Protection against Canine Distemper Virus in Dogs after Immunization with Isolated Fusion Protein

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Canine distemper virus attachment (hemagglutinin [H] equivalent) and fusion (F) antigens were purified by affinity chromatography with monoclonal antibodies. The purified antigens were used to immunize groups of three dogs. Radioimmune precipitation assays with sera from these animals showed that the F antigen preparation was pure and induced only an F polypeptide-specific antibody response but that the H antigen preparation had a slight contamination by the F antigen. Immunized animals were challenged with virulent canine distemper virus. Two animals in each group developed pronounced humoral and cellular immune responses after challenge. Among these infected animals, only the dogs immunized with H antigen developed symptoms, albeit mild. In contrast, three nonimmunized control animals developed severe disease, with a fatal outcome in two cases. The complete resistance against challenge in two dogs was interpreted to reflect in one case anti-F immunity and in the other case most likely a high level of anti-H immunity. It is suggested that the F antigen may be of particular interest for the development of morbillivirus and possibly other paramyxovirus subunit or synthetic vaccines, because it can induce immunity capable of blocking virus infection and in situations of virus replication prevent the emergence of symptoms.

Paramyxovirus envelopes contain two main surface glycoproteins, which are targets for host immune defense mechanisms. Evaluation of the relative immune-protective role of these components is a prerequisite for development of subunit or synthetic vaccines. In general the attachment, hemagglutinin (H) protein seems to be the major antigen involved in *in vitro* neutralization, but *in vivo* an antibody response to the fusion (F) component appears to be required for the prevention of spread of infection and disease development (6, 12).

In a previous study (2), we used infection with canine distemper virus (CDV) in dogs to evaluate the role of morbillivirus peplomers in immune protection. In the previous study dogs were immunized with live attenuated CDV (CDV-L), heat-inactivated CDV, or live measles virus (MV). Only the CDV-L vaccine gave complete protection, whereas the two other immunogens gave partial immunity. The dogs showed mild signs of infection after challenge and gave strong anamnestic cellular and humoral immune responses. Because CDV and MV have closely related F antigens but essentially distinct H antigens (9), the experience of immunization with measles vaccine indirectly may illustrate the efficacy of immunization with the CDV F antigen. To further analyze the consequences of immunization with different surface components, the H and F antigens of CDV were isolated and used to immunize groups of dogs.

MATERIALS AND METHODS

Virus and cell culture. The Convac vaccine strain (11) and the Onderstepoort CDV strain (5) were propagated in Vero cells by a previously described technique (13). At an advanced stage of cytopathic effects, Convac CDV-infected cultures were harvested, and a 10% (vol/vol) cell suspension was prepared for the purification of H and F proteins. The

Snyder Hill strain of virulent CDV in dog spleen suspension was used to challenge dogs (16).

Purification of H and F antigens by affinity chromatography with monoclonal antibodies. Affinity chromatography with monoclonal antibodies was used as previously described for the purification of MV envelope components (17). CDV H antigen-specific (13) and MV F antigen-specific (7) monoclonal antibodies were used for the isolation of the respective peplomers. Antigen preparations were made by cell lysis as previously described (17). The composition of the lysis buffer was 10 mM Tris hydrochloride (pH 7.8)–150 mM NaCl–600 mM KCl–0.5 mM MgCl₂–2% Triton X-100 with 1 mM phenylmethylsulfonyl fluoride and 1% Aprotinin.

Monoclonal immunoglobulin G was purified from mouse ascites by ion-exchange affinity chromatography on DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) columns. The immunoglobulin was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at a concentration of 5 mg/g of gel by the procedure recommended by the manufacturer.

Immunosorbent gels were washed with lysis buffer, mixed with antigen, and held on ice for 2 h with frequent agitation. The gels were then transferred to columns and washed with lysis buffer followed by 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–0.1% Nonidet P-40 (TEN buffer; 3). H protein was desorbed with 100 mM glycine-hydrochloride (pH 2.8)–0.1% Triton X-100. Pooled fractions were neutralized with 1 M Tris hydrochloride. F protein was desorbed by the use of 3 M KSCN in TEN buffer. Pooled fractions were desalted on a PD-10 column (Pharmacia). Antigens for immunization were analyzed for protein concentration by the method of Bradford (4). The final concentration was about 100 µg/ml. The efficiency of purification was controlled by mixing ³⁵S-labeled virus-infected cell lysate into the starting material and performing a radioimmune precipitation assay (RIPA) with the final product. The final product was also examined by electron microscopy.

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TABLE 1. Effect of immunization with purified envelope glycoproteins on clinical signs after exposure of dogs to virulent CDV

Immunization	Dog no.	No. of days after inoculation with virulent CDV for:				
		Depression	Wt loss	Elevated body temperature ^a	Lymphopenia ^b	Virus isolation ^c
None	1	4-14	6-14	4-5, 10-13	7-14	4-10
	2 ^d	4-16	6-16	4-6, 12-16	7-16	4-10
	3 ^d	4-16	6-16	4-5, 10-16	4-16	4-10
CDV-H antigen	4	ND ^e	ND	ND	ND	ND
	5	4-10	6-9	4-5, 8-10	4-7	4-7
	6	4-9	6-9	4, 8, 9	4-10	4-7
CDV-F antigen	7	ND	ND	ND	ND	ND
	8	ND	ND	ND	ND	ND
	9	ND	ND	ND	ND	ND
CDV-L	10	ND	ND	ND	ND	ND
	11	ND	ND	ND	ND	ND

^a Above 39.5°C.^b Less than 2×10^3 lymphocytes per mm³.^c Isolated from buffy coat cells cocultivated with dog lung macrophages.^d Dog became moribund 16 days postinfection.^e ND, Not detected.

Immunization experiment in dogs. Eleven 3-month-old, specific-pathogen-free beagle dogs from the James A. Baker Institute colony were studied. Blood samples were collected at the initiation of the experiment and repeatedly thereafter. Groups of three dogs were immunized with two injections of purified peplomers at 42-day intervals (days -63 and -21). The first injection was 0.5 ml of antigen mixed with Freund complete adjuvant inoculated subcutaneously, and the second injection was 0.5 ml of antigen administered intravenously. A third group of two animals were given CDV-L vaccine 21 days before challenge. All animals plus three control dogs were each challenged intranasally with 5×10^3 (50% tissue culture infective dose) of the Snyder Hill strain of virulent CDV (day 0). The clinical reactions of the dogs were determined by daily observations and recording of body temperature and by biweekly monitoring of weight gain. Circulating blood lymphocytes were counted weekly. Blood samples for serological tests were collected on days 7, 10, 14, 21, and 28 after challenge. Blood samples for virus isolation from buffy coat cells were collected on days 4, 7, 10, and 14 after challenge. Buffy coat cells were cocultivated with dog lung macrophages for virus isolation.

Serological tests. The techniques used for serological testing were previously described in detail. They included CDV neutralization (CDV-N) tests in Vero cells with the adapted Onderstepoort strain of CDV (1), CDV enzyme-linked immunosorbent assay (ELISA) with Convac strain-infected cells in microtiter plates as previously described for other paramyxoviruses (10), and MV hemolysis-inhibition (HLI) tests (8).

Immune lymphocyte-mediated cytotoxicity assays. The immune lymphocyte-mediated cytotoxicity assay procedure was described in detail previously (14). Briefly, peripheral blood lymphocytes purified by carbonyl-iron treatment and centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) were incubated for 6 h at 37°C in an atmosphere of 5% CO₂ in air with ⁵¹Cr-labeled CDV-infected dog testicle fibroblasts at a ratio of 100 lymphocytes to 1

target cell. Thereafter, supernatant fluids and the remaining cells were harvested separately with the Titertek collection system (Flow Laboratories, Inc., McLean, Va.), and the radioactivity in these samples was determined in a gamma counter (LS 6800; Beckman Instruments, Inc., Palo Alto, Calif.). The percent lymphocyte-mediated cytotoxicity of a sample was computed by subtracting the percent cytotoxicity caused by lymphocytes from a dog before immunization from the percent cytotoxicity detected after immunization and after virus challenge. The background cytotoxicity generally was less than 5%. The lymphocytes and target cells were matched for dog leukocyte antigens. This testing was performed by M. St. John of the Mary Imogene Bassett Hospital, Cooperstown, N.Y.

RIPA. Vero cells were infected with the Convac strain of CDV, and at the stage of advanced cytopathic effects the culture was labeled with [³⁵S]methionine for 24 h. The infected cells were harvested in cold phosphate-buffered saline and disrupted on ice in the cell lysis buffer presented in the description of immunoaffinity chromatography. Thereafter, the RIPA was performed as described in detail previously (11, 15).

Electron microscopy. Immunoaffinity-purified H and F proteins were negatively contrasted on carbon-coated grids by using 2% sodium tungstosilicate at pH 6. The grids were examined in a Philips EM300 electron microscope at 60 kV accelerating voltage.

RESULTS

Clinical reactions. The three nonimmunized dogs contracted severe disease after challenge with virulent virus (Table 1). All three dogs became severely depressed, developed biphasic elevated body temperature, and showed weight loss. Two of the animals became moribund on day 16 after infection. In contrast, dogs immunized with CDV-L vaccine did not show any symptoms after challenge. The two groups of dogs immunized with purified H and F antigen, respectively, showed different reactions to challenge with virulent virus. None of the dogs which had received the F antigen showed any clinical reaction, but two of the three

TABLE 2. CDV and MV antibody titers in response to vaccination and after challenge with virulent CDV 21 days postvaccination

Immunization	Dog no.	Reciprocal antibody titer					
		At day of challenge			After challenge (14 days)		
		CDV-N	CDV-ELISA	MV-HLI	CDV-N	CDV-ELISA	MV-HLI
None	1	<6	<100	<4	220	1,600	8
	2	<6	<100	<4	26	1,600	16
	3	<6	<100	<4	110	1,600	64
CDV-H antigen	4	<6	6,400	4	<6	1,600	<4
	5	<6	1,600	4	220	25,600	128
	6	<6	1,600	4	900	6,400	64
CDV-F antigen	7	10	25,600	8	20	6,400	8
	8	10	6,400	<4	2,560	25,600	64
	9	13	25,600	4	10,240	25,600	64
CDV-L	10	900	6,400	8	450	6,400	16
	11	1,800	6,400	16	2,560	6,400	16

FIG. 1. RIPA with dog sera. Before challenge with virulent CDV (day 0), the animals were immunized as follows: 1 to 3, none; 4 to 6, purified H antigen; 7 to 9, purified F antigen; and 10 to 11, modified CDV-L vaccine. The day of serum sample collection is indicated. The positions of different CDV-specific polypeptides are indicated on the left. The samples to the right contain molecular weight markers. Two minor bands were occasionally observed in the background, a 43K protein presumably representing actin and an unidentified 76K protein.

FIG. 2. Affinity chromatography-purified F protein in TEN buffer. Negative contrasting by 2% sodium tungstosilicate. Club-shaped monomeric peplomers and dimeric peplomer aggregates are predominant. Magnification, $\times 160,000$.

dogs which had received the H antigen showed symptoms (Table 1). The symptoms were of moderate intensity, and both dogs had fully recovered by day 14 after infection.

Virus isolation. Virus was isolated from buffy coat cells of the three nonimmunized dogs between days 4 and 10 after challenge and from two of the three H antigen-immunized dogs between days 4 and 7 after challenge (Table 1).

Antibody responses. The CDV-challenged nonimmunized animals showed rising antibody titers (Table 2). At day 14 after challenge, moderate titers of CDV-N and MV-HLI antibodies were present, whereas the CDV-ELISA antibody titers were comparatively low. Dog 1, which survived infection, had a relatively high titer of CDV-N antibodies. In the RIPA (Fig. 1a), the only antibody response identified was against the nucleoprotein (NP) polypeptide and its degradation products except in dog 1, which in addition showed a weak anti-H antibody response. The possible occurrence of an anti-F antibody response could not be determined, because the MV-HLI activity found may have been due to the presence of antibodies to the H protein (also reflected in the occurrence of CDV-N antibodies), and furthermore, any weak F1 band in the RIPA would have been hidden by NP breakdown products.

Dogs immunized with modified CDV-L vaccine developed high titers of CDV-N and CDV-ELISA antibodies, and there were no significant changes in antibody titers after exposure to virulent virus. In the RIPA (Fig. 1b), a pronounced antibody response to H, NP (and degradation products), and probably F1 polypeptides was identified.

The three dogs immunized with purified H antigen developed moderate and low CDV-ELISA and MV-HLI antibody titers, respectively. No neutralizing antibodies could be detected. The one dog (no. 4) without clinical reaction to virus exposure did not show any change in antibody titers after challenge. As expected, the two dogs which showed moderate symptoms developed high antibody titers in all

tests. In the RIPA (Fig. 1a), it can be seen that hyperimmunization with purified H antigen gave a weak to moderate antibody response to the 85K H polypeptide. However, a weak 41K F1 protein band also was precipitated, indicating that the preparation used for immunization had a slight contamination with F antigen. The RIPA with 14-day-post challenge sera confirmed the absence of any alteration of the antibody response in dog 4 but a marked antibody response to H, NP, and F1 plus F2 polypeptides in dogs 5 and 6. In dog 5, traces of an antibody response to the matrix (M) antigen (36K) were also seen.

The three dogs immunized with purified F antigen developed high CDV-ELISA, low MV-HLI, and low CDV-N antibody titers. After challenge with virulent virus, the antibody titers remained unaltered in one dog (no. 7), whereas in the two other symptomless dogs, virus replication occurred, leading to the development of high antibody titers demonstrable in all tests.

The RIPA (Fig. 1b) showed that the preparation used for immunization contained only F immunogen. The peplomere morphology of this material is shown in Fig. 2. After hyperimmunization, a distinct 41K F1 band was seen just below the contaminating band of cellular actin (Fig. 1b). In dog 7, the selective anti-F antibody response remained essentially unaltered after challenge. The other two dogs mounted pronounced antibody responses to the different structural polypeptides, including a weak response to the M protein.

Lymphocyte-mediated cytotoxic antibody responses. Among the three nonimmunized dogs, only the animal (no. 1) surviving challenge developed a detectable cell-mediated immunity (CMI; Fig. 3). The two dogs which received modified CDV-L vaccine mounted a pronounced but shortlasting CMI and were completely protected against disease and infection after challenge. Four of the six animals immunized with isolated peplomers in Freund complete adjuvant developed a low CMI titer. After challenge, one of these four dogs (no. 4) was resistant to infection without an anamnestic CMI response, but another H antigen-immunized dog among the four (no. 5) became infected and developed disease despite an anamnestic CMI response. The infection established by virulent virus challenge in dogs 5, 6, 8, and 9 were in all cases associated with the evolution of CMI. However, the peak of CMI response in animals preimmunized with H and F peplomers occurred on days 10 and 7, respectively. Furthermore, the most pronounced responses were seen in the two H antigen-immunized dogs, which developed symptoms. In one F antigen-immunized dog (no. 7), CMI was not demonstrated in the samples collected after immunization or after challenge. However, the dog was protected from infection and disease.

DISCUSSION

In previous studies (2), dogs were immunized with heat-inactivated CDV and live MV and then challenged with virulent CDV. After immunization, these animals showed a weak antibody response and demonstrated no CMI. They showed mild symptoms after challenge and responded with pronounced both humoral and cellular immune responses. This partial protection was interpreted to reflect anti-H immunity in the case of dogs receiving inactivated CDV and anti-F immunity in the case of animals immunized with live MV. The aim of the present study was to further evaluate the degree of protection established after immunization with different surface antigens. For this purpose, affinity chromatography-purified H and F CDV antigens were used.

FIG. 3. CDV immune lymphocyte-mediated cytotoxicity in response to vaccination and after challenge (arrows; day 0) with virulent CDV (Snyder Hill strain). (a) Unvaccinated control dogs 1 (□), 2 (▼), and 3 (○). (b) Dogs vaccinated with CDV-H antigen on days postinoculation -63 and -21; dogs 4 (○), 5 (▼), and 6 (□). (c) Dogs vaccinated with CDV-F antigen on days postinoculation -63 and -21; dogs 7 (▼), 8 (○), and 9 (□). (d) Dogs vaccinated with modified live CDV-L on day postinoculation -21; dogs 10 (□) and 11 (▼).

The F antigen preparation induced an exclusive antibody response to this antigen, but the H antigen gave both a homologous and a slight anti-F antibody response. The influence of the latter response on the immunity of dogs is difficult to evaluate. However, there was a certain difference in the degree of protection of animals in each group. Two dogs of three in each group contracted an infection, but only animals immunized with H antigen showed symptoms, and virus from buffy coat cells could be isolated only from these animals. Thus, it is possible that immunity against F antigen is relatively more efficient in restricting the spread of a virus infection and, therefore, the development of symptoms. An alternative explanation can be that the relatively higher degree of protection against disease in F antigen-immunized dogs may be a reflection of a more potent immunogenicity as indicated by the higher titers of CDV-ELISA antibodies. This titer difference may also explain why CDV-N antibodies were detectable in F antigen- but not in H antigen-immunized dogs. No clear guidance to interpretation of degree of resistance to challenge could be obtained from analyses of MV-HLI antibody titers, which generally were low, and CMI responses. Weak CMI responses were seen in four of the animals after primary immunization. Possibly, a weak and short-lasting response in the two remaining animals was missed with the schedule of sampling used. The

specificity of the weak responses recorded is uncertain, and they may reflect natural killer cell effects due to the use of Freund complete adjuvant with the material used for injection 1 (day -63). CMI responses were also seen after challenge but only in the four dogs with anamnestic immune responses, and these responses occurred later and were most pronounced in the dogs immunized with H antigen which displayed symptoms. A relatively more extensive virus replication in these dogs may have been the cause of the accentuated responses. In dogs 4 and 7 (Fig. 3), anti-F and anti-H antibody presumably sufficed to prevent infection after challenge, because no CMI response was detectable before infection.

There was no detectable replication of the virulent CDV used for challenge in two dogs, one immunized with H antigen and the other immunized with F antigen. The dog immunized with H antigen showed the highest titer of CDV-ELISA antibodies and may have been protected by a pronounced anti-H antibody response despite the fact that no neutralizing antibodies were detectable. An anti-F immunity resulting from contaminating F peplomer antigen in the H antigen preparation may also have been involved in the protection of this animal, although this appears less likely. The dog protected by injection of F antigen is of special interest, because the preparation used for immunization

appeared pure and morphologically homogeneous (Fig. 2), and the only detectable antibody response was directed against this antigen (Fig. 1b). In view of the fact that immunity to F antigen has been shown to block the replication of challenge virus and prevent the emergence of symptoms in animals after virus replication, we suggest that the F antigen may suffice as an immunogen for protection against disease produced by CDV. Furthermore, this consideration may also be applicable in attempts to make subunit or synthetic vaccines for other morbilliviruses.

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