
<td>CF1</td>
<td>CF</td>
</tr>
<tr>
<td>1</td>
<td>1:≥256</td>
<td>&lt;1:2</td>
<td>1:16</td>
</tr>
<tr>
<td>2</td>
<td>1:128</td>
<td>&lt;1:2</td>
<td>1:8</td>
</tr>
<tr>
<td>3</td>
<td>1:64</td>
<td>&lt;1:2</td>
<td>1:2</td>
</tr>
<tr>
<td>4</td>
<td>1:32</td>
<td>&lt;1:2</td>
<td>1:4</td>
</tr>
<tr>
<td>5</td>
<td>&lt;1:2</td>
<td>1:16</td>
<td>&lt;1:2</td>
</tr>
<tr>
<td>6, 7, 8</td>
<td>1:16(3)</td>
<td>&lt;1:2(3)</td>
<td>&lt;1:2(3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sephadex (Pharmacia) - equilibrating buffer: 0.4M NaCl in 0.01M phosphate buffer, pH 7.2.

<sup>b</sup> DEAE (Sigma) - starting buffer: 0.05M NaCl in phosphate buffer, pH 8.0; limiting buffer: 0.6M NaCl in phosphate buffer, pH 8.0.

<sup>c</sup> Serum fraction containing IgM after elution with the limiting buffer.

<sup>d</sup> One of 3 fractions tested had a CF titer of 1:4.

<sup>e</sup> Number of sera tested.
Table 4. Comparison of the Complement-fixation Test and Indirect Immunofluorescence for Myelin Antibodies in Canine Distemper (CD) Sera and Controls.

<table>
<thead>
<tr>
<th>CD Serum Number</th>
<th>Complement-Fixation (CF) Titer</th>
<th>Complement-Fixation Inhibition (CFI) Titer</th>
<th>Myelin Specific Immunofluorescencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\geq 1:256$</td>
<td>$&lt; 1:2$</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>1:128</td>
<td>$&lt; 1:2$</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>1:64</td>
<td>$&lt; 1:2$</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>1:32</td>
<td>$&lt; 1:2$</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1:16</td>
<td>$&lt; 1:2$</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>$&lt; 1:2$</td>
<td>1:16</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>$&lt; 1:2$</td>
<td>1:16</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>$&lt; 1:2$</td>
<td>1:8</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>$&lt; 1:2$</td>
<td>1:4</td>
<td>-</td>
</tr>
<tr>
<td>Controls (7)b</td>
<td>$&lt; 1:2$ (7)</td>
<td>$&lt; 1:2$ (7)</td>
<td>- (7)</td>
</tr>
</tbody>
</table>

a Sera prediluted 1:10 in 3% rhodamine albumin (Difco) counterstain.

b Number of sera tested.
Figure 1. Electron micrograph of CNS myelin (x 57,500).
Figure 2. A stained cross section of canine spinal cord that demonstrates myelin-specific immunofluorescence by the indirect method. Non-myelin tissues stained nonspecifically (arrows) only with rhodamine-albumin counterstain (x 520). Insert: immunofluorescent positive isolated CNS myelin (x 840).
Figure 3. Mean antimyelin antibody titers in gnotobiotic dogs infected with R252-CDV versus mean antibody titers of 9 control dogs. Criteria for group separation are given in the text.
Fig. 3
CHAPTER II

SEROLOGICAL RESPONSE TO CANINE DISTEMPER VIRAL ANTIGENS
IN GNOTOBIOTIC DOGS INFECTED WITH R252-CANINE DISTEMPER VIRUS

Introduction

Canine distemper virus (CDV) infection is the most common disease affecting dogs. It is a pantropic infection in that the virus replicates readily in epithelial, lymphoid and nervous tissues (1). Although the clinical signs of active infection in dogs are well recognized and an effective vaccine is available, little is known about the factors which limit CDV infection in some susceptible animals and permit fatal encephalitis in others.

Previous publications from this laboratory have described the isolation and in vitro characteristics of a CDV isolate designated R252-CDV and the clinical and pathological features of the disease in gnotobiotic dogs (9,10,11 and McCullough, B., Krakowka, S., Koestner, A. and Shadduck, J., J. Inf. Diseases, in press). Salient features which tended to distinguish the clinical picture in gnotobiotic dogs infected with R252-CDV from that of Snyder Hill (SH-CDV) were: 30% fatality vs. 100% fatality with SH-CDV, lymphopenia of 6-7 weeks duration in clinically recovered pups, and relatively high incidence of central nervous system demyelination with R252-CDV. In those studies, serum neutralizing (SN) antibodies were present in R252-CDV infected dogs showing chronic demyelinating encephalitis.
This observation suggested that the presence of antiviral antibodies in
CDV-infected dogs cannot always be interpreted as a sign of recovery
from infection.

The objective of the present study was to further explore the
development of antivirus antibodies in R252-CDV infected gnotobiotic
dogs for their predictive value on the course of the disease. A second
objective was to characterize the viral antigens involved by thermal
stability, lipid solvent extraction and serologic cross reactivity with
measles virus and canine brain tissue.

Both serum neutralization (SN) and complement-fixation (CF) tests
were used. Other workers have shown that CF antibodies to CDV are found
for only 1-3 months after infection whereas SN antibodies persist for
much longer (4,5).

In contrast, we have found that CF was as sensitive and reliable as
SN. Of these two tests, CF was easier to perform and more adaptable to
testing serial samples from many infected dogs. Further, by CF, it was
possible to detect differences between immune and persistently infected
dogs in immunoreactivity to CDV internal and envelope antigens.

**Materials and Methods**

**Experimental Canine Sera**

Preinoculation and weekly samples to 12 weeks postinfection (PI)
from 25 R252 canine distemper virus (CDV)-infected and 5 uninfected con-
tral gnotobiotic dogs were used. Details concerning methods of viral in-
oculation, sample collection, and clinical and pathological evaluations
have been published (9,10).
Viruses

The origin and passage history of the canine distemper viruses used in this investigation have been described previously (9,10,13). R252-CDV was originally recovered from the brain of an infected gnotobiotic dog by co-cultivation of primary cultures of cerebellum with African green monkey kidney (Vero) cells.

Vero cell-adapted Onderstepoort strain (Ond-CDV) originally obtained from Dr. M. J. G. Appel, has been maintained in this laboratory for 3 years.

Edmonston strain of measles virus (MV) was purchased from Flow Laboratories, Rockville, Maryland. The virus, (Lot No. M944040) after passage through several established human cell lines, was passaged once in Vero cells and supplied in 5.0 ml frozen aliquots without pre-treatment with Tween-80 and ether.

Cell Culture

A cell culture line of African green monkey kidney (Vero) cells was used. Details concerning passage history have been described (13). Cells were maintained in Eagle's minimal essential medium (MEM) with 3% bovine fetal serum, 1% NaHCO₃ (8.8% w/v), and 1% antibiotics (penicillin 20,000 units/ml; streptomycin, 10 mg/ml; and nystatin, 5000 units/ml).

Virus Neutralization

Vero cells were seeded onto 16 mm plastic disposable tissue culture plates (Linbro Chemical Co., New Haven, Conn.) at a concentration of 1.8 x 10⁵ cells/well. Sera were diluted 4-fold in MEM. 0.025 ml
of serum samples were then mixed with an equal volume of Ond-CDV suspension containing 100 TCID₅₀ and incubated at 37°C for 1 hour. One-tenth ml of the mixtures were then inoculated onto the previously prepared Vero cell suspensions using 4 wells per dilution of serum. Neutralizing capacity of the sera was determined by inhibition of Ond-induced cytopathic effect, i.e. inhibition of giant cell formation after 3 days incubation 37°C, 10% CO₂.

Complement enhanced neutralizations on sera obtained 3 weeks post infection (PI) and 12 weeks PI were performed by the above procedure except that the sera were diluted 4-fold in MEM containing sterile 10% reconstituted guinea pig complement.* Twenty-four hours after inoculation of the virus-antibody mixtures onto Vero cells, the medium was replaced by MEM without guinea pig complement. Neutralization endpoints were determined as in the regular neutralization assays. Controls for nonspecific toxic effects of sera on monolayers, virus alone and complement and virus together were included with each serum tested. In addition positive (hyperimmune anti-CDV serum) and negative (uninoculated control serum) sera were tested periodically to insure sensitivity and specificity of the procedures.

Preparation of CF Antigens and the Complement-Fixation Test

Roller bottle cultures were seeded with Vero cells and maintained to confluency at 37°C, 10% CO₂. Three thousand TCID₅₀ doses of R252-CDV in 10 ml of medium was added and allowed to adsorb to the monolayer for

* Baltimore Biological Laboratories, Cockeysville, Md.
1 hour at 37°C before the addition of 90 ml maintenance media. The supernatant was harvested and replaced with fresh MEM daily. After 5 days when CPE was generalized, infected cells were scraped from the glass, pooled with supernatant medium, and disrupted by 3 cycles of freeze-thawing. The resultant material after low speed centrifugation (3000XG) was concentrated 10-fold by forced dialysis against polyethylene glycol, reclarified by centrifugation and stored in 1.0 ml aliquots at -70°C until use in the CF test.

Complement-fixing antigen from Ond-CDV infected roller bottles infected with $3 \times 10^4$ TCID$_{50}$/ml was prepared through a 4-day harvest schedule in a similar manner. Measles virus antigen was used as supplied by the manufacturer.

All CF tests were performed in plastic disposable micro-test plates (Cooke Engineering, Alexandria, Virginia) as described (12). In order to validly compare results, all reagents were standardized in preliminary titration experiments. Block titrations of 3 different reference antisera were made against the 3 different viral antigens used (eg. Ond-CDV, R252-CDV, and MV). These experiments were repeated using 2, 3 and 4 CH$_{50}$ hemolytic complement units of guinea pig origin.

Plates with antigen, antibody and complement were incubated at 37°C for 1 hour. Sensitized sheep red blood cells (SRBCs) were then added and the plates incubated at 37°C for 30 minutes. Absence of lysis of SRBCs indicated that a viral antigen-antibody complement-fixing reaction had occurred. One CF antigen-antibody unit was defined as that final dilution of serum or antigen that permitted less than 50% lysis of indicator SRBCs. Negative controls for anti-complementary activity of test sera
and antigens as well as positive lytic controls for complement and SRBCs together were included in every test.

**Characterization of the CF Antigens**

The thermal stability of the MV and CDV antigens involved in the reaction was examined by heat inactivation experiments. Equal volumes of CF antigen were incubated in a waterbath at 4°C, 37°C, and 56°C. Aliquots were removed at various intervals and titrated for residual activity with reference antibody.

Viral concentrates were mixed with an equal volume of reagent grade ether and 1/20 volume of 0.1% aqueous Tween-80. The mixture was shaken for 2 hours at room temperature (8). The aqueous phase was titrated for antigenic activity by complement-fixation. Heat inactivation experiments were also conducted on the disrupted antigen.

Cross reaction with CNS myelin in convalescent sera was examined by absorption experiments. Sera were absorbed with 2.5 mg lyophilized CNS myelin per ml of serum for 12 hours at 4°C. Absorbed and unabsorbed sera were tested for CDV antibodies and CNS myelin antibodies (12). These dogs had been previously immunized with keyhole limpet hemocyanin (KLH). Complement-fixing antibodies to KLH were determined before and after absorption as a control for the absorption procedure.

**Results**

**Clinicopathological Features of R252-CDV in Gnotobiotic Dogs:**

In gnotobiotic dogs, as in conventional dogs, infection with virulent canine distemper virus results in a spectrum of illness ranging
from asymptomatic infection to fulminant fatal encephalitis. In general, 3 degrees of involvement can be distinguished and have been described in detail by McCullough, et al. (9,10).

Gnotobiotic dogs of Group 1 develop acute encephalitis 15-40 days after infection. They exhibit lesions of thymic atrophy, systemic lymphoid depletion and focal central nervous system (CNS) involvement characterized by minimal or absent mononuclear inflammatory cell infiltrates. Dogs of Group 2 in contrast are characterized by chronic encephalitis with a clinical cause of at least 3 months duration. Lymphoid depletion is not an outstanding feature at necropsy. Central nervous system demyelination is often extensive and characterized by prominent inflammatory cell infiltrates. These animals remain persistently infected with CDV throughout the experiment. Dogs of Group 3, except for a transient lymphopenia, do not develop overt disease during the observation period, have no lesions of active distemper infection at necropsy and are considered to be recovered immune animals.

**Serum Neutralizing and Complement-Fixation Test for Distemper Antibodies:**

Sera from 5 representative dogs from each of the 3 groups were tested for viral antibodies by serum neutralization (SN). The intervals tested were: pre-inoculation samples and 2, 6 and 12 weeks postinfection (PI). The results are illustrated in figure 4. Antibody titers varied inversely with severity of disease. Dogs of Group 1 affected fatally with R252-CDV had little or no neutralizing antibody in their serum. Immune dogs of Group 3 produced the highest antibody titers. Persistent-
ly infected dogs of Group 2 were intermediate in antibody response.

The complement-fixation (CF) test was employed to further study these antibodies. All sera were tested with 2 units of viral antigen and 2 hemolytic units of guinea pig complement \( (\text{CH}_50) \). The effect of varying amounts of guinea pig complement on measurable anti-CDV antibodies in 1 immune dog is shown in figure 5. Two \( \text{CH}_50 \) units of complement was the most sensitive in demonstrating seroconversion. Twice that amount (eg. 4 \( \text{CH}_50 \) units) completely obscured the reaction between antibody and antigen.

Weekly serum samples from 25 gnotobiotic dogs were tested by CF with one lot of titrated R252-CDV antigen. Results given in figure 6 were consistent with those seen by serum neutralization. The 12 immune dogs of Group 3 demonstrated a vigorous antibody response which was first detected 2 weeks after infection and was maximal 4-5 weeks after infection. Titers persisted at this level throughout the observation period. Mean antibody titers in 8 persistently infected dogs of Group 2 appeared 3-4 weeks after infection and remained about half the level as that of immune dogs. Five fatally infected dogs of Group 1 contained little or no antibody in their serum. No difference in antibody titers was seen when these sera were retested with 2 units of Ond-CDV antigen.

Heterologous reaction with measles virus (MV) was examined by CF tests using 2 units of titrated MV. Results are summarized in figure 7. Antibody titers measured with MV were 2 to 4-fold lower than corresponding values obtained with homologous CDV antigen. Although the same pattern of group responses that were seen with CDV was evident with MV differences in mean titers between immune (Group 3) and persistently in-
fected dogs (Group 2) were minimal.

Qualitative differences in virus-neutralizing capacity between groups of dogs were demonstrated by complement-enhanced neutralization. The results are given in Table 5. Neutralizing titers of sera obtained 3 weeks after infection were increased 2 to 4-fold in both groups when exogenous complement was included in the reaction mixture. Neutralizing titers in sera 12 weeks PI were increased only 1 to 2-fold in Group 2 dogs. In contrast, exogenous complement resulted in a 2.5 to 3.5 increase in SN titer of 12 week PI sera from immune animals.

Preliminary Characterization of Viral Antigen(s):

In a third series of experiments, the heat labile nature of the CF antigens as measured by titration with reference antibody was investigated. Results are summarized in Table 6. At 56°C, most of the antigenicity in crude CDV preparations is lost within 1 hour. A small portion is, however, retained as intact antigen for at least 3 hours. Heat inactivation at 37°C shows a similar but less precipitous decline in antigen titer. Maintenance of CDV at 4°C for 3 hours did not affect CF titers. Under similar test conditions, a parallel decline in MV antigenic titer following heat treatment was not seen.

Treatment of MV with Tween-80 and ether (T-80E) results in disintegration of the viral particles, loss of infectivity, and release of both internal core antigen and MV-hemagglutinin (8). The effect of these agents on antigenicity of the various viral preparations was examined and results are summarized in Table 7. This reagent caused a significant decrease in CDV antigen titers. Measles virus CF antigen was unaffected by
treatment. When compared to untreated antigen, T-80E-disrupted CDV was heat resistant as no further loss of titer was seen when this preparation was heated at 56C for 3 hours (Table 6). Despite the drop in antigen titer in the aqueous phase following T-80E treatment, further decline in antigenicity was heat resistant. This suggests that 2 types of antigens are involved in the CF reaction. The first is lipid-like in that it is heat labile and solubilized with T-80E. The second antigen is heat stable, and ether resistant. It probably represents the internal viral component which cross reacts with MV.

Complement-Fixing Antibodies to Tween-80 Ether-Extracted CDV Antigen

Four ml of crude antigen was divided into 2 ml aliquots and 1 aliquot was subsequently treated with T-80E. The resultant 2 ml aqueous phase was used along with untreated CDV as antigens in parallel antibody titration experiments. The results are given in Table 8. Disrupted CF antigen always gave lower antibody titers than did the untreated antigen. A 4-fold decline in antibody activity was seen in Group 2 sera. In contrast, use of T-80E-disrupted antigen resulted in a 4 to 32-fold loss of antibody titer in immune Group 3 sera.

Serologic Cross Reactivity Between Viral Antigens and Canine Central Nervous System (CNS) Myelin

Characterization of CF antiviral antibodies facilitated the search for evidence of serological cross reactivity between viral antigens and canine central nervous system (CNS) myelin.
Five convalescent sera taken from R252-CDV infected dogs previously immunized with keyhole limpet hemocyanin (KLH) were tested for anti-myelin and CDV antibodies, absorbed with lyophilized CNS myelin, and re-tested. Absorption with myelin removed significant amounts of anti-myelin activity. Antibody titers to CDV and KLH were relatively unaffected (Table 9).

In addition, 3 hyperimmune canine antimyelin sera and 3 hyperimmune canine anti-vaccine CDV sera were tested for the simultaneous presence of antimyelin and antiviral antibodies by CF and SN. Only homologous antibodies were detected (Table 10).

Discussion

Canine distemper virus (CDV) infection in gnotobiotic dogs has been described as a mild infection (16). Few if any clinically detectable alterations are detectable in the incubation stages of the disease. Nonetheless, mortality rates seen in gnotobiotic dogs approach that seen with CDV infection in conventional animals.

In our experience (unpublished data), approximately 30% of gnotobiotics infected with the viral isolate, R252-CDV, die of encephalitis. In the past, this variation in clinical picture in conventional dogs was in part explained by debilitation and intercurrent infection with opportunistic pathogens (1). Since the pattern is evident in gnotobiotic dogs devoid of potentially harmful pathogens, other explanations for this variability in clinical picture must be sought. If the environment can be discarded as a major influence on the course of disease, 3 broad factors may remain to be considered: i) variability due to subpopulations
of virulent virus in the inoculum or following passage through suscep-
tible dogs, ii) variability due to the intrinsic susceptibility of indi-
vidual dogs, iii) combinations of the above two. That subpopulations of
CDV differing not in antigenicity, but in biological behavior exist has
been suggested by some (2), demonstrated in vivo by others (17); and
shown in vitro in our laboratory (13). This factor can be appreciated
by comparing fatality rates seen when susceptible dogs are given avirulent
vaccine virus or neurovirulent Snyder-Hill virus.

Undoubtedly dogs vary in their genetic susceptibility to CDV but
exploration of this phenomenon is frustrated by the lack of identifying
characteristics which distinguish these animals. In inbred experimental
laboratory animal systems, immunological responsiveness to chemically de-

dined antigens appears to be determined by simple Mendelian genetics
(18,19). In man, an outbred system, susceptibility or predisposition to
certain diseases are being linked to restricted histocompatibility types
which, in turn, have a genetic basis. Undoubtedly, a parallel situation
exists in the canine population with regard to general susceptibility to
distemper. The products of an intact immune response reflect not only
the genetic makeup of the individual animal but also the cumulative
effects of secondary factors such as direct interaction between the
virus and immunocytes. In this regard, we have shown that distemper
virus infection has a direct effect on the ability of peripheral blood
lymphocytes to respond to mitogenic stimulation (S. Krakowka et al.,
manuscript in preparation). Despite the inability to separate primary
(genetic) factors from secondary modifying influences in our outbred
system, humoral antibody response to viral antigens in CDV-infected gno-
tobiotic dogs may have predictive value and was therefore investigated in the present study.

Our experiments have shown that both the serum neutralizing and CF tests will differentiate immune (Group 3) dogs from acutely infected (Group 1) dogs 2-3 weeks after infection on the magnitude (titer) of anti-CDV antibodies. This represents a marginal improvement on clinical observations since these animals die 3-5 weeks after infection. Persistently infected (Group 2) animals present a different and even more complex prognostic problem. In this instance neutralizing and CF antibodies are found 3 weeks after infection. It is clear from our experiments that mean titers in this group are lower than those of immune animals but only marginally so early in infection. We were unable to determine precisely an early (3 weeks PI) antibody titer that reliably separated Group 3 from Group 2 dogs.

The morphological effects of CDV on the canine lymphoid system, e.g. cytolytic necrosis, giant cell formation, and resultant lymphoid depletion have been described repeatedly (1,9,10,11,16). This direct viral effect on immunocytes may have not only quantitative (i.e. magnitude of response as reflected in titer) effects but also may result in qualitative deficiencies in the range and specificity of anti-CDV immunoglobulins produced. Despite the inherent limitations of the CF test as a secondary test, (e.g. dependent upon events following primary antigen binding) evidence was found to support this concept. This hypothesis was developed from results obtained when measles virus (MV) was used as test antigen.

MV and CDV share common core antigen which is not associated with neutralizing sites on the virion (1,3,6,7,8). If the only difference be-
tween Group 2 and Group 3 was in magnitude of response, then titer differences with MV between the groups would be as great as that seen in the homologous (CDV) system. This was not the case. Both groups responded to approximately the same degree against the shared core antigen. The vigorous response to envelope determinants in addition to the core determinants distinguishes immune from persistently infected dogs.

This same phenomenon was evident when complement was tested as a promoter of viral neutralization. Antibodies from persistently infected Group 2 dogs were less able to activate complement during neutralization than were antibodies from immune dogs. That no differences were seen shortly after infection could be attributed to the predominance of complement-fixing IgM neutralizing antibodies in these sera. Although no experimental evidence was sought, it may be that the orderly switch from IgM to IgG or other defects in IgG production occur in CDV-infected dogs and that this could account for the poor neutralizing capacity of sera obtained 12 weeks after infection. Thus, qualitative defects in SN antibodies in Group 2 dogs may contribute to the virulence of CDV in these dogs.

Treatment of crude viral preparations with Tween-80 and ether disrupts the virion and partitions envelope lipids in the nonaqueous phase (8). Accordingly, the aqueous phase should contain predominantly non-lipid core antigens. Antigenicity of MV was unchanged following disruption confirming that envelope antigens were not involved in the heterologous reaction. In contrast, a significant decline in antigenic titer was seen with CDV antigens following treatment. This, along with the heat inactivation data suggests that 2 types of viral antigens contribute
to the antigenic titers obtained in these experiments. The first antigen is lipid-like in that it is heat labile and solubilized with T-80E. The second antigen, being heat stable and ether resistant probably represents the internal viral component which cross reacts with MV.

When the disrupted aqueous phase CDV was used as antigen and tested with representative sera from the Groups 2 and 3, a difference in loss of titer was evident. More activity was "lost" when the antigen was titrated with immune sera (Group 3) than with sera from infected dogs (Group 2). These results again emphasize the ability of immune dogs to produce antibodies reactive with envelope antigen.

One of the features of canine distemper infection that is of great comparative medical importance is the pathogenetic mechanisms involved in the demyelinating form of encephalitis seen with this disease. Viral, immunological and host factors must be considered in investigation of this phenomenon. We have shown that sera from CDV-infected dogs contain antibodies that react with CNS myelin and that appearance of these antibodies are correlated with development of disease (12). Although the stimulus for this production and in vivo effects are unknown, their in vitro myelinotoxic ability suggests a pathogenic role for these antibodies in CNS demyelination. Since their pattern of development coincides with antiviral responses it was possible that antibodies of both antimyelin and antiviral specificity in fact reflect antigenic overlap between viral and CNS determinants. Cross reaction with MV and CNS myelin basic protein has been reported by others (15). The results of absorption and cross neutralization experiments indicate that the antigenic stimuli for antibody production in these dogs are different and
that the appearance of antiviral and antimyelin antibodies in serum are parallel but unrelated events.

In summary, our results have shown that the CF test is a reliable serological tool to use in the exploration of the immune response of dogs to CDV. Complement-fixing antibodies are produced against both internal and external antigens and a detectable CF response should persist for longer periods of time than has been indicated in the literature. Immune dogs can be distinguished from persistently and acutely infected dogs not only by titers but also by differences in reaction with envelope and core antigens. Further investigation of these apparent differences should determine if they are a consequence of chronic infection with CDV or in fact reflect subtle genetic deficiencies which originally encouraged the establishment of persistent infection with CDV.
Summary

The humoral immune response to canine distemper virus (CDV) antigens in experimentally CDV-infected gnotobiotic dogs was studied by the complement-fixation and serum neutralization tests. It was found that antibody titers obtained in both serologic methods varied inversely with the severity of disease produced. Immune dogs demonstrated the highest antibody titers whereas fatally infected dogs show little or no antibody activity in their serum. A third group of dogs characterized by chronic persistent infection had intermediate anti-CDV values.

Preliminary characterization of the viral antigens involved in the complement-fixation test indicated that at least two antigenic components were involved. One antigen was soluble in ether and heat labile while the other was relatively heat stable and unaffected by ether treatment. No evidence for serologic cross reaction between viral and CNS components was found.

A vigorous antibody response to envelope antigen determinants in addition to core determinants distinguished immune from persistently infected dogs. The results of this study suggest that the inability to produce antibodies to these envelope antigens may be a crucial factor in the establishment of a persistent CDV infection in these dogs.
Table 5. The Effect of Complement on Serum Neutralizing Antibody Titers in R252-Canine Distemper Virus (CDV) Infected Gnotobiotic Dogs.

<table>
<thead>
<tr>
<th>R252-CDV Infected Dogs</th>
<th>Post-Infection Week 3</th>
<th>Post-Infection Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Complement</td>
<td>With Complement</td>
</tr>
<tr>
<td>Group 2 Dogs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Persistently Infected)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Group 3 Dogs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Immune)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.0</td>
<td>8.0 (2.0)</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>6.0 (1.0)</td>
</tr>
<tr>
<td>3</td>
<td>&lt;4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>4.0</td>
<td>8.0 (4.0)</td>
</tr>
</tbody>
</table>

a 1 part reconstituted guinea pig complement (Becton, Dickinson and Co., Cockeysville, Md.) to 9 parts tissue culture medium.

b Expressed as log₂ antibody titer.

c Log₂ increase in antibody titer in the presence of guinea pig complement.
Table 6. The Effect of Heat on Complement-Fixing Antigen Titers\textsuperscript{a,b} of Untreated Tween-80 and Ether disrupted R252-CDV Antigens.

<table>
<thead>
<tr>
<th>Time of Inactivation (Minutes)</th>
<th>Untreated R252-CDV Antigen</th>
<th>T-80E Treated\textsuperscript{c} R252-CDV Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textsuperscript{4C}</td>
<td>\textsuperscript{37C}</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>45</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>120</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>180</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} One antigenic unit defined as one log\textsubscript{2} dilution.

\textsuperscript{b} Antigen titer determined with one lot of undiluted reference anti R252-CDV antiserum.

\textsuperscript{c} Antigen mixed with equal volume of reagent grade ether and 1/20 volume of 0.1% aqueous Tween 80.
Table 7. The Effect of Tween-80 and Ether Disruption on Various Viral Antigen Titers Determined by Titration with Reference Anti-R252-CDV Antiserum.

<table>
<thead>
<tr>
<th>Viral Antigen</th>
<th>Tween-80 and Ether Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>R252-CDV</td>
<td>8³</td>
</tr>
<tr>
<td>Ond-CDV</td>
<td>8</td>
</tr>
<tr>
<td>MV</td>
<td>3</td>
</tr>
</tbody>
</table>

³ Titer expressed as log₂ dilution.
Table 8. The Effect of Disruption of R252-CDV Antigen with Tween-80 and Ether on Antibody Titers\textsuperscript{a}

In Persistently Infected (Group 2) and Immune (Group 3) Gnotobiotic Dogs

<table>
<thead>
<tr>
<th>Tween-80 and Ether Extraction of R252-CDV Antigen</th>
<th>Persistently Infected Dogs (Group 2)</th>
<th>Immune Dogs (Group 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4</td>
<td>1  2  3  4  5</td>
</tr>
<tr>
<td>Before Extraction</td>
<td>5\textsuperscript{b}  4  4  2</td>
<td>8  8  5  5  5</td>
</tr>
<tr>
<td>After Extraction</td>
<td>3  2  2  0</td>
<td>4  5  0  2  3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Serum samples tested were drawn 12 weeks after infection.

\textsuperscript{b} Antibody titers are expressed as log\textsubscript{2} of dilution.
Table 9. Serological Cross Reactivity Between CNS Myelin and Tissue Culture Origin R252-CDV.

Evaluation by CNS Myelin Absorption.

<table>
<thead>
<tr>
<th>R252-CDV Infected Dogs</th>
<th>Loss of complement-fixing antibody units(^a) to various antigens after absorption(^b) with canine CNS myelin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CNS Myelin</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) One complement-fixing antibody unit defined as \(1 \log_2\) dilution of serum.

\(^b\) Absorbed with 2.5 mg of lyophilized canine CNS myelin per 1.0 ml of serum.

\(^c\) Keyhole Limpet Hemocyanin.

\(^d\) KLH was employed as an internal control to ensure against nonspecific loss of titer due to the absorption procedure.
Table 10. Comparison of Complement-Fixing and Serum Neutralizing Antibody Titers\textsuperscript{a} in Gnotobiotic Dogs Hyperimmunized with Either CNS Myelin\textsuperscript{b} or Canine Distemper Virus\textsuperscript{c}.

<table>
<thead>
<tr>
<th>Sera</th>
<th>Complement-Fixing Antibody Titer to: \begin{tabular}{c} CNS Myelin \ R252-CDV \end{tabular}</th>
<th>Serum Neutralizing Antibody Titer to CDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimyelin Sera</td>
<td>\begin{tabular}{ccc} 1  &amp; 5  &amp; &lt;1  \ 2  &amp; 7  &amp; &lt;1  \ 3  &amp; 4  &amp; &lt;1  \ \end{tabular}</td>
<td>\begin{tabular}{c} 1  \ 1  \ 1  \ \end{tabular}</td>
</tr>
<tr>
<td>Anti-CDV Sera</td>
<td>\begin{tabular}{ccc} 1  &amp; &lt;1  &amp; 7  \ 2  &amp; &lt;1  &amp; 7  \ 3  &amp; &lt;1  &amp; 5  \ \end{tabular}</td>
<td>\begin{tabular}{c} 10  \ 10  \ 9  \ \end{tabular}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Expressed as \( \log_2 \) antibody titer.

\textsuperscript{b} Six bi-monthly injections of 10.0 mg lyophilized myelin in incomplete and complete Freund's adjuvant.

\textsuperscript{c} Multiple injections of D-VAC vaccine (Bio-Ceutic Laboratories, Inc., St. Joseph, Mo.).
Figure 4. Serum neutralizing antibody titers to canine distemper virus (CDV) in R252-CDV infected gnotobiotic dogs.
Fig. 4

Log$_2$ Antibody Titer

Weeks after infection

Group 1

Mean ± SE

Group 2

Group 3

12
6
2
0
-2
-4
-6
0 3 6 9

Fig. 4
Figure 5. Effect of varying amounts of guinea pig complement (GPC) on complement-fixing antibody titers to R252-canine distemper virus antigen.
Fig. 5
Figure 6. Complement-fixing antibody titers to R252 canine distemper virus (CDV) antigen in CDV-infected gnotobiotic dogs.
Fig. 6

MEAN±SE

• GROUP 1

•• GROUP 2

○○ GROUP 3

LOG₂ ANTIBODY TITER

WEEKS AFTER INFECTION

Fig. 6
Figure 7. Complement-fixing antibody titers to measles virus antigen in R252 canine distemper virus infected gnotobiotic dogs.
Fig. 7

- **GROUP 1**
- **GROUP 2**
- **GROUP 3**

**MEAN ± SE**

**LOG2 ANTIBODY TITER**

**WEEKS AFTER INFECTION**
CHAPTER III

EFFECTS OF CANINE DISTEMPER VIRUS INFECTION ON LYMPHOID FUNCTION: IN VITRO AND IN VIVO CORRELATES

Introduction

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) immunodeficiency, primary or acquired. With other viruses such as murine and avian oncogenic RNA 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 following antigenic challenge (11,30).

The suppressive effects of measles virus (MV) on in vitro and in vivo manifestations of delayed-type hypersensitivity (DTH) are well recognized, although the mechanism is unclear (13,26,30,31,35). Intact DTH is generally considered essential for recovery from MV infection (6). Fatal infection with conventional MV is seen in patients with genetic or drug-induced immunodeficiency and various lymphoreticular malignancies (2,13,23). Reports of immunocompetence in patients with subacute sclero-
sing panencephalitis (SSPE), a chronic demyelinating encephalitis associated with an MV-like agent are conflicting. Some studies indicate SSPE is accompanied by subtle immunological abnormalities (15,18,28,29). Other reports find no relationship (1,27,34).

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 following infection with distemper virus (19,25) and more extensively, lesions of and pathogenic 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 cause 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, et al., manuscript in preparation). In the present study, in vitro and in vivo tests for cell-mediated immune function in CDV-infected dogs are sequentially evaluated. A disparity between in vitro and in vivo data was encountered and this was evaluated in terms of the final outcome of the infection in experimental animals.

Materials and Methods

Dogs

A total of 23 colostrum-deprived gnotobiotic dogs from 3 litters were used in these experiments. Nineteen dogs (10 infected and 9 control) were used in experiments correlating in vitro lymphocyte blast transforma-
tion (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 (16). 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 (7,19,25). The inoculum is stored as a clarified 10% cerebellar suspension in liquid nitrogen. In a typical experiment, 2 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-VacR was purchased from Pittman-Moore Corporation and administered to 4 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 (25). Two ml of serum was also collected at that time for subsequent analysis of complement-fixing antibodies to canine distemper virus.

Ten ml of heparinized blood containing 20 units preservative-free heparin* per ml were drawn once a week throughout the observation period as a source of lymphocytes for the microlymphocyte blast transformation (LBT) assay. Four ml of blood was mixed with an equal volume of sterile 5% dextran in a syringe and allowed to sediment for 1 hour at 37°C. The leukocyte-rich plasma was aspirated and the leukocytes were washed once in Hank's balanced salt solution containing 50 mg % ethylene diamine tetracetic acid (HBSS-EDTA). Contaminating erythrocytes in the cell pellet were lysed by the addition of 9.0 ml sterile distilled H2O for 10 seconds. Isotonicity was re-established by the addition of 2.9 ml of 3.5% NaCl. The leukocytes were pelleted by centrifugation at 800 rpm x 10 minutes and resuspended in Eagle's minimum essential medium (MEM) containing 20% fetal calf serum, 1% sodium bicarbonate (8.8% w/v) and 1% antibiotics (penicillin, 20,000 units/ml; streptomycin, 10 mg/ml; and nystatin, 5,000 units/ml) to a final cell concentration of $1 \times 10^6$ leukocytes/ml.

The remaining 6.0 ml of heparinized blood was diluted to 30 ml with HBSS-EDTA and lymphocytes were isolated by centrifugation over a ficoll-hypaque gradient, specific gravity 1.0805 (22). The final preparation

*Panheprin, Abbott Laboratories, Chicago, Ill. 60064.
was adjusted to $1 \times 10^6$ lymphocytes/ml with MEM.

Both dextran sedimented (DS) and ficoll-hypaque (FH) purified lymphocyte preparations were cultured in quadruplicate in flat bottomed microplates (Falcon Plastics, #3041. Oxnard, Ca. 93030) at $1 \times 10^5$ cells/well with the plant mitogens e.g. pokeweed mitogen at 50 ug/ml MEM and phytohemagglutinin-P at 0.1 ul stock/ml MEM for 3 days at 37°C, 10% CO$_2$.

During the last 18 hours 0.5 uCi 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, Wisconsin). Radioactivity incorporated into cellular nucleic acid was trapped on glass filter paper and subsequently quantitated in scintillation vials filled with a toluene-base cocktail* 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 canine distemper virus ($>1:256$) was fractionated by 50% saturated ammonium sulphate. The resultant globulin was conjugated to fluorescein isothiocyanate (antiCDV-FITC) after which the unbound dye was removed by passage through a Sephadex G-25 column as described (20). Over-labelled globulin was removed by passage through a DEAE cellulose column equilibrated with 0.1 M Tris 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-

* Permablend II, Packard Instrument Co., Downers Grove, Ill.
infected spleen sections by preincubation with unconjugated anti-CDV serum (21).

Leukocytes from both FH and DS preparations were washed once in saline, smeared on glass coverslips, and air dried. Coverslips were fixed in acetone at room temperature for 30 minutes. The smears were stained with anti-CDV-FITC for one hour, 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 cm² 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 (10). The grafts were applied on the 14th day after infection. The graft sites were bandaged with sterile vasoline-impregnated gauze and surgical tape for 7 days postoperatively. Thereafter, sites were examined twice daily and the color and flexibility of the allograft was 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

A total of 10 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 No. 1 along with littermate con-
trols is shown in fig. 8.

Four of the 10 infected dogs developed signs of acute encephalitis 14-28 days postinfection (PI). A rapid progression to death within 48 hours of onset was seen in those dogs. The remaining 6 dogs survived the 10 week observation period without overt signs of disease except for prolonged lymphopenia. No gross or histologic evidence of active distemper infection was seen in these dogs at necropsy.

Characterization of Lymphocyte Culture Preparations

Ficoll-hypaque (FH) prepared lymphocyte cultures from uninfected dogs contained 80-95% 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 40-75% 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 per cent. In both cases, a significant degree of contamination with non-sedimented erythrocytes and platelets were noted. Differential counts of dextran-sedimentation (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 employed in this study regularly and consistently induced DNA synthesis as measured by the uptake of $^{3}H$-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 indices (SI) as well as counts per minute (cpm) than did FH cultures from the same blood sample. The degree of stimulation measured in cpm, in individual dogs was variable.

A further source of variation was encountered when gross cpm or SI was compared between litters of normal dogs. Litter number 2 usually gave higher cpm than did litters number 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 5-fold) in R252-CDV infected dogs limited the number of lymphocytes available for weekly sequential study. A cell culture concentration of $1 \times 10^6$ cells/ml ($1 \times 10^5$ cells/culture) gave adequate stimulation. Stimulation decreased as the number of cells cultured decreased to a threshold concentration of $0.5 \times 10^6$ cells/ml.

Since the degree of lymphocyte transformation is also a function of time in culture, this parameter was investigated. Ficoll-hypaque prepared lymphocytes responded maximally at 3 or 4 days in culture. In con-
trast, DS cultures showed peak stimulation at 3 days with cpm falling off rapidly after this time. Data from 2 representative dogs is given in Table 11. 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 phytohemagglutinin-P, reconstituted according to the manufacturers instructions, gave optimal stimulation at a concentration of 0.1 ul stock/ml culture media (Table 12). Likewise, a concentration of 50.0 ug pokeweed mitogen per ml of culture media was found optimal (Table 13).

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 (Table 14). 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 cpm for mitogen-induced lymphocyte blast transformation (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 14 showed that no return of lympho-
cyte mitogenic activity was observed in fatally infected dogs. In con­
trast, a trend toward return to preinoculation values 4 to 6 weeks after
infection was seen in dogs which were destined for recovery as illus­
trated in fig. 9. Lymphocytes from several of these animals remained in­
capable 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 $10^6$ lympho­
cytes/ml, showed a comparable depression of stimulation. Further, de­
pression of mitogen response persisted into the convalescent period, a
time in which absolute lymphocyte returned to 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, 40-70% 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 mito­
gens were tried and did not affect our results (Tables 15 and 16).

A direct viral effect on lymphocytes in cultures was considered a
likely possibility as measles virus, another paramyxovirus, will depress
lymphocyte transformation in vitro (26). Immunofluorescent staining of
lymphocytes from infected dogs revealed the presence of viral antigen in the cytoplasm of cells of both lymphocyte and monocyte morphology. The proportion of immunofluorescent 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 differentiated fatally infected from recovered animals. Leukocytes from fatally infected dogs contained viral antigen from 1 week PI until death. In contrast, fluorescent positive cells were seen for only 1-3 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 4 normal gnotobiotic dogs were tested for mitogen-induced blast transformation before and after vaccination with a modified live distemper virus vaccine. 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 Lymphocyte Blast Transformation (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 17.

Despite the high incidence of surgical and post-operative failures in infected (4/10) and control (3/9) animals in these experiments, the results are unequivocal. No significant retention of allografts were seen in R252-CDV infected gnotobiotic dogs. Only 1 dog demonstrated prolonged graft survival as compared to controls (infected dog 1, Table 17). This animal died of acute encephalitis 16.5 days after grafting. Control autografts remained viable throughout the observation period (fig. 10). In contrast, allografts placed on control uninfected as well as infected dogs exhibited changes in color and loss of pliability 11 to 14 days after application (fig. 11). Biopsies of these grafts at this time revealed histologic features typical of graft rejection (9,36) including epithelial and dermal necrosis, cellular infiltration of mononuclear inflammatory cells and neutrophils, and vascular thrombosis and necrosis (fig. 12). Although allografts from CDV-infected animals showed similar overall histologic changes, the cellular inflammatory reaction in both the allograft and underlying dermis was less prominent than in uninfected control allografts.
Discussion

The present experiments describe results of infection with a paramyxovirus, canine distemper virus, on lymphoid function in gnotobiotic dogs. Previous studies in our laboratory and elsewhere have emphasized the morphological effects of this virus on lymphoid tissue (3,19,25,37). A consistent gross pathologic finding in fatally infected dogs is thymic atrophy. Generalized lymphoid depletion is recognized. Demonstration of virus in both lymphocytes and macrophages by immunofluorescence and electron microscopy suggests that depletion is a direct viral effect and not the result of endogenous corticosteroid secretion or seclusion in non-lymphoid sites. Despite these morphological data, little is known about the functional capabilities of the immune system as reflected in peripheral blood lymphocyte activity during the course of the disease.

The in vitro response of peripheral blood lymphocytes to plant mitogens is characterized as a nonantigen specific polyclonal transformation to immature blast cells. Although immunospecificity is lacking, the lymphocyte-blast transformation (LBT) assay is widely studied because the intracellular events involved in lymphocyte responses to either specific antigen or mitogen appear to be identical (17). For this reason, the blast transformation assay was used as one in vitro parameter of cellular immunocompetence.

The response to mitogenic stimulation in pre-inoculation and control samples from gnotobiotic dogs is similar to the expected response in conventional dogs reported in the literature although absolute values in cpm or stimulation indices naturally vary with culture conditions used by different investigators. Five normal gnotobiotic control dogs were evalu-
uated weekly along with the CDV-infected animals. A cyclic periodicity in the amount of $^{3}$H-thymidine incorporated, as reflected in cpm was observed in these controls. Dionigi et al. observed this phenomenon in PHA-stimulated peripheral blood lymphocyte cultures from normal individuals sampled biweekly for several months (12).

Despite any contributing effects of cyclic phenomena, infection with CDV has a profound and prolonged suppressive effect on canine peripheral blood LBT. The effect is seen at the onset of lymphopenia but may persist after lymphocyte counts return to the normal range.

Several different in vitro and in vivo possibilities were considered as possible mechanisms of lymphocyte suppression. An active infection with virulent CDV is necessary to observe this effect since inoculation of dogs with a modified live virus vaccine failed to affect LBT. Such things 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.

The simplest hypothesis which would be consistent with the previous morphological data is that mitogen unresponsiveness is a consequence of direct viral effect on either lymphocytes or macrophages. Viral antigen, as detected by direct immunofluorescence was found in leukocytes of all infected dogs 1-2 weeks postinfection (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. It is possible that the immunofluorescent probe was not sufficiently
sensitive to detect persistent intracellular virus after this time. Viral antigen disappears from leukocytes as neutralizing antibodies appear in the serum (3). Failure to detect virus after this time may be the result of an in vivo blocking effect of anti-CDV antibodies. This would be similar to in vitro methods used to confirm the specificity of fluorescein-conjugated reagents whereby pre-inoculation with unconjugated antiserum blocks immunospecific fluorescence of the subsequently added conjugated antiserum.

An effect analogous to antigenic competition on mitogen responsiveness has been noted by Gershon (14). He found that spleen cells taken from mice immunized 1-2 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 depletion of mitogen-sensitive lymphocytes occurs as a consequence of infection with distemper. Although the sequential morphologic events of non-lethal infection on the lymphoid system have not been studied in detail, this hypothesis would be consistent with the lymphocyte depletion effects of distemper on central lymphoid organs.

Loss of mitogen reactivity was seen in all CDV-infected dogs. Early in the disease, animals which eventually died of generalized infection and encephalitis could not be distinguished from immune dogs by LBT. Mortality in these animals is 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 loss of mitogen responsiveness.

It is apparent that an effective immune response insofar as recovery from infection with CDV was not suppressed as dogs surviving the infection produced adequate levels of antiviral antibodies despite their lymphocytes' inability to react to mitogens in vitro. Since LBT is an in vitro test results obtained are not readily transposed to in vivo immune events. This is particularly true in an outbred system where adaptive cell transfer experiments cannot be performed. Consequently a reliable in vivo measure of cellular immunity in R252-CDV infected dogs 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's adjuvant. Skin reactions using both Koch's old tuberculin and purified protein derivative were minimal in 1/3 of the dogs and undetectable in the remaining animals. Further, results in positive animals were not reproducible upon re-testing. Interpretation of delayed-type hypersensitivity to a soluble antigen keyhole limpet hemocyanin was frustrated by the simultaneous presence of intense immediate-type reactions induced by antibodies in the skin and subcutis of immunized animals.

Rejection of skin allografts is a reflection of histocompatibility differences between donor and recipient. Thymic dependent lymphocytes are the cells responsible for detecting those antigenic differences and for initiating the rejection reactions. Further, the ability of lympho-
cytes to respond to mitogenic stimulation is positively correlated with the capacity to reject foreign allografts (8). In their experience with renal transplants Rivard et al. state that mongrel dogs with active distemper retain renal allografts which otherwise would have been rejected (33). 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, the possibility of a coincidental "good" histocompatibility match was considered as likely an explanation for this exception as any suppressive effects of CDV infection. In our experiments, 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.

Two possibilities were entertained as explanations for the apparent discrepancy between in vitro and in vivo data. As to the first alternative, differences between the two tests in their sensitivity to immunodepressive influences may exist. The LBT assay may be very responsive to subtle immunological defects while allograft rejection is not. In man, rejection of foreign transplants often occurs in spite of procedures known to affect in vitro lymphocyte function such as immunodepressive drugs and anti-thymocyte globulin. Lubaroff found in passive transfer experiments that as little as 500,000 lymph node cells given to isogenic x-irradiated rats restored immunocompetence in recipients (24). In these reconstituted rats, median skin graft survival times were the same regardless of the numbers of cells transferred once the threshold dose had been attained. By analogy to the present studies significant graft survival in
CDV-infected dogs would only occur if the number of potentially competent lymphocytes is reduced below a certain low level. The paucity of mononuclear inflammatory cells in rejected allografts from CDV-infected animals suggest that skin graft rejection is not sensitive enough to detect less than total interference with immunological function.

An alternative explanation was also considered. Skin graft rejection was correlated with the development of effective antiviral immunity; LBT, on the other hand, was not. In LBT, poorly understood variables such as culture media effects, cell concentration effects and the obligatory requirements for viable phagocytic cells affect the quality of mitogenic stimulation. This along with the uncertainty of whether or not the in vitro behavior of peripheral lymphocytes truly reflects central lymphoid function combine to limit interpretation of results obtained in the test (14). It is possible that suppression of lymphocyte activity represents an in vitro artifact which inaccurately reflects in vivo events.

An important corollary of this conclusion relates to the participation of lymphocytes in autoimmune-like phenomena observed in the central nervous system demyelinating phase of canine distemper or other paramyxovirus related demyelinating diseases such as subacute sclerosing panencephalitis and multiple sclerosis. Failure to demonstrate brain antigen autosensitivity in vitro cannot be construed as final evidence against the pathogenic role of lymphocytes in any of these diseases.

In conclusion, we have investigated the effects of CDV on the functional parameters of the immune system. Although immunosuppressive effects could be demonstrated in vitro, parallel in vivo evaluation sug-
gested that less than complete suppression exists in the CDV-infected animals.

Summary

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, in some cases, 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. Early differentiation between those animals which eventually died of generalized infection with encephalitis and those immune dogs was not possible by this assay.

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. Several likely explanations for this apparent discrepancy between in vitro and in vivo data were considered. It was concluded from the results of this study, that although immunodepressive effects of CDV could be demonstrated in vitro, parallel in vivo experiments indicate that less than complete suppression of immune functions occurs during the course of infection.
Table 11. The Effect of Varying Times in Culture\(^1\) on Mitogen-Induced Peripheral Blood Lymphocyte Transformation\(^2\) in Two Normal Gnotobiotic Dogs.

<table>
<thead>
<tr>
<th>Days</th>
<th>Dog No. 1</th>
<th></th>
<th></th>
<th>Dog No. 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PHA-P(^3)</td>
<td>PWM(^4)</td>
<td>Control</td>
<td>PHA-P(^3)</td>
<td>PWM(^4)</td>
</tr>
<tr>
<td>2</td>
<td>71 ± 3</td>
<td>1042 ± 43(14.7) (^5)</td>
<td>296 ± 18(4.2)</td>
<td>102 ± 6</td>
<td>1527 ± 73(15.0)</td>
<td>669 ± 17(6.6)</td>
</tr>
<tr>
<td>3</td>
<td>55 ± 4</td>
<td>5855 ± 254(106.4)</td>
<td>2925 ± 98(53.2)</td>
<td>68 ± 8</td>
<td>7759 ± 232(114.1)</td>
<td>5844 ± 51(85.9)</td>
</tr>
<tr>
<td>4</td>
<td>63 ± 6</td>
<td>1955 ± 34(31.0)</td>
<td>1531 ± 62(24.3)</td>
<td>116 ± 32</td>
<td>2339 ± 132(20.2)</td>
<td>3493 ± 196(30.1)</td>
</tr>
<tr>
<td>5</td>
<td>86 ± 9</td>
<td>524 ± 23(6.1)</td>
<td>776 ± 26(9.0)</td>
<td>62 ± 7</td>
<td>747 ± 25(12.0)</td>
<td>2225 ± 288(35.9)</td>
</tr>
<tr>
<td>6</td>
<td>52 ± 5</td>
<td>176 ± 5(3.4)</td>
<td>224 ± 19(4.3)</td>
<td>73 ± 3</td>
<td>464 ± 61(6.4)</td>
<td>809 ± 40(11.1)</td>
</tr>
</tbody>
</table>

\(^1\) Cultures adjusted to \(10^6\) white blood cells/ml (\(10^5\) white blood cells/culture).

\(^2\) Data expressed in counts per minute (CPM) \(\pm\) standard error of quadruplicate cultures.

\(^3\) Phytohemagglutinin - P (Difco, Detroit, Mich.) at 0.1 ul stock/culture.

\(^4\) Pokeweed Mitogen (Gibco, Long Island, N.Y.) at 50 ug/culture.

\(^5\) Stimulation index calculated by dividing CPM in mitogen-stimulated cultures by CPM in control culture.
Table 12. Comparison of Ficoll-Hypaque and Dextran-Sedimentation Prepared Lymphocyte Cultures: Response to Phytohemagglutinin-P\(^1\) Following Infection with R252-Canine Distemper Virus (CDV).

<table>
<thead>
<tr>
<th>Weeks Post Infection</th>
<th>Ficoll-Hypaque Prepared Lymphocytes(^2)</th>
<th>Dextran-Sedimentation Prepared Lymphocytes(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R252-CDV Infected Dog No.</td>
<td>R252-CDV Infected Dog No.</td>
</tr>
<tr>
<td>-1</td>
<td>494±22 (10.5) (^4)</td>
<td>1972±93 (28.6)</td>
</tr>
<tr>
<td>1</td>
<td>77±13 (0.8)</td>
<td>49±6 (1.0)</td>
</tr>
<tr>
<td>5</td>
<td>81±6 (1.4)</td>
<td>95±8 (1.9)</td>
</tr>
<tr>
<td>10</td>
<td>237±11 (6.1)</td>
<td>296±29 (2.2)</td>
</tr>
</tbody>
</table>

\(^1\) Difco, Detroit, Michigan: 0.1 ul stock/culture

\(^2\) Adjusted to 10\(^6\) lymphocytes/ml (10\(^5\) lymphocytes/culture)

\(^3\) Adjusted to 10\(^6\) leukocytes/ml (10\(^5\) leukocytes/culture)

\(^4\) Data expressed as counts per minute (cpm) ± standard error of quadruplicate cultures. Stimulation Index (in parentheses) is calculated by dividing cpm in mitogen-stimulated cultures by cpm in control cultures.
Table 13. Comparison of Ficoll-Hypaque and Dextran-Sedimentation Prepared Lymphocyte Cultures: Response to Pokeweed Mitogen\(^1\) Following Infection with R252-Canine Distemper Virus (CDV).

<table>
<thead>
<tr>
<th></th>
<th>Ficoll-Hypaque Prepared Lymphocytes(^2)</th>
<th>Dextran-Sedimentation Prepared Lymphocytes(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks</td>
<td></td>
<td>R252-CDV Infected Dog No.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R252-CDV Infected Dog No. 2</td>
<td>2516±121(36.5)</td>
</tr>
<tr>
<td>-1</td>
<td>644±42(13.7)(^4)</td>
<td>834±44(17.4)</td>
</tr>
<tr>
<td>1</td>
<td>56±10(0.6)</td>
<td>80±5(1.7)</td>
</tr>
<tr>
<td>5</td>
<td>98±3(1.7)</td>
<td>153±12(3.0)</td>
</tr>
<tr>
<td>10</td>
<td>461±21(11.8)</td>
<td>908±35</td>
</tr>
</tbody>
</table>

\(^1\) Gibco, Long Island, N.Y. - 50 ug/culture

\(^2\) Adjusted to \(10^6\) lymphocytes/ml (\(10^5\) lymphocytes/culture)

\(^3\) Adjusted to \(10^6\) leukocytes/ml (\(10^5\) leukocytes/culture)

\(^4\) Data expressed as counts per minute (cpm) ± standard error of quadruplicate cultures. Stimulation Index (in parentheses) is calculated by dividing cpm in mitogen-stimulated cultures by cpm in control cultures.
Table 14. Effect of Infection with R252-Canine Distemper Virus (CDV): Mitogen-Induced Lymphocyte Blast Transformation of Peripheral Blood Lymphocytes\(^1\) in Fatally Infected\(^2\) Gnotobiotic Dogs of Group One.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Phytohemagglutinin-P(^3) R252-CDV Infected:</th>
<th>Pokeweed Mitogen(^4) R252-CDV Infected:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI 1 2 3</td>
<td>1 2 3</td>
</tr>
<tr>
<td></td>
<td>0.1 ul stock (Difco, Detroit, Mich.)/culture.</td>
<td>50.0 ug (Gibco, Grand Island, N.Y.)/culture.</td>
</tr>
<tr>
<td></td>
<td>50 - 100</td>
<td>50 - 100</td>
</tr>
<tr>
<td></td>
<td>37 ± 3 (1.0)</td>
<td>199 ± 3 (2.9)</td>
</tr>
<tr>
<td></td>
<td>50 ± 2 (1.1)</td>
<td>856 ± 95 (5.6)</td>
</tr>
<tr>
<td></td>
<td>38 ± 2 (0.9)</td>
<td>417 ± 21 (9.3)</td>
</tr>
<tr>
<td></td>
<td>47 ± 4 (1.3)</td>
<td>397 ± 63 (2.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1</td>
<td>584 ± 22 (12.7)</td>
<td>2629 ± 219 (20.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3043 ± 72 (13.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>214 ± 9 (4.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3862 ± 204 (30.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7656 ± 553 (34.3)</td>
</tr>
<tr>
<td>0</td>
<td>2045 ± 147 (35.9)</td>
<td>657 ± 42 (16.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>756 ± 66 (11.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2140 ± 192 (37.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4489 ± 162 (112.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3886 ± 158 (56.3)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Dextran-sedimentation prepared lymphocytes.

\(^2\) The dogs died on postinfection day 29, 21, and 14 respectively.

\(^3\) 0.1 ul stock (Difco, Detroit, Mich.)/culture.

\(^4\) 50.0 ug (Gibco, Grand Island, N.Y.)/culture.

\(^5\) Data expressed as mean counts per minute (cpm) ± standard error of quadruplicate cultures. The cpm in cultures without mitogen ranged from 50-100.

\(^6\) Stimulation index, given in parentheses, was calculated by dividing cpm in mitogen-stimulated cultures by cpm in control cultures.
Table 15. Effect of Phytohemagglutinin-P (PHA-P) Concentration on $^3$H-Thymidine Incorporation\(^1\) by Lymphocytes\(^2\) from Normal and R252-Canine Distemper Virus (CDV) Infected Gnotobiotic Dogs.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Unstimulated Control Cultures</th>
<th>Concentration of PHA-P/ml</th>
<th>2.5 ul</th>
<th>0.5 ul</th>
<th>0.1 ul</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected control</td>
<td></td>
<td></td>
<td>39 ± 2</td>
<td>81 ± 5</td>
<td>709 ± 33</td>
</tr>
<tr>
<td>dogs (2)</td>
<td></td>
<td></td>
<td>62 ± 4</td>
<td>45 ± 2</td>
<td>121 ± 7</td>
</tr>
<tr>
<td>R252-CDV infected</td>
<td></td>
<td></td>
<td>107 ± 33</td>
<td>53 ± 9</td>
<td>60 ± 7</td>
</tr>
<tr>
<td>dogs (3)</td>
<td></td>
<td></td>
<td>30 ± 4</td>
<td>77 ± 18</td>
<td>68 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37 ± 2</td>
<td>50 ± 5</td>
<td>46 ± 5</td>
</tr>
</tbody>
</table>

\(^1\) Data expressed as the mean counts per minute (cpm) of quadruplicate samples ± standard error. The stimulation index is given in parentheses.

\(^2\) 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 16. Effect of Pokeweed Mitogen (PWM) Concentration on $^3$H-Thymidine Incorporation\(^1\) by Lymphocytes\(^2\) from Normal and R252 Canine Distemper Virus (CDV)-Infected Gnotobiotic Dogs.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Unstimulated Control Cultures</th>
<th>Concentration of PWM/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>250 ug</td>
</tr>
<tr>
<td>Uninfected control dogs</td>
<td>39 ± 2</td>
<td>1494 ± 92 (38.3)</td>
</tr>
<tr>
<td></td>
<td>62 ± 4</td>
<td>916 ± 46 (14.8)</td>
</tr>
<tr>
<td>R252-CDV infected dogs</td>
<td>107 ± 33</td>
<td>111 ± 14 (1.0)</td>
</tr>
<tr>
<td></td>
<td>30 ± 4</td>
<td>85 ± 1 (2.8)</td>
</tr>
<tr>
<td></td>
<td>37 ± 2</td>
<td>75 ± 6 (2.0)</td>
</tr>
</tbody>
</table>

\(^1\) Data expressed as the mean counts per minute (cpm) of quadruplicate samples ± standard error. The stimulation index is given in parentheses.

\(^2\) 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 17. Comparison of Skin Allograft Survival Time, Mitogen-Induced Lymphocyte Blast Transformation and Antibody Response to R252-CDV in Control and R252-CDV Infected Gnotobiotic Dogs.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Allograft Rejection Time (Days)</th>
<th>Depression of Mitogen-Induced Lymphocyte Blast Transformation</th>
<th>Complement-Fixing Antibody Response to R252-CDV; Sero-Conversion After Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected control dogs (6)</td>
<td>11.75 ± 0.5²</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>R252-CDV infected dogs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>16.5³</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>14.0</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>11.5</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>12.0</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>11.5</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>11.0</td>
<td>Yes⁴</td>
<td>Yes</td>
</tr>
</tbody>
</table>

¹ Two controls from litter 1 and 4 control animals from litter 3.

² Mean survival time ± standard error.

³ This dog died of acute encephalitis 16.5 days after grafting. At that time, the allograft was still viable by both gross and histologic criteria.

⁴ Mitogen response in this dog was significantly depressed for 2 weeks only. Lymphocyte reactivity returned to normal levels during the grafting experiment.
Figure 8. Absolute peripheral lymphocyte levels in gnotobiotic dog litter No. 1 during infection with R252 canine distemper virus.
Fig. 8

- LYMPHOCYTES PER MM$^3 \times 10^3$
- WEEKS AFTER INFECTION

- CONTROLS (3)
- INFECTED (4)

MEAN ± SE
Figure 9. The effect of nonfatal infection of 6 gnotobiotic dogs with R252-CDV on the incorporation of $^3$H-thymidine into peripheral blood lymphocyte cultures stimulated with phytohemagglutinin-P and pokeweed mitogen.
Fig. 9
Figure 10. Control autograft on postgrafting day 11. The graft is of normal color and pliability.
Figure 11. The appearance of a rejected allograft on uninfected control dog 11 days after placement. The graft is dark brown in color, has begun to retract from the edges of the grafting site and is no longer pliable.
Figure 12. Photomicrograph of rejected skin allograft demonstrating the histologic features of graft rejection such as epithelial necrosis and infiltration of the graft by mononuclear inflammatory cells and neutrophils. (350 x)
BIBLIOGRAPHY

Chapter I


Chapter II


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Chapter III


