Humoral Immune Response in Dogs with Old Dog Encephalitis and Chronic Distemper Meningo-encephalitis

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(Accepted 6 February 1987)

SUMMARY

The humoral immune response in sera and cerebrospinal fluids (CSFs) of dogs with various forms of canine distemper virus (CDV)-induced encephalitis was assessed by immunoprecipitation of radiolabelled nucleocapsid, phosphoprotein, membrane (M), haemagglutinin and fusion proteins. Sera from vaccinated dogs and hyperimmune sera contained antibodies to all the above antigens. In two cases of old dog encephalitis the sera and CSFs showed a restricted response to the M protein of CDV, whilst in three other cases of old dog encephalitis, two cases of chronic distemper (meningo-)encephalitis and experimentally induced encephalitis the humoral immune response appeared to be directed primarily to the nucleocapsid, phosphoprotein and the M protein but not the haemagglutinin or fusion proteins. Precipitation of the M protein by most of the sera was observed only when the antigen had been prepared by in vitro translation.

INTRODUCTION

Canine distemper virus (CDV) has been shown to be involved in neurological disorders of the dog (ter Meulen et al., 1972; Appel et al., 1981). The importance of the humoral immune response in these is indicated by the fact that the initial infection of dogs by CDV frequently leads to fatal complications, when the dog does not mount an effective early neutralizing antibody response (Appel et al., 1981). When a demyelinating encephalitis develops months or even years after the primary infection, the disease is referred to as chronic distemper meningo-encephalitis (CDE). When no preceding illness occurs and symptoms of encephalitis develop in aged or immunized dogs the disease is called old dog encephalitis (ODE). The similarity between subacute sclerosing panencephalitis (SSPE), caused by measles virus (MV) which is a related member of the morbillivirus genus (Kingsbury et al., 1978), and ODE has prompted comparative studies of the two diseases. In both, virus can occasionally be isolated from the affected host, but in many cases this has proved impossible (Katz & Koprowski, 1973). Isolation of virus from cases of SSPE requires cocultivation of tissues with susceptible cells, but in ODE and CDE cases CDV can be isolated directly from a variety of tissues of the affected dog (Imagawa et al., 1980). The MV viruses isolated from SSPE cases show differences from standard laboratory strains, although none of these has been found to be characteristic for the disease (Rima, 1983). The CDV strains isolated from cases of ODE and CDE show differences in the nucleocapsid (N), haemagglutinin (H) and fusion (F) antigens analysed by limited proteolysis (Shapshak et al., 1982). Molecular studies directly on brain tissues of SSPE patients (Hall & Choppin, 1981; Baczko et al., 1984, 1986; Haase et al., 1985; Norrbyn et al., 1985; Liebert et al., 1986) indicate that virus maturation in the brain may be restricted by defects in transcription and translation of one or several of the viral envelope proteins. No such studies have as yet been reported for ODE or CDE.
An earlier comparative study on SSPE, ODE and CDE (Hall et al., 1979b) reported that the humoral immune response to the membrane (M) protein in sera and cerebrospinal fluids (CSFs) was restricted only in SSPE patients and not in ODE and CDE (Hall et al., 1979a; Stephenson & ter Meulen, 1979; Wechsler et al., 1979; Machamer et al., 1980; Hall et al., 1979b). With improved methodology of immunoprecipitation and with the aid of an unambiguous assignment of CDV antigens by the use of monoclonal antibodies to CDV (Örvell et al., 1985) we have reassessed the humoral immune response in ODE, CDE, experimentally induced encephalitis and field cases as well as in vaccinated and hyperimmunized dogs.

METHODS

Virus and cells. The Onderstepoort strain of CDV was grown in African green monkey kidney cells (Vero) in Eagle’s medium supplemented with 5% newborn calf serum in the presence of antibiotics as described before (Campbell et al., 1980).

Sera and CSFs. The ODE and CDE sera and CSFs were those described earlier (Hall et al., 1979b). Two sera from dogs vaccinated with the Rockborn strain of CDV, a serum and CSF from a field case of distemper encephalitis and from experimental encephalitis induced by the A75-17 strain of CDV, a hyperimmune dog serum and pre-immune serum of a dog were obtained from Professor M. J. Appel (Cornell University, Ithaca, N.Y., U.S.A.). Experimental encephalitis sera and CSFs 15463, 15470, 15471 and 15477, a commercial dog anti-CDV serum (dCDVcom) and serum from a dog convalescing from experimentally induced encephalitis were obtained from Professor M. Vandevelde (Bern, Switzerland). A rabbit hyperimmune serum raised against the M protein of MV were obtained from Dr. C. Örvell and Dr E. Norrby (Karolinska Institute, Stockholm, Sweden). Monoclonal antibodies to the M protein of CDV are not yet available.

Preparation of antigen radiolabelled in vivo. Petri dishes (5 cm diameter) infected with the Onderstepoort strain of CDV at an m.o.i. of 3 to 5 were labelled 18 h post-infection (p.i.) when 50% of the cells were in syncytia. The cell monolayer was washed twice with prewarmed phosphate-buffered saline (PBS) and a mixture of PBS and methionine-free medium (3:1) was added. After 15 min preincubation, 1 ml of fresh methionine-free medium/PBS mixture and 0.3 mCi of [35S]methionine (Amersham) were added to the dishes and they were further incubated, with gentle rocking, at 37 °C. After 4 h the cells were washed with appropriate immunoprecipitation buffers (see below) without detergents, scraped into a centrifuge tube and lysed by addition of 0.1 vol. concentrated detergent mixtures, i.e. 10% (v/v) Triton X-100 with Machamer buffer, 5% (v/v) Triton X-100 and 5% (w/v) sodium deoxycholate (DOC) in the case of Saleh buffer and 10% (v/v) Triton X-100, 10% (w/v) DOC and 1% SDS in the case of RIPA buffer. The lysate was then centrifuged at 50,000 g for 20 min at 0 °C in a precooled rotor. The supernatant was divided into samples and stored at −20 °C for use in immunoprecipitation experiments.

Preparation of antigen radiolabelled in vitro. Poly(A)+ RNA extracted from CDV-infected Vero cells as described by Baczko et al. (1986) was added to rabbit reticulocyte lysates (kindly donated by Dr S. Siddell, Würzburg, F.R.G.). In vitro translation was carried out as described by Baczko et al. (1986). After incubation at 32 °C for 50 min the lysate was diluted fivefold with Machamer buffer (see below) and the incorporation into TCA-precipitable material was assayed using standard techniques. Samples containing 200,000 c.p.m. of 35S-labelled protein were used directly in the immunoprecipitation experiments.

Immunoprecipitation. Immunoprecipitation was carried out essentially as described before (Baczko et al., 1986).

Frozen lysates were thawed on ice, mixed with 1/20 vol. of aprotinin solution (Sigma) and centrifuged for 4 min in a microfuge at 2 °C. The lysate supernatant was removed and 50 to 100 μl amounts were transferred to microfuge tubes. To these, 1/20 vol. of aprotinin solution and 2 μl of serum or 10 μl of CSF were added and they were incubated for 2 h on ice. Then 10 μl of a slurry of Protein A-Sepharose (Pharmacia) in the appropriate buffer was added and a further incubation on ice for 45 min was allowed with frequent shaking of the tubes. The immunoprecipitates linked to Protein A-Sepharose beads were collected by microcentrifugation and the pellets were washed four times with buffer, leaving about 20 μl of buffer behind at each step to ensure that the pellets were not disturbed. After the last wash the beads were suspended in about 20 μl double-strength solubilizer for SDS-PAGE, i.e. 4% SDS, 4% 2-mercaptoethanol, 12% (w/v) urea in 125 mM-Tris–HCl buffer pH 6.7, boiled for 2 min and stored at −20 °C for electrophoretic analysis. In this investigation three different buffer systems, described by Ohara et al. (1985), were used. These included Machamer buffer [1% Triton X-100, 0.15 M NaCl, 5 mM-EDTA, 0.1 mM-phenylmethylsulphony fluoride (PMSF) in 50 mM-Tris–HCl pH 8.0 (Machamer et al., 1980)], Saleh buffer [0.5% Triton X-100, 0.5% DOC, 5 mM-NaCl, 1 mM-PMSF, 5% v/v aprotinin solution (Sigma) in 25 mM-Tris–HCl pH 8.0 (Saleh et al., 1979)] and RIPA buffer [1% Triton X-100, 1% DOC, 0.1% SDS, 0.15 M NaCl, 1 mM-PMSF, 5% v/v aprotinin solution (Sigma) in 10 mM-Tris–HCl pH 8.0 (Lamb et al., 1978)]. When mouse
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Fig. 1. Immunoprecipitation of CDV antigens with various monoclonal antibodies and hyperimmune sera against CDV. (a) infected cell lysate, (b) uninfected cell lysate. Abbreviations of sera used: hrCDV, hyperimmune rabbit serum raised against the Onderstepoort strain of CDV; anti-N, -P, -F, -H, monoclonal antibodies against respectively the nucleocapsid, phospho, fusion proteins and H glycoprotein of CDV; Mpoly, a monospecific serum raised against the M protein of MV; no s., no serum; lysate, radiolabelled cell lysates used for immunoprecipitation; mark., ^14C marker proteins. The positions of the L, P, H, N, M and F proteins of CDV are indicated.

RESULTS

Characterization of antigens

Previous studies on CDV proteins induced in Vero cells (Campbell et al., 1980) indicated that the H and P proteins of CDV migrate closely together in the SDS–PAGE system that we used earlier. Therefore, the main objective of the experiment in Fig. 1 was to characterize CDV antigens found in infected cell lysates, using a different SDS–PAGE system which allowed better separation of the H and P proteins. Radiolabelled infected and mock-infected cell lysates were incubated with monoclonal antisera specific for the N, P, F and H proteins of CDV as well as with hyperimmune sera.
as with a monospecific serum raised against the M protein of MV and rabbit hyperimmune sera to precipitate the viral antigens. The monoclonal N antibody precipitated predominantly the 60K nucleocapsid protein. The P monoclonal antibody precipitated the P protein and a 30K P-related protein (Fig. 1) and coprecipitation of the N and particularly the L protein were observed. These coprecipitations were not unexpected since it is known that the transcriptive complex of paramyxoviruses involves the N, P and L proteins (Hamaguchi et al., 1983).

The F monoclonal antibody precipitated three proteins with respective masses of 62K, 41K and 20K, which were respectively the F0, F1 and F2 proteins of CDV. The H monoclonal antibody precipitated the H protein only. It should also be noted that the M protein although abundantly present in the lysates was not precipitated by the hyperimmune dog or rabbit serum and the polyclonal monospecific serum raised against the M protein of MV, nor did it react with any of the monoclonal antibodies to the M protein of MV at our disposal (data not shown). In contrast, a report published earlier indicated that this antigen was precipitated by most sera. However, in the published profiles there was proteolysis, particularly of the N protein of CDV and it was not clear whether the band designated M indeed represented this protein or breakdown products (Hall et al., 1979b). Precipitation of the M protein was only observed in our study when the antigen had been prepared in vitro (see below).

Comparison of sera and CSFs of dogs with ODE and CDE

The presence of antibodies to various CDV antigens in sera and CSFs from dogs with ODE and CDE was assessed by their ability to immunoprecipitate 35S-labelled CDV antigen. It has recently been shown that the results obtained by immunoprecipitation tests are very dependent on the balance between the solubilization of the antigens and their ability to be precipitated in any specific buffer system. Ohara et al. (1985), for example, demonstrated that the M protein of MV could be precipitated by sera and CSFs obtained from SSPE patients when some buffer systems were used, whereas in others no precipitation was observed. This led us to investigate precipitation in RIPA buffer (Lamb et al., 1978) or Saleh buffer (Saleh et al., 1979). The latter was declared most effective for precipitation of MV antigens with SSPE sera (Ohara et al., 1985). In each set of experiments we included SDS-PAGE analysis of the lysate in order to ascertain that all CDV antigens had been solubilized and were present in the lysate for precipitation. Also precipitations with pre-immune serum were carried out to detect non-specific binding.

Fig. 2 and 3 show the results of precipitation experiments with RIPA and Saleh buffers, respectively, for different sera and CSFs in cases of ODE (dogs 1, 7, 8, 10, 15 and 16), CDE (dogs 6 and 9), a hyperimmunized dog serum raised against the virulent Snyder-Hill strain of CDV, rabbit hyperimmune sera, sera from dogs vaccinated with the Rockborn strain of CDV, a field case and an experimental encephalitis as well as two high titre hamster sera. The results show that in general the P protein was better precipitated in Saleh buffer than in RIPA buffer, but that otherwise the N, H and F antigens were precipitated in both buffer systems by the various sera containing antibodies to these proteins. The results of these precipitation experiments indicated that the ODE cases fell into at least two groups. In ODE-1 and ODE-7, antibodies to the N, P, H and F antigens were detected in serum and CSF and in the cases of ODE-8 and ODE-10 N- and P-specific antibodies were predominant and no response to the H and F antigens was detected. In control experiments with sera from vaccinated dogs and hyperimmune dog and rabbit sera the N, P, F and H proteins were precipitated. In the case of ODE-15 the response in the CSF was very weak and only antibodies to the N protein could be demonstrated while in the serum N-, P-, F- and H-specific antibodies were observed (Table 1). In dogs with chronic encephalitis, experimentally induced encephalitis and in a field case the N and P proteins appeared to be the major antigens to which antibodies were found in both the serum and CSF. In the cases of the experimental encephalitides induced by CDV-17 (15463 to 15477) the response in the CSF was weak when compared with that in a dog convalescing from
Fig. 2. Immunoprecipitation of CDV antigens with various sera and CSFs in RIPA buffer. Abbreviations of sera used: ODE1s, serum from dog ODE1; ODE1c, CSF from dog ODE1 etc.; hdCDV, hyperimmune dog serum raised against the Snyder-Hill strain of CDV; vac. 1s and vac. 2s, sera from two dogs vaccinated with the Rockborn strain of CDV; field enc. s and c, serum and CSF of a field case of CDV encephalitis; exp. enc. s and c, serum and CSF from a dog infected with the A75-17 strain of CDV; hrCDV, as in Fig. 1; ham. 87s and 92s, two high titre sera from hamsters infected with the Onderstepoort strain of CDV; pre.s, pre-immune dog serum.
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Fig. 3. Immunoprecipitation of CDV antigens with various sera and CSFs using the Saleh buffer. Abbreviations as in legend to Fig. 2. Also included are sera and CSFs from dogs 15463, 15470, 15471 and 15478 with experimental encephalitis induced by strain A75-17; dCDV5s, serum from a dog convalescing from experimental encephalitis; dCDVcom.s, commercial dog serum against CDV (Behringwerke, Marburg, F.R.G.); inf.cells, a lysate from infected cells solubilized directly with electrophoresis sample buffer.

Table 1. Antibody responses in ODE sera and CSFs*

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<th>N</th>
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* Data were obtained from Fig. 2, 3 and 4.
† vi, Antigen synthesized in vivo.
‡ vt, Antigen synthesized in vitro.
§ + +, Strong band in immunoprecipitation; +, weak band in immunoprecipitation; -, no band observed in immunoprecipitation.
### Table 2. Antibody responses in CDV encephalitides sera and CSFs*

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* Data were obtained from Fig. 2, 3 and 4.
† vi, Antigen synthesized in vivo.
‡ vt, Antigen synthesized in vitro.
§ + +, Strong band in immunoprecipitation; +, weak band in immunoprecipitation; -, no band present in immunoprecipitation.

The previous sets of experiments did not allow any conclusions to be reached about the presence of antibodies to the M protein as there was no evidence of precipitation of this antigen with any of the sera. We also attempted to obtain precipitation of the M protein in Machamer buffer (Machamer *et al.*, 1980) and a buffer used by Sheshberadaran & Norrby (1986), but without success. We noted however, that, when in vitro translation products made from CDV-infected cell RNA were allowed to react with the hyperimmune dog serum, the N, P and M were precipitated (Fig. 4a). The designations of the N, P and M proteins in the in vitro translation lysates have been confirmed by comparing the limited proteolysis products of the proteins synthesized in vivo and in vitro and were further confirmed by hybrid selected translation experiments with cDNA clones of the mRNAs coding for these proteins (Russell *et al.*, 1985).

Designation of the L, F and H proteins in the in vitro translation products was unreliable and therefore they were not further analysed. Thus it appeared that the hyperimmune dog serum for example was able to precipitate the M protein when it was prepared in vitro. Therefore, we analysed the ability of all the sera and CSFs used in this study to precipitate the M protein synthesized in vitro. The results (Fig. 4b, c) demonstrated that the sera and CSFs of ODE cases 1 and 7 contained few if any antibodies to the M protein whereas these were detected in all the other sera and CSFs. It was also interesting to note that, although ODE-8 serum and CSF precipitated the N protein efficiently when the antigen was labelled in vivo they were not able to precipitate the same antigen prepared in vitro (Fig. 4c). A similar observation was made for the CSF of the experimental encephalitis case used. The sera from dogs 15470, 15471 and 15477 had a very different pattern of precipitation of the N and P proteins made in vivo as compared to the same proteins made in vitro (Fig. 4b).
DISCUSSION

This paper highlights some problems inherent in immunoprecipitation of antigens of morbilliviruses, problems first reported for MV by Ohara et al. (1985). The N, F and H proteins of CDV are readily precipitated but precipitation of the P protein of CDV is facilitated by Saleh buffer rather than RIPA buffer. The major difference between these two buffers is the presence of low concentrations of SDS in RIPA and the presence of this ionic detergent appears to affect the ability of the P protein to precipitate. This may be a reflection of sensitivity of the antigenic sites on this protein to denaturation by SDS or of the low average avidity of the P-specific antibodies present in the sera since high avidity monoclonal antibodies precipitate the P protein well in RIPA buffer. In contrast, the M protein, when labelled in vivo, was not precipitated in either buffer by any of the sera. However, it became apparent that certain sera did contain antibodies to the M protein of CDV, as they precipitated antigen which had been prepared in vitro. We do not believe that this effect is dependent on the buffer since antigen labelled in vivo was not precipitated by either of the sera in the same buffer (Machamer et al., 1980) used for precipitation of the antigen labelled in vitro. This may possibly reflect different folding of the M protein or, alternatively, the association between the M protein and another cellular protein present in the antigen preparations labelled in vivo (such as actin; Giuffre et al., 1982) may alter antigenic sites which are available for reaction with antigen labelled in vitro. It is also interesting to note that to date no monoclonal antibodies to the M protein of CDV have been reported and this may reflect the difficulty of immunoprecipitation of the M protein synthesized in vivo. This is usually the ultimate test for specificity of monoclonal antibodies (Orvell et al., 1985).

The protein of CDV designated L is probably not immunoprecipitated specifically since its precipitation correlates completely with that of the P protein. Coprecipitation of the L and P proteins probably accounts for the presence of the L protein in the precipitates.

The comparison of the sera indicates that there are specific patterns of precipitation for the different antigens. It appears that the hyperimmune dog sera, the rabbit sera and the vaccinated dog sera contain antibodies to the N, P, M, H and F proteins of CDV. The hamster sera have high neutralizing titres and appear to contain a large amount of antibodies to the H protein. Two of the ODE sera (1 and 7) appear different from the others since they appear to contain H and F antibodies but not M-specific antibodies. The sera and CSF of these cases thus appear to be similar to those of SSPE patients where an antigenic response to all the MV antigens except the M protein is abundant (Hall et al., 1979a; Stephenson & ter Meulen, 1979; Wechsler et al., 1979; Machamer et al., 1980). In the ODE-8 and -10 cases antibodies to only the N, P, and M proteins have been found. In most cases the responses in the serum and CSF are the same except in the case of ODE-15 where the serum contains H and F antibodies but the CSF conforms more to the response obtained with the other encephalitic CSFs. It is interesting to note that in those cases of ODE where H and F antibodies were not present, virus isolations were successful whereas in cases ODE-1 and ODE-7 no virus could be isolated (Imagawa et al., 1980). Thus virus isolation was correlated with the absence of H and F antibodies in ODE. The observation that in some cases (for example in ODE-8) the serum and CSF contained antibodies to N protein synthesized in vivo but not in vitro suggests that the observed immune response was largely directed against conformational epitopes or against N protein molecules complexed with other proteins.

In the other cases of encephalitis investigated here, i.e. CDE 6 and CDE 9 as well as the field case and the experimental encephalitides, the pattern of precipitation of the antigens is the same as that in the ODE-8 and -10 cases and is restricted to the N, P and M proteins. Our data show interesting differences between some ODE cases in which the immune response to the M protein is restricted while antibodies to the F and H proteins are present and other cases of ODE and CDE in which antibodies to the N, P and M proteins predominate. Earlier observations (Appel et al., 1981) that encephalitis follows the failure of dogs to mount a neutralizing immune response would predict the lack of antibodies to the F and primarily the H protein which we observe in this study in encephalitic dogs. The reasons why certain dogs fail to mount such a response and the observed existence of biotypes of CDV which are especially encephalitogenic strains, are questions of interest in which now at least the role of the humoral immune response can be monitored adequately using the techniques described here.
"lysate" 

hdCDV pre,s

15463s 15463c 15470s 15470c 15471s 15471c 15477s 15477c dCDV5

dCDVcom

pre,s

field enc,c

lysate
Fig. 4. Immunoprecipitation of CDV antigens prepared by in vitro translation. (a) Immunoprecipitation of $1 \times 10^6$ c.p.m. $^{35}$S-labelled in vitro translation product by hyperimmune dog serum. (b) and (c) Immunoprecipitation of $2 \times 10^5$ c.p.m. of labelled antigen prepared by in vitro translation, with various sera and CSFs. Abbreviations as in legend to Fig. 3.
In conclusion, we have confirmed and extended the observations of Ohara et al. (1985) that the methodology can influence the assessment of antibody responses to morbilliviruses by immunoprecipitation. The discrepancies between our results and those reported earlier (Hall et al., 1979b) in which the same sera and CSFs have been shown to precipitate all the antigens of CDV are not easily explained. It is possible, assuming that RIPA buffer was used in the earlier study, that non-specific adsorption (Ohara et al., 1985) is the explanation for the different results. We have had the advantage of using monoclonal antibodies to better define the various bands observed in immunoprecipitations as representing CDV structural proteins. In the earlier report several extra bands were present and the designation of the F1 and F2 proteins was based only on the migration of bands in SDS–PAGE; the presence of proteolytic products of the larger structural proteins was not ruled out.

Special thanks are due to Drs M. J. Appel, S. L. Cosby, E. Norrby, C. Örvell and M. Vandeveld for generous gifts of sera and CSFs and to Ms Jeanette Hintermeier for technical support in this project. We thank the Deutsche Forschungsgemeinschaft (SFB 105) for financial support.

REFERENCES


Immune responses in distemper encephalitides


(Received 12 December 1986)