Polypeptides and Functions of Antigens from Human Coronaviruses 229E and OC43

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Coronaviruses possess three major size classes of polypeptides as judged by molecular weight: ~180,000, ~50,000, and ~23,000. Human coronaviruses 229E and OC43 possess not only three similar size classes of polypeptides but also three distinct antigens, none of which cross-react with the heterologous strain. Polypeptides separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were reacted in rocket immunoelectrophoresis with antiserum monospecific to each of the three strain-specific antigens (excised precipitin lines from crossed immunoelectrophoresis profiles were used for immunogens). Monospecific antiserum with neutralizing ability reacted with a polypeptide of 186,000 daltons for 229E and a polypeptide of 190,000 daltons for OC43. The antigen which elicited neutralizing antibody response was located at the surface, associated with the corona of the virion, glycosylated, and bound by concanavalin A. Another less prominent surface antigen was represented by size classes of 23,000 daltons for 229E and 24,000 for OC43. The core antigens of the viruses had molecular weights of 49,000 for 229E and 52,000 for OC43 virus. Thus, the molecular weights and functions of the antigens of human coronaviruses are similar to those of animal coronaviruses. The polypeptides of coronaviruses 229E and OC43 are nearly identical as judged by molecular weight, but the similar polypeptides of the two viruses represent different immunological specificities.

Considerable knowledge concerning the biological and biophysical properties of coronaviruses has accumulated during the past few years. In contrast, knowledge concerning the antigenic composition of coronaviruses, particularly human strains, has advanced little because studies have been limited by two major obstacles: lack of potent reagents (serological test antigens and antisera) and the difficulties of differentiating host cell and serum antigens from viral components (6, 13, 14, 19). Our previous study showed that as many as 10^9 PFU of coronaviruses 229E and OC43 per ml can be obtained in human rhabdomyosarcoma cell cultures (22). As a consequence of the improved growth, we have prepared antiserum which resolved three viral antigens in serological test antigens prepared for each virus: 229E and OC43 (23). Although each of the three antigens was specific to the virus of origin without evidence of cross-reactions, the electrophoretic mobilities were highly similar, suggesting similar biological function. In addition, we have pre-

pared antisera monospecific to individual viral antigens which have permitted the demonstration that one antigen (with the slowest electrophoretic mobility of the three antigens) elicits neutralizing antibody response in the case of 229E and both neutralizing and hemagglutination-inhibiting antibody responses in the case of OC43 virus.

The purpose of this paper is to describe the characteristics of the three coronavirus antigens in terms of localization and biochemical characteristics including determination of the molecular weights of the antigens.

MATERIALS AND METHODS

Viruses and antisera. Coronaviruses 229E and OC43 were propagated in human rhabdomyosarcoma cell cultures (22). Viruses were purified by rate zonal and isopycnic centrifugation and concentrated to 4 mg of protein per ml as described previously (23). The preparation of monospecific antisera and antiserum to whole virus was also described previously (23).

Immunological identification of antigens. Techniques and conditions employed in two-dimensional immunoelectrophoresis and methods used to identify virus specific precipitin peaks were described previously (23). The method of Bog-Hansen and Brogren (4) for crossed affino-immunoelectrophoresis was used to

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identify glycoproteins with lectin binding sites. Absorption of antiserum for demonstration of viral surface antigens was carried out by absorbing 1 ml of antiserum with 10^{10} PFU of purified virus at 37°C for 2 h and at 4°C for 8 h. Precipitates were removed by centrifugation. The profiles obtained with the absorbed antiserum were compared with those with unabsorbed antiserum. Antigens which showed significant increases in peak height (i.e., significant antibody) were judged to be surface antigens (1). Charge-shift immunoelectrophoresis (2, 3, 12) was carried out as described by Alexander and Kenny (2) by using concentrations of deoxycholate ranging from 0.05 to 0.3% in the first-dimension gel.

Immunoabsorbent chromatography. Gamma globulins were purified from rabbit monospecific antiserum by fractionation DEAE-Sephadex A-50 (9). The purity of fractionated gamma globulin was established by immunoelectrophoresis, and protein concentration was determined by the method of Lowry et al. (16). The methods described by Caldwell and Kuo (7) were used to couple 15 ml of gamma globulin (80 mg of protein) in 0.1 M PO_4 buffer (pH 7.0) to 1 g of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif.) One milliliter of Triton X-100-solubilized viral antigens (2 to 3 mg of protein) was adsorbed to the column and eluted with 2 M NaI in 0.05 M PO_4 buffer containing 0.1% Triton X-100.

Polyacrylamide gel electrophoresis. High-pH discontinuous buffer polyacrylamide gel electrophoresis was performed by the method of Laemmli (15). Gels were cast in a Hoefer dual vertical slab gel electrophoresis cell, model SE 500 (Hoefer Scientific Instruments, San Francisco, Calif.), and consisted of a 5% separating gel and an 8.5% resolving gel. Samples (80 to 100 μg of protein) were solubilized in sample buffer so that the mixture contained 0.05 M Tris-hydrochloride (pH 6.8), 1% sodium dodecyl sulfate (SDS), 5% beta-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue. Gels were electrophoresed at 15 mA per slab gel until stacking had occurred; the current was then increased to 30 mA per slab gel. Gels were stained for 1 h at 60°C by using 0.25% Coomassie brilliant blue stain in 45% methanol-10% acetic acid-water solvent and destained in 20% ethanol-7% acetic acid-water solvent. The following standard markers (and molecular weights) were used: myosin (200,000), beta-galactosidase 130,000, phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300). Molecular weights of viral polypeptides in gels were estimated by the method of Shapiro et al. (24).

Polyacrylamide rocket immunoelectrophoresis. SDS-treated proteins were analyzed by the method of Schafer-Nielsen and Bjerrum (21). Polyacrylamide strips were embedded on 5 by 7-cm glass slides in 7 ml of 0.6% agarose in buffer containing 0.3% sodium deoxycholate and 1% Triton X-100. After the agarose solidified, agarose was removed from $\frac{3}{4}$ of the slide (away from the embedded polyacrylamide strip) and replaced by agarose (4 ml) containing 20 to 30% antiserum and 1% Triton X-100. The electrophoresis buffer was supplemented with a final concentration of 0.1% sodium deoxycholate and 1% Triton X-100. Electrophoresis was carried out at 3.5 V/cm for 10 h.

RESULTS

Correlation of coronavirus antigens with virion structure. When purified virions were subjected to mild proteolysis with bromelain (1 mg/ml) at 37°C for 1 h (8), the viral projections were removed, but the virions remained completely enveloped as judged by electron microscopy. Their density shifted from 1.18 to 1.15 g/ml when treated virions were rebanded by equilibrium centrifugation on preformed renografin gradients in the presence of 1 mM *p*-chloromercuribenzoate to stop the enzymatic reaction. Analysis of the bromelain-treated virions by two-dimensional immunoelectrophoresis revealed that antigens 229E 0.25 and OC43 0.23 were removed from the electrophoretic profile (Fig. 1). Harsher proteolytic treatment of virions with bromelain (2 mg/ml) at 37°C for 2 h eliminated the slow-migrating antigens and, in addition, reduced the peak height of the intermediate-migrating antigens as compared with untreated virus controls, whereas the fast-migrating antigens were unaffected. Finally, when virions were subjected to exhaustive proteolysis with pronase (2 mg/ml) at 37°C for 8 h, we observed a 30% reduction in the peak height of

FIG. 1. Effect of limited proteolysis on the antigenic composition of 229E and OC43 virus. Virions were subjected to limited proteolysis with bromelain (1 mg/ml) at 37°C for 1 h and banded in density gradients before electrophoresis. The antigenic profiles of 229E and OC43 virus, untreated (a, c) and after proteolytic treatment (b, d) are shown for comparison. Sixty micrograms of protein was electrophoresed in the first phase. The second-dimension gel contained 15% rabbit 229E antiserum (a, b) and 10% OC43 antiserum (c, d). Peaks appearing at the origin in frames b, c, and d are artifacts of incomplete virus solubilization because they were removed by centrifugation of the samples (not shown).

the fast-migrating antigens and elimination of all other virus precipitin peaks, whereas in untreated virus controls a 1:4 dilution of antigen resulted in a 75% reduction in the peak height of the antigens, but still allowed for detection of the other two antigens. In addition, the fast-migrating antigens were associated with a component containing nucleic acid since after exhaustive proteolysis with pronase they could be pelleted through a renografin cushion of 1.50-g/ml density, but were no longer detectable when virions were treated with 0.1% SDS for 15 min before enzymatic digestion.

The surface nature of the antigens also was demonstrable by absorption of antisera with intact homologous virus. The precipitin peaks of the slowest-migrating antigens were almost entirely removed in antigenic profiles obtained with absorbed antisera as compared with untreated sera and sera absorbed with heterologous virus, whereas the peak areas of the fastest antigens remained unchanged. The intermediate antigens had increased peak areas and decreased intensities of staining.

Membranes, or membrane-associated proteins, are amphiphilic; their electrophoretic mobility is increased under charge shift conditions (2, 3, 12) where immunoelectrophoresis is carried out in gels containing both a nonionic and a charged detergent (Triton X-100 and sodium deoxycholate, respectively, for these experiments). The electrophoretic mobility of antigen OC43 0.23 was increased to 0.43 when electrophoresis was carried out in the presence of 0.5% Triton X-100 and 0.1% deoxycholate (Fig. 2). Similar results were obtained for antigen 229E 0.25. The fastest-migrating antigens of both viruses did not show charge shift. A moderate charge shift was observed with antigens OC43 0.41 and 229E 0.44, but this required a higher concentration of deoxycholate (0.3%) which has been shown to provide ambiguous results in

FIG. 2. Electrophoretic mobility of antigen 0.23 under normal and charge shift conditions. Antigen (60 μ g of protein of solubilized whole virus) was electrophoresed in the first phase in the presence of 0.5% Triton X-100 (a) or 0.5% Triton X-100 and 0.1% sodium deoxycholate (b). For second dimension electrophoresis, gels contained 15% of monospecific anti-serum to antigen 0.23.

FIG. 3. Crossed affino-immunoelectrophoresis of OC43 virus. Solubilized virus (60 μ g of protein) was electrophoresed in the first phase (anode to the left). For second-dimension electrophoresis, the intermediate gel contained 750 μ g of unbound concanavalin A and the top gel contained 25% rabbit OC43 antiserum (anode at top of figure).

determining the amphiphilic nature of mycoplasmic antigens (2).

When Triton X-100-solubilized whole virus was tested with unbound lectins by crossed affino-immunoelectrophoresis (4), antigens OC43 0.23 (Fig. 3) and 229E 0.25 (not shown) formed a precipitin peak with concanavalin A, indicating that they contain carbohydrate residues with at least two ConA binding sites. The two other antigens for each virus were not affected by the ConA intermediate gel, nor were any of the antigens bound by peanut or wheat germ lectin.

These results indicate that with either virus the slowest-migrating antigen which elicits neutralizing antibody response in rabbits is associated with the corona of the virion, whereas the fastest-migrating antigen is internal and appears associated with a substructure containing nucleic acid. The antigen of intermediate electrophoretic mobility appears associated with the membrane; however, its exact location could not be ascertained.

Virion structural polypeptides. Three major size classes of structural polypeptides were resolved with either virus by polyacrylamide gel electrophoresis with apparent molecular weights of 186,000, 49,000 and 23,000 for 229E virus (Fig. 4A) and of 190,000, 52,000 and 24,000 for OC43 virus (Fig. 5A). The only major polypeptides which did not contain carbohydrate as judged by Alcian blue staining (29) were the intermediate size polypeptides. Boiling of 229E virus samples for 2 min before electrophoresis in the presence of 1% SDS and 5% beta-mercaptoethanol had no effect on the polypeptide pattern (Fig. 4B). In contrast, under the same conditions the small polypeptides of OC43 virus disappeared, and a large-molecular-weight aggregate appeared on top of the gel (Fig. 5B). Furthermore, when the reducing conditions or

FIG. 4. Polypeptide composition of 229E virus. A, Unboiled; B, boiled. Virus samples (100 μ g of protein) were examined on 8.5% SDS-polyacrylamide slab gels as described in the text. The electrophoretic profiles of molecular weight standards are shown in the two tracks to the left of the virion polypeptides.

duration of boiling (or both) was varied, new polypeptides appeared with molecular weights from 30,000 to 48,000 with a concomitant decrease in the small polypeptides. Subjection of virus to limited proteolysis with bromelain resulted in loss of the large polypeptide (Fig. 5C), whereas only the medium-size polypeptide was resistant to exhaustive digestion with pronase (Fig. 5E). Similar results for bromelain and pronase treatment were observed with 229E virus (data not shown).

Correlation of specific antigens with virion structural polypeptides. The antigen which elicited neutralizing antibody response in rabbits was isolated from Triton X-100-solubilized OC43 virus by immunosorbent chromatography with monospecific neutralizing antiserum. When tested by SDS-polyacrylamide gel electrophoresis, two polypeptides with apparent molecular weights of 190,000 and 105,000 were observed (Fig. 5D), but only one precipitin peak was resolved in two-dimensional immunoelectrophoresis against either monospecific neutralizing or whole virus antiserum with an electrophoretic mobility of 0.23 (Fig. 6).

The antigenicity of OC43 viral polypeptides was tested by rocket immunoelectrophoresis (21). Each size class of polypeptides formed a precipitin peak with antiserum to whole virus (Fig. 7A), but did not react with antiserum to host cells or calf serum. Removal of the large

polypeptide by limited proteolysis resulted in loss of the corresponding precipitin peak (Fig. 7E). In contrast, monospecific neutralizing antiserum formed a precipitin peak only with the largest polypeptide (190,000 daltons) in highly purified virus preparations (Fig. 7B). Additionally, a component of 105,000 daltons was evident in less purified samples and also reacted with antiserum to complete virus (Fig. 7A). Apparently, those polypeptides share a common antigenic determinant. Monospecific antiserum prepared against antigen 0.44 formed a major precipitin peak with the small polypeptide of 24,000 daltons. However, precipitin peaks with other polypeptides of the small size class formed and fused (observed also with antiserum to complete virus). Therefore, the small polypeptides share common antigenic determinants (Fig. 7A and E). The intermediate size polypeptide reacted with monospecific antiserum prepared against antigen 0.56 (Fig. 7D). It also reacted with whole virus antiserum which was absorbed with whole virus. Results with 229E virus were similar: whole virus antiserum reacted with each size class of polypeptides (Fig. 8A); removal of the large polypeptide by limited proteolysis resulted in loss of the corresponding precipitin peak (Fig. 8E); whereas monospecific antisera

FIG. 5. Polypeptide composition of OC43 virus. A, Unboiled; B, boiled; C, after limited proteolysis and isopycnic rebanding on renografin gradient; D, antigen purified from Triton X-100-solubilized virus by immunoadsorption with monospecific neutralizing antiserum; E, after exhaustive proteolysis. Virus samples (100 μ g of protein in A through D, unknown in E) were examined on 8.5% SDS-polyacrylamide gels as described in the text. The molecular weights of standards are indicated.

FIG. 6. Electrophoretic profile of antigen 0.23 purified from Triton X-100-solubilized OC43 virus by immunoabsorption with monospecific neutralizing antiserum. The second-dimension gel contained 10% antiserum to whole virus.

to antigens 0.25, 0.44 and 0.57 reacted with the large, small, and intermediate-size polypeptides, respectively (Fig. 8B, C, and D).

DISCUSSION

The assignment of specific antigens to virion substructures is difficult since the fine structure of human coronaviruses is little understood. Our studies show that antigens OC43 0.23 and 229E 0.25, which elicit neutralizing antibody response, are clearly associated with the corona of the virus since they can be removed by mild proteolysis which leaves virions fully enveloped. Similar findings were reported for porcine coronavirus by Bohac and Derbyshire (5). A prominent surface location of these antigens was also evidenced by absorption of antisera with whole virus. In addition, lectin binding and charge shift reactivity are properties in line with surface glycoproteins of enveloped viruses. The fastest electrophoretic antigen (0.56 for OC43 and 0.57 for 229E) appeared to be associated with an internal component containing nucleic acid as judged by resistance to exhaustive proteolysis and sedimentation properties after proteolysis in the presence or absence of SDS. Furthermore, absorption of antiserum with purified virus did not remove antibody to these antigens. No direct evidence was provided in our experiments as to the location in the virion of the antigen with intermediate mobility. However, studies of antiserum absorption, charge shift reactivity, and sensitivity to proteolytic treatment suggest that this antigen is associated with the viral membrane, a notion also suggested by polypeptide studies.

The polypeptide composition and association with virion structure has been reported for several coronaviruses; therefore, correlation of virion structural polypeptides with viral antigens might provide further information as to the location of antigens in the virion. The polypeptide

patterns shown by OC43 and 229E virions in our study, when grown, purified, and analyzed under identical conditions, were almost the same and closely resembled those reported by Macnaughton (17) as well as those reported for three animal coronaviruses (10, 20, 25): the largest polypeptides, ranging from 180,000 to 200,000

FIG. 7. Association of OC43 viral polypeptides with specific antigenic activity. Electropherograms of virus (rate zonally and isopycally purified) were prepared on 8.5% SDS-polyacrylamide slab gels. Gel strips (shown at base of profiles) were excised and reacted in rocket immunoelectrophoresis with antisera. A, Whole virus antiserum; B, monospecific antiserum to antigen 0.23 (corona); C, monospecific antiserum to antigen 0.44 (membrane); D, monospecific antiserum to antigen 0.56 (core). Major viral polypeptides are indicated by large arrows; minor viral polypeptides are indicated by small arrows. E, Highly purified virus was subjected to limited proteolysis with bromelain (1 mg/ml at 37°C for 1 h), rebanded isopycally, and then electrophoresed on 8.5% SDS-polyacrylamide slab gel. The electropherogram (shown) was reacted in rocket immunoelectrophoresis with whole virus antiserum.

FIG. 8. Association of 229E viral polypeptides with specific antigenic activity. Viral electropherograms were prepared on 8.5% SDS-polyacrylamide slab gels. Gel strips (shown at base of profiles) were excised and reacted in rocket immunoelectrophoresis with antisera. A, Whole virus antiserum; B, monospecific antiserum to antigen 0.25 (corona); C, monospecific antiserum to antigen 0.41 (membrane); D, monospecific antiserum to antigen 0.57 (core). Major viral polypeptides are indicated by arrows. E, Highly purified virus was subjected to limited proteolysis with bromelain (1 mg/ml at 37°C for 1 h), rebanded isopycally, and then electrophoresed on 8.5% SDS-polyacrylamide slab gel. The electropherogram (shown) was reacted in rocket immunoelectrophoresis with whole virus antiserum.

daltons, were glycosylated and could be removed from the virions by mild proteolytic treatment; an intermediate-size nonglycosylated polypeptide of about 50,000 daltons, which was identified as core protein; and small glycoproteins of 23,000 to 30,000 daltons, associated with membrane protein. However, these polypeptide patterns do not fully agree with those shown by other laboratories for OC43 and 229E viruses (13, 14). In these studies, an additional small polypeptide of about 16,000 daltons was identified which could be removed by mild proteolysis. On the other hand, the largest polypeptide of about 195,000 daltons was found to contain lipid and was not affected by mild proteolytic treat-

ment. In addition, a minor polypeptide of 165,000 daltons was found.

These discrepancies might reflect differences in virus growth and purification methods and different conditions in polypeptide analysis. Several problems became apparent during polypeptide analysis: preparation of "pure virus," assignment of minor polypeptides, and presence of viral polypeptides in aggregated forms. The polypeptide patterns of virions grown in either diploid or heteroploid cells were identical. However, with cell-associated virus, problems of purification were greater, and additional polypeptides were found, possibly due to immature virions. For that reason, virions released from infected cells into growth medium were used in our studies. The use of specific antisera to differentiate viral from host cell components was found important in our studies: major polypeptides of 200,000 and 15,000 daltons in less purified virus samples and minor polypeptides in more highly purified samples were identified as host cell components. Finally, with a mouse coronavirus, Sturman et al. (25, 27) have shown that solubilization conditions employed in peptide analysis affected the polypeptide profile due to aggregate forms of the envelope glycoprotein. In our study, solubilization conditions (particularly heating with reducing agents) markedly affected the overall pattern as well as the number of polypeptides seen with OC43 virus.

The effect of mild enzymatic treatment on the polypeptide composition of OC43 and 229E virions was similar to results reported for other coronaviruses (11, 20, 25): the largest polypeptides were removed. These polypeptides reacted with whole virus or monospecific neutralizing antiserum. In addition, a polypeptide of about 105,000 daltons was found in OC43 virus which also reacted with these antisera. This polypeptide was found more often in less purified virus samples but also in antigen purified from Triton X-100-solubilized virions by immunoabsorption with monospecific antiserum. We do not know whether the smaller polypeptide is derived from the larger one as is the case with mouse coronavirus (26); however, the two polypeptides share a common antigenic determinant.

A non-glycosylated polypeptide of about 50,000 daltons was found in all animal coronaviruses studied (10, 13, 14, 17, 18, 20, 25) and is generally agreed to represent core protein (28). The polypeptide is not degraded when virions are subjected to exhaustive proteolysis. In our studies, a polypeptide with similar properties was found in both OC43 and 229E virions; this polypeptide reacted with antiserum absorbed with whole virus and, importantly, with monospecific antiserum prepared against the internal, fast-migrating antigen.

TABLE 1. Characteristics of coronavirus antigens as measured by quantitative immunoelectrophoresis

Viral antigen ^a	Charge shift ^b	Lectin reactivity ^c	Bromelain sensitivity ^d	Pronase sensitivity ^e	Adsorption ^f	Mol wt ^g
OC43 0.56	—	—	—	—	—	52,000
OC43 0.44	±	—	—	+	+	24,000
OC43 0.23 ^h	+	+	+	+	++	190,000
229E 0.57	—	—	—	—	—	49,000
229E 0.41	±	—	—	+	+	23,000
229E 0.25 ^h	+	+	+	+	++	186,000

^a Strain of virus and electrophoretic mobility (23) of specific antigen.

^b +, Significant change of electrophoretic mobility of indicated antigen on addition of 0.1% deoxycholate; ±, charge shift only on addition of 0.3% deoxycholate; —, no change in mobility at 0.3% deoxycholate.

^c +, Deletion of peak by concanavalin A on immuno-affinoelectrophoresis; —, peak unchanged (i.e., does not contain carbohydrates which concanavalin A binds).

^d Whole virus subjected to limited proteolysis with bromelain; +, antigen removed by treatment.

^e Whole virus subjected to exhaustive proteolysis; +, antigens removed by treatment.

^f +, Peaks which show significant increases in peak height upon adsorption of serum with intact purified virus (i.e., components which have a surface location).

^g Values from polyacrylamide gel electrophoresis.

^h Antigen elicits neutralizing antibody response in rabbits and humans.

Finally, the smallest polypeptides of OC43 and 229E viruses were in the size range of membrane-associated polypeptides of animal coronaviruses (28). In the case of mouse coronavirus, they exhibited anomalous behavior on heating in presence of reducing agents and were shown to give rise to new polypeptide species (25, 27). Similar properties were observed in our studies with the small polypeptides of OC43 virus. These polypeptides reacted with whole virus antiserum and monospecific antiserum prepared against the intermediately migrating antigen which appeared membrane associated and were shown to share a common antigenic determinant. Multiple bands in the region of the small size class of polypeptides whose relative proportions varied with different preparation were also observed with 229E virus. They were shown to share a common antigenic determinant when tested against whole virus antiserum or monospecific antiserum prepared against the intermediately migrating antigen. With both viruses, these antigens showed erratic migration behavior during two-dimensional immunoelectrophoresis (23), suggesting the presence of aggregated forms which might correlate with the observed properties of the small polypeptides.

Thus, the position of coronavirus antigens in the virion, as determined by two-dimensional immunoelectrophoresis, was substantiated by polypeptide studies (Table 1). The model proposed by Sturman et al. for mouse coronavirus (27) would fit our data well, with the largest polypeptide being analogous to their component E2, the peplomer glycoprotein; the smallest being analogous to E1, the membrane protein; and the intermediate being analogous to N, the nucleoprotein. Although our studies were limited by the resolving power of the antisera, it is

nevertheless striking that the polypeptides of the two human coronavirus strains 229E and OC43, although highly similar in molecular weight, should represent six different antigenic specificities with no common components between the viruses.

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