ANALYSIS OF STRUCTURAL PROTEINS OF MEASLES, CANINE DISTEMPER, AND RINDERPEST VIRUSES

TAKESHI A. SATO, MASANORI HAYAMI and KAZUYA YAMANOUCHI*

Department of Measles Virus, National Institute of Health, Musashi-Murayama, Tokyo 190-12 and *Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108

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SUMMARY: Serological relationships among measles virus (MV), canine distemper virus (CDV), and rinderpest virus (RV), which constitute morbillivirus subgroup of paramyxoviridae, were investigated by immunoprecipitation and SDS-polyacrylamide gel electrophoresis for their major structural proteins, i.e., hemagglutinin (H), nucleocapsid (NC), fusion (F), and matrix (M) proteins. The molecular weights of the four structural proteins of MV and CDV were confirmed to correspond to those previously reported by several investigators. Structural proteins of RV were analyzed for the first time in the present study and found to have molecular weights of 74,000, 62,000, 44,000, and 40,000 for H, NC, F, and M proteins, respectively. By labeling with glucosamine, the presence of carbohydrate moiety was found in H protein for all the three viruses and in F protein of CDV. The sera from the convalescent animals infected with respective virus disclosed one-way cross pattern depending on the combinations of virus and antisera, but failed to show the reciprocal cross reactivity. On the other hand, hyperimmune sera to respective virus showed the reciprocal cross-reactivity with the four structural proteins indicating that each of the major structural proteins possesses the antigen common to all three morbilliviruses.

INTRODUCTION

Measles virus (MV), canine distemper virus (CDV), and rinderpest virus (RV) are classified in morbillivirus subgroup of paramyxoviridae (Kingsburry et al., 1978), and their serological relationships are demonstrated by neutralization test, immunofluorescent technique, and complement fixation test (Adams and Imagawa, 1957; Plowright, 1968; Imagawa, 1968; Yamanouchi et al., 1970; Orvell and Norrby, 1974; Yamanouchi, 1980). Among the three viruses, MV and CDV have potential to cause slowly progressing central nervous disease such as subacute sclerosing panencephalitis (SSPE) (ter Meulen, Katz and Muller, 1973; Yamanouchi, 1980) and old dog encephalitis (Imagawa et al., 1979; Yamanouchi, 1980). Recent demonstration of the lack of antibody to M protein in the serum of SSPE patients is implicated for the establishment of persistent infection of SSPE (Hall, Lamb and Choppin, 1979; Wechsler, Weiner and Fields, 1981).
1979). On the other hand, the possibility of CDV as a causative agent for multiple sclerosis is still controversial because of the difficulty in serological approaches due to the cross-reaction between CDV and MV (Norrby et al., 1974; Burridge, 1978).

The structural proteins of MV and CDV were analyzed by several investigators; the molecular weights of H, NC, F, and M proteins of MV were shown to be 78,000–80,000, 58,000–60,000, 40,000–44,000, and 36,000–38,000, and those of CDV 76,000–77,000, 58,000–60,000, 40,000, and 34,000–35,000, respectively (Bussell, Waters and Seals, 1974; Graves, Silver and Choppin, 1978; Wechsler and Fields, 1978; Stallcup, Wechsler and Fields, 1979; Campbell et al., 1980). Serological relationship between CDV and MV by Hall et al. (1980) disclosed the reciprocal cross-reaction at the level of structural proteins was demonstrated except one-way cross-reaction of H protein, i.e., anti MV serum precipitated H protein of both MV and CDV but anti CDV serum precipitated that of only CDV. Thus far, analysis of structural proteins of RV has not been reported, and their serological relationship with the other two morbilliviruses was conducted only with anti RV serum.

In the present study, major structural proteins, i.e., H, NC, F, and M proteins, of MV, CDV and RV were compared by immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1979; Lamb, Etkind and Choppin, 1978). By the use of hyperimmune serums to the respective viruses, reciprocal cross-reactions were demonstrated with H, NC, F, and M proteins among MV, CDV, and RV.

Materials and Methods

Virus and cells: Edmonston strain of MV was used after two cycles of plaque cloning in Vero cells (Shishido et al., 1973). Onderstepoort strain of CDV (Yamanouchi et al., 1979) and LA strain of RV (Yamanouchi et al., 1974) were previously described. All the three viruses were grown in Vero cells which were maintained in Eagle's minimum essential medium (EMEM) supplemented with 5% calf serum, 0.11% sodium bicarbonate, 100 units of penicillin and 100 μg per ml of spectomycin.

Antiserums: As convalescent anti MV serum, the serum of a monkey naturally infected with MV was collected approximately one month after the infection and was coded as S-22. The serum of a patient with atypical measles (A.S.) was used as hyperimmune anti MV serum. Serum (D-16) was collected from a dog one month after sc inoculation with Lederle strain of CDV and served as convalescent anti CDV serum. Hyperimmune anti CDV dog serum gifted by Dr. M. Appel, Cornell University, had been prepared by immunization with formalin-inactivated Snyder Hill strain followed by challenge by intranasal inoculation with the same virus 3 weeks later. The serum collected 2 weeks after the challenge inoculation was used as hyperimmune anti CDV serum (G-2). Convalescent anti RV serum (A-140) was collected from a rabbit 14 weeks after
iv inoculation with LA strain of RV. Hyperimmune anti RV serum (C-185) was supplied by Dr. T. Furutani, National Institute of Animal Health. This serum was detailed previously (Yamanouchi et al., 1970). In brief, cattle were immunized with live attenuated vaccine followed by challenge with virulent virus.

**Serological tests:** Micro-neutralization tests were conducted on semimicroplates (Linbro FB-16-24-TC) by the procedure described previously (Yamanouchi et al., 1979) in Vero cells by use of 100 TCID\(_{50}\) of Edmonston strain of MV, Onderstepoort strain of CDV and LA strain of RV were used as challenge virus. The reciprocal of the highest dilution of serum protecting 50% of the cell-culture wells from the viral cytopathic effect was taken as the neutralizing antibody titer.

**Hemagglutination-inhibition (HI) tests** were conducted with MV hemagglutinin. The procedures were detailed previously (Yamanouchi et al., 1969).

**Preparation of virus-infected cell extract:** Vero cells were grown in plastic dishes (60 mm, Falcon 3002) and inoculated with virus at the multiplicity of infection (moi) of 0.1 plaque forming unit (PFU)/cell. When 50% of the cells showed cytopathic effect (CPE), i.e., in 48 hr for MV, 72 hr for CDV, and 100 hr for RV, the infected cells were incubated with EMEM depleted of valine and tyrosine for 1 hr at 37 C, and the medium was replaced with EMEM containing 100 \(\mu\)Ci/ml \(^3\)H-valine and 100 \(\mu\)Ci/ml \(^3\)H-tyrosine. After further incubation for 10 hr at 37 C, the cells were extracted for 15 min at 4 C with 4 ml/dish of lysis buffer consisting of 0.15 M NaCl, 1% sodium deoxycholate (DOC), 1% Triton X-100, 0.1% sodium dodecylsulfate (SDS), 0.01 M Tris-HCl, pH 7.4, and 1 mM phenylmethylsulfonylfluoride.

The lysates were clarified by centrifugation at 10,000 rpm for 15 min and at 35,000 rpm for 60 min in a Beckman SW 50.1 rotor, and the supernatants were stored at -80 C as antigens for immunoprecipitation. Labeling with \(^3\)H-glucosamine (100 \(\mu\)Ci/ml) instead of \(^3\)H-amino acids was similarly carried out. As a control, the lysates of normal Vero cells were prepared by labeling with either \(^3\)H-amino acids or \(^3\)H-glucosamine.

**Purification of RV:** Vero cells grown in roller-bottles were infected with RV at a moi of 0.1 PFU/cell. Two days later, normal growth medium was replaced with EMEM depleted of valine, tyrosine and leucine, and containing 5 \(\mu\)Ci/ml of \(^3\)H-valine, \(^3\)H-tyrosine, and \(^3\)H-leucine. After further incubation for 3 days, culture fluid and cells were sonicated and clarified by centrifugation at 5,000 rpm for 20 min. Supernatant was then pelleted by centrifugation at 7,500 rpm for 20 hr in a Hitachi 18PR centrifuge with an RPR-9 rotor. The pelleted virus was resuspended in a small volume of 0.15 M NaCl-0.01 M Tris-HCl-0.001 M EDTA (NTE) buffer pH 7.4, applied to a 20 to 50% discontinuous sucrose density gradient, and centrifuged in an SW 27 rotor at 26,000 rpm for 60 min. Virus recovered at the interphase was pelleted at 100,000 \(\times\)g for 1 hr in an SW 50.1 rotor and suspended in NTE buffer.

**Immunoprecipitation:** Ten microliters of antiserum was added to 50 \(\mu\)l of cell lysate or a purified virus suspension and left standing overnight at 4 C. Immune complexes were precipitated by adding 25 \(\mu\)l of a 50% suspension of
protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in 0.01 M PBS, pH 7.2, for 2 hr at 4°C. The protein A-Sepharose beads were pelleted at 1,500 rpm for 5 min and washed 4 times in 1 ml of lysis buffer. The resulting precipitate was suspended in 50 μl of sample buffer consisting of 0.0625 M Tris-HCl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue, and boiled at 100°C for 4 min.

Electrophoresis: Either the cylindrical gel or slab gel system was employed for PAGE. The former was carried out by the method of Laemmli (1970) in 9% polyacrylamide cylindrical gel in electrophoresis buffer consisting of 0.05 M Tris-glycine, pH 8.8, and 0.1% SDS at a constant current of 4 mA per gel column for about 3 hr. After electrophoresis, gel slices of 1-mm thickness were solubilized with Soluene-350 (Packard Instrument Co., Inc.) and incubated at 60°C for 1 hr. After adding 2 ml of chilled toluene-scintillation mixture, the isotope was counted in Aloka scintillation counter.

Electrophoresis on slab gel was carried out on 9 or 10% acrylamide gel (2 mm thick) with the electrophoresis buffer described above at a constant current of 10 mA per gel slice overnight. The gel slabs were dehydrated with dimethyl sulfoxide (DMSO) at room temperature for 60 min, followed by treatment with 2,5-diphenyloxazole dissolved in DMSO for 3 hr. After washing in water for 60 min to remove DMSO, gel slabs were dried and exposed to X-ray film (Sakura A type) at −70°C.

Molecular weights were calculated using 14C-methylated protein mixture as standard (myosin, 200,000; phosphorylase-b, 92,500; bovine serum albumin, 69,000; Ovalbumin, 46,000; carbonic anhydrase, 30,000; lysozyme, 14,300) (The Radiochemical Center, Amersham, England).

RESULTS

Serological Test with the Antiserums

The convalescent and hyperimmune serums to MV, CDV, and RV were examined for antibody reactivities to homologous and heterologous viruses by neutralization and HI tests. The results are summarized in Table I. The convalescent serums neutralized homologous virus but did not react with heterologous viruses or did so only at low titers. With hyperimmune serums and atypical measles serums, cross neutralization to various extents was found among three viruses. Anti CDV and anti RV hyperimmune serums cross-reacted with MV also in HI test.

Determinations of Molecular Weights of Structural Proteins

Analysis of major structural proteins of MV, CDV, and RV was carried out on gel slabs by treating the virus-infected cell lysates with hyperimmune serum to homologous virus. Molecular weights of the four major structural
TABLE I
Serological test of anti MV, CDV and RV serums

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Animal</th>
<th>Serum code</th>
<th>Immunization</th>
<th>Antibody titers</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV</td>
<td>Monkey</td>
<td>S-22</td>
<td>Natural infection</td>
<td>VN&lt;sup&gt;1) &lt;/sup&gt; 200 &lt;2 6</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>A.S.</td>
<td>Atypical measles</td>
<td></td>
</tr>
<tr>
<td>CDV</td>
<td>Dog</td>
<td>D-16</td>
<td>Experimental infection</td>
<td>2 2,660 &lt;2 &lt;8</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>G-2</td>
<td>Hyperimmunization</td>
<td>20 10,000 2</td>
</tr>
<tr>
<td>RV</td>
<td>Rabbit</td>
<td>A-140</td>
<td>Experimental infection</td>
<td>16 &lt;2 1,600 &lt;8</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>C-185</td>
<td>Hyperimmunization</td>
<td>1,600 26 2,500 512</td>
</tr>
</tbody>
</table>

1) VN: Virus neutralization test.
2) HI: Hemagglutination inhibition test.

Fig. 1. Comparison of major virus proteins. Virus-infected cells labeled with <sup>3</sup>H-amino acids (a-1: MV, a-2: CDV, A-3: RV) or purified virion of RV (b) were precipitated with homologous antiserum. <sup>3</sup>H-glucosamine-labeled cells were similarly examined for glycoproteins (a-4: MV, a-5: CDV, a-6: RV). Electrophoresis was carried out on 9% (a) or 10% (b) acrylamide gels.
proteins, H, NC, F, and M, were found to be 76,000, 60,000, 42,000, and 38,000 for MV (Fig. 1a-1), 73,000, 59,000, 42,000, and 36,000 for CDV (Fig. 1a-2), and 74,000, 62,000, 44,000, and 40,000 for RV (Fig. 1a-3), respectively. The values for MV and CDV corresponded to those previously reported by other investigators. As regards to RV, H protein possessed an intermediate molecular weight between those of MV and CDV, whereas the molecular weights of NC and M proteins were larger than those of MV and CDV. The same result was obtained by immunoprecipitation of purified virion of RV with hyperimmune serum (Fig. 1b).

By labeling with $^3$H-glucosamine, H proteins of all three viruses were found to be glycosylated. Glycosylation was also found in F protein of CDV, but not in F protein of MV or that of RV (Fig. 1a-4, 5, 6).

Cross Reactions of Structural Proteins by Convalescent Serums

The three convalescent serums, i.e., anti MV (S-22), anti CDV (D-16) and anti RV (A-140), detected four structural proteins of corresponding homologous viruses except for a low level of reaction with NC protein of MV and with M protein of CDV and RV.

In heterologous combinations, irregular patterns of mainly one-way cross reaction to structural proteins of heterologous viruses were observed depending on the combination of virus and antigens except F protein with which reciprocal cross-reactions were noticed among the three viruses (data not shown). Therefore, hyperimmune serums were employed in the following experiments to clarify the cross-reacting antigens.

Cross Reactions of Structural Proteins by Hyperimmune Serums

Serum of a patient with atypical measles (A.S.) clearly detected H, NC, F, and M proteins of MV (Fig. 2a). Heterologous hyperimmune serum, i.e., anti CDV (G-2) and anti RV (C-185), also demonstrated the four structural proteins of MV. It is noteworthy that anti CDV hyperimmune serum reacted with all the four proteins in contrast to the result of Hall et al. (1980) who claimed the absence of cross reaction with H protein of MV with anti CDV serum.

The four structural proteins of CDV were demonstrated with heterologous antiserum to MV and RV as well as with homologous anti CDV serum (Fig. 2b).

The cross reactions with structural proteins of RV was observed with both homologous and heterologous antiserums with similar patterns described for CDV (Fig. 2c).

Thus, reciprocal cross reactions with H, NC, F, and M proteins were clearly demonstrated among MV, CDV, and RV with hyperimmune serums.

**DISCUSSION**

The presence of cross-reacting antigens among the three morbilliviruses, MV, CDV, and RV, has been shown by ordinary serological techniques such as
Fig. 2. Cross reactivity of major structural proteins examined by hyperimmune serums (a: MV, b: CDV, c: RV).
virus neutralization test, complement fixation test, and indirect immunofluorescent technique (Yamanouchi et al., 1970; Orvell and Norrby, 1974). Recently, common antigenicities were also shown for their structural proteins (Stephenson and ter Meulen, 1979; Hall et al., 1980). However, polypeptide analysis of RV has not been reported thus far. Therefore, reciprocal antigenic relationships at structural protein level among all the three morbilliviruses are unknown.

In the present study, four major structural proteins, H, NC, F, and M proteins, were examined as the other two proteins, L and P proteins, were not detected under the present experimental conditions. The four structural proteins of MV and CDV were confirmed to resemble to each other as reported previously by other investigators (Waters and Bussell, 1973; Bussell et al., 1974; Campbell et al., 1980; Hall et al., 1980; Orvell and Norrby, 1980). In addition, RV was found to consist of the structural proteins with similar molecular weights to those of MV and CDV. As a remarkable feature of RV structural proteins compared with those of MV and CDV, a larger molecular weight of M protein of RV can be pointed out. H protein of RV was shown to be glycosylated like that of the other two morbilliviruses.

Glycosylation of F protein of morbilliviruses has not been fully studied. It is known that F₀, the precursor of F protein, is glycosylated in both MV and CDV. F protein consists of F₁ and F₂ which are produced by proteolytic cleavage of F₀, and F₁ of MV was shown not to be glycosylated (Mountcastle and Choppin, 1977; Tyrrell and Norrby, 1978; Hardwick and Bussell, 1978; Anttonen et al., 1980). On the other hand, glycosylation of F₁ of CDV is still controversial. Bussell et al. (1974) and Hall et al. (1980) reported glycosylation of F₁ of CDV, whereas Orvell (1980) recently claimed that F₁ of CDV is not glycosylated. Our results favor those of Hall et al. (1980). Glycosylation of F₁ of RV is unknown. We demonstrated that F₁ of RV (expressed as F protein in the text since F₂ not detected under the present experimental conditions) is not glycosylated as in MV. F₂ protein of both MV and CDV has been shown to be glycosylated. Analysis of F₂ of RV remains to be done.

Cross antigenic reactions at structural protein level among morbilliviruses were demonstrated reciprocally between MV and CDV. Antigenic relationship with RV was examined with two viruses, i.e., MV and CDV, and antisera to MV, CDV, and RV. The present study was designed to use all three viruses as well as their respective antisera raised in their natural hosts. Although the convalescent serums showed one-way cross reactions in some combinations of antisera and viruses, the hyperimmune serums clearly demonstrated the presence of cross antigenic relationships among the four structural proteins of the three viruses. It should be pointed out that the hyperimmune serums demonstrated reciprocal cross reactions of H proteins between MV and CDV. In contrast to the report by Hall et al. (1980), in which anti MV serum reacted with H protein of CDV, whereas anti CDV serum failed to react with that of MV.

Thus far, only one serological type has been known for each virus of
morbillivirus subgroup. The demonstration of common antigenicity of each structural protein among MV, CDV, and RV may have taxonomical significance, indicating stable antigenicity of each structural protein of these viruses.

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