ANTIGENIC RELATIONSHIPS OF CORONAVIRUSES DETECTABLE BY PLAQUE NEUTRALIZATION, COMPETITIVE ENZYME LINKED IMMUNOABSORBENT ASSAY, AND IMMUNOPRECIPITATION

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ABSTRACT

The antigenic relationships of mouse coronaviruses JHM and A59, human viruses OC43 and 229E, and multiple sclerosis (MS) isolates SD and SK have been investigated by plaque neutralization, competitive enzyme linked immunoabsorbent assay, and immunoprecipitation.

A59, SK, or SD plaques are neutralized by antiserum prepared against homologous as well as heterologous virus. Plaque neutralization also demonstrated weak reactivity between SD or SK and mouse virus JHM but no reactivity with human coronavirus 229E. An antiserum prepared against human virus OC43 neutralized viruses SD and SK but not mouse viruses A59 or JHM.

In a competitive enzyme linked immunoabsorbent assay (cELISA) the binding of antiserum prepared against MS isolate SK to bound SK antigen was inhibited to a comparable degree using OC43, SD, or A59 viral antigens. Coronavirus 229E or uninfected cell antigens did not block the binding of anti-SK serum to bound antigen. However, a cELISA utilizing OC43 as bound antigen and competing an anti-OC43 serum suggests that virus OC43 may be more closely related to SK than A59.

Specific viral polypeptides that share antigenic determinants have been identified by immunoprecipitation of S^{35} methionine labeled viral infected cell extracts. Polypeptides of similar molecular weight were precipitated from A59, SD, or SK infected cell extracts

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by SD, SK, OC43, or A59 antisera. Our data suggests that the mouse coronavirus A59, human coronavirus OC43, and MS isolates SD and SK contain antigenically related polypeptides of similar molecular weight.

INTRODUCTION

We recently reported the isolation of two coronaviruses from fresh, unfrozen multiple sclerosis (MS) autopsy material (3). One of these viruses (SD) was detected following the intracerebral (IC) inoculation of weanling BALB/c mice. The second isolate (SK) was evident on the twelfth subculture of mouse 3T3 (17Cl-1) cells inoculated with homogenized MS tissue. Chronic or latent infections by murine coronaviruses are frequently encountered in mouse breeding colonies (4,7,18). Therefore, careful evaluation of the human or murine origin of these MS isolates is necessary. In an attempt to identify species specific antigenic markers, we have compared the antigenic properties of human coronaviruses 229E and OC43 and murine coronaviruses JHM and A59 with MS isolates SK and SD. The viruses are compared by plaque neutralization, competitive enzyme linked immunoabsorbent assay (cELISA), and by immunoprecipitation utilizing homologous and heterologous antisera.

MATERIALS AND METHODS

Cells and virus. The source of known human and mouse coronaviruses has been previously described (5). Coronaviruses SD and SK are viruses isolated from multiple sclerosis patients as previously described (3). Mouse viruses, A59 and JHM, and MS isolates, SD and SK, were grown in 17Cl-1 cells and purified from supernatant fluids by polyethylene glycol (PEG) precipitation and sucrose density gradients (14,19). Human virus 229E was grown in WI38 cells. Human virus OC43 was grown in suckling mouse brain utilizing C57 black mice (15). Virus concentrations of OC43 was estimated by hemagglutination of chicken erythrocytes (11).

Antiserum. Antiserum directed against coronaviruses A59, SK, and SD were produced utilizing sucrose gradient purified virus injected into rabbits, guinea pigs, and mice according to protocol previously described (5). Antiserum to human coronaviruses are reference antisera obtained from the Center for Disease Control and kindly supplied by Dr. Harold Kaye. Antisera directed against OC43 virus was titered by hemagglutination inhibition (9).

Plaque neutralization. Coronaviruses SD, SK, A59, and JHM produced plaques on confluent monolayers of DBT cells (8) grown in 60 mm petri dishes. Details of the method will be published elsewhere (5). Human virus 229E was plaque assayed on confluent monolayers of WI38 cells utilizing a similar technique. All antisera used in plaque neutralization studies were heat inactivated at 56^{o}C for 30 minutes. Plaque

neutralizations for the various antisera were determined by a comparison of the degree of neutralization of the test antisera compared to control plaque numbers. Control neutralizations utilized fetal calf serum or preimmune serum diluted to a comparable level to that of the test antiserum dilution.

cELISA. The ELISA technique utilized alkaline phosphatase conjugated anti-IgG of the appropriate species according to the methods described by Voller (20). Purified viral antigen was bound to Cooke M29AR (Dynatech Laboratories) microELISA plates overnight at 4°C in a humid chamber. After antigen binding, the plates were washed with phosphate buffered saline pH 7.2 (PBS) containing 0.5% TWEEN-20 for 10 min., then rinsed twice with distilled H_2O to prevent nonspecific binding. Viral antigens utilized as competitors were solubilized in a buffer containing 0.02 M Tris pH 7.4, 0.05 M sodium chloride, 0.5% deoxycholic acid (DOC), and 0.5% NP-40 (lysis buffer). Serial two-fold dilutions of competing antigen were incubated overnight at 4°C with a constant amount of antibody. Absorbed antisera was then reacted for 60 minutes at 37°C with antigen bound to the microELISA plate, washed, and reacted with conjugated anti-IgG and substrate. The colorimetric reaction was read utilizing a Titertek multiskan microELISA reader (Flow Labs).

The competing antigens for viruses SK and A59 were purified by sucrose density gradients prior to disruption with NP40 lysis buffer. Virus OC43 was partially purified from infected suckling mouse brain (20% homogenates) by adsorption and elution from human "O" erythrocytes according to the methods of Kaye et al (10). Control competitors included supernatants or cytoplasmic extracts of uninfected cells.

Immunoprecipitation. A detailed description of procedures for infected cell radiolabeling and immunoprecipitation will be published elsewhere (5). Briefly, uninfected or virus infected monolayers (multiplicity of infection 0.1-1.0 pfu/cell) of 17Cl-1 cells were radiolabeled with S^{35} methionine in the presence of 1 ug/ml of actinomycin D. Sixteen to twenty hours post-infection, cytoplasmic extracts were prepared utilizing the lysis buffer described above and nuclei were removed by centrifugation. Immunoprecipitation of viral specific peptides from these cytoplasmic extracts utilized formalin fixed and washed staphylococcus aureus, Cowan I strain, as described by Kessler (12). Precipitated antigen-antibody-Staph A complexes are washed five times with 1% Tritom-X 100, 1% DOC, and 0.15 M sodium chloride pH 7. The final pellet is resuspended in 50 microliters of sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 0.001% bronphenol blue, 62.5 millimolar Tris hydrochloride pH 6.8, 1.2 M Urea, 0.1% Beta mercaptoethanol. The proteins are solubilized at 37°C for 60 minutes and analyzed by SDS polyacrylamide gel electrophoresis (13). Polyacrylamide gels consisted of a 10-20% acrylamide gradient in Tris glycine cross-linked with N,N'-diallyl-tartardiamide (DATD) (6). Gels

were impregnated with PPO (2,5-diphenyloxazole) utilizing the methods of Bonner and Laskey (1). Molecular weights were estimated relative to radiolabeled VSV markers (21).

RESULTS

Reciprocal plaque neutralizations. Neutralization of plaque formation using antisera prepared against different coronaviruses is shown in Table 1. The degree of homology of the viruses can be measured by a comparison of neutralization values. These are defined as the highest serum dilution capable of 50% neutralization of 60-150 plaques. The results reveal that MS isolates SK and SD are antigenically related to A59 and more distantly to mouse virus JHM. Human coronavirus 229E appears to be distinct from the other viruses. However, antiserum prepared against OC43 neutralizes SD and SK but shows no neutralization at a 1:20 dilution of mouse viruses A59 or JHM. This result can be interpreted to indicate that MS isolates SD and SK are more closely related to the human virus OC43 than are the mouse viruses.

Hemagglutination and hemagglutination inhibition. The human coronavirus OC43 is known to hemagglutinate chicken and human "O" erythrocytes. We, therefore, investigated the ability of viruses SD and SK to hemagglutinate these cells. OC43 was the only virus capable of hemagglutinating erythrocytes. In addition, only antisera directed against OC43 inhibited this hemagglutination. Therefore, although viruses SK and SD are antigenically related to OC43, they differ from this virus in that they do not hemagglutinate erythrocytes and they replicate in mouse cell lines.

RECIPROCAL PLAQUE NEUTRALIZATIONS[1]

	Mouse		MS Isolates		Human
Antisera	JHM	A59	SK	SD	229E
Anti-SK	500	1,200	10,000	10,000	<20
Anti-SD	40	100	2,000	2,000	<20
Anti-OC43[2]	<20	<20	80	160	<20
Anti-229E	<20	< 20	<20	<20	1,000
Anti-A59	40	500	320	320	<20

[1]Numbers given are the highest serum dilution causing greater than 50% neutralization of approximately 100 plaques

[2]Hemagglutination inhibition titer for this antiserum utilizing chicken erythrocytes was 1:640

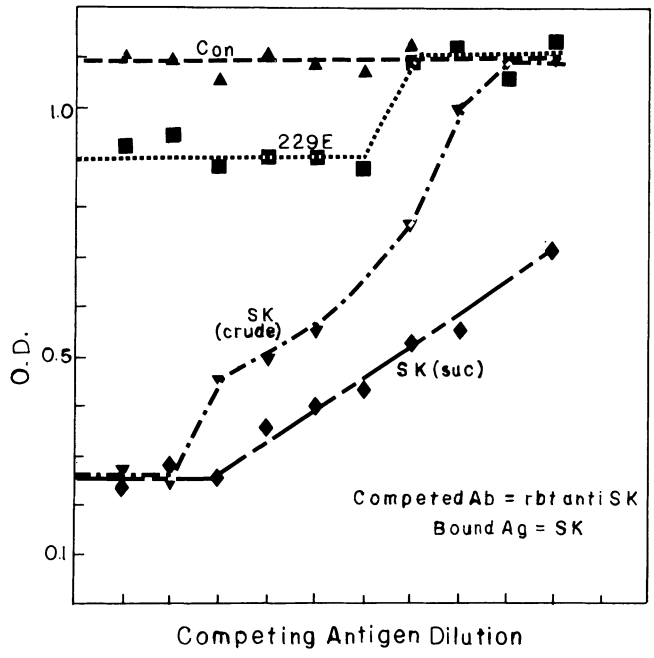

Figure 1: cELISA demonstrating absorption of rabbit antisera directed against coronavirus SK by NP40 extracts of homologous (SK) or heterologous (229E) viral antigen. Purified SK virus is the antigen bound to the microELISA plate. SK (crude) = virus obtained directly from infected 17Cl-1 cell supernatants used as competing antigens. SK (suc) = purified virus used as competing antigen. Con = uninfected cell supernatant as competing antigen.

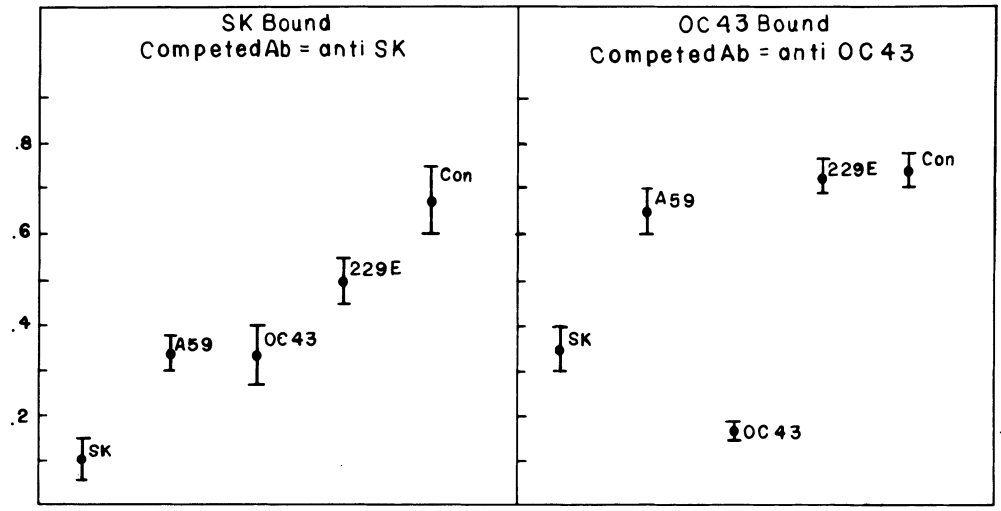

Figure 2: cELSIA using SK bound antigen (left) or OC43 bound antigen (right). Mean absorbance values ± one standard deviation are given for each competitive antigen at saturation.

Competitive enzyme linked immunoabsorbent assay (cELISA). This assay measures the relatedness of different viral antigens by their capacity to block the binding of specific antibody to viral antigen bound on the plastic surface of microELISA plates. For this assay purified viral antigen is bound to plastic using standard ELISA techniques (20). Serial dilutions of NP40 disrupted, competing viral antigens are incubated 10-24 hours at 4°C with a constant amount of antisera. This absorbed antisera is then reacted with the bound antigen. Utilizing homologous virus as the competing antigen results in a nearly complete inhibition of antibody binding to the antigen bound on the plastic (Figure 1). However, heterologous antigen inhibits binding to an extent proportional to the degree of homology. A representative experiment utilizing virus SK as the bound antigen and adsorbing rabbit anti-SK antiserum with the homologous (SK) antigen or heterologous (229E) antigen is shown in Figure 1. A linear relationship is observed for competing antigen concentrations capable of saturating the antibody. Extrapolation of this linear line results in a Y-intercept that is characteristic of the degree of homology. As is shown in Figure 1, this intercept is not altered by the degree of purity of the competing antigen. The heterologous antigen (229E) results in very little competition relative to that of the uninfected cell control.

In Figure 2, this method is used to determine the relationship between viruses SK, A59, OC43, and 229E. Figure 2A demonstrates that when SK is bound to plastic, both viruses A59 and OC43 compete at a comparable level for the antibody directed against SK while virus 229E does not compete. In Figure 2B, OC43 is bound to the microELISA plates and anti-OC43 serum is the competed antibody. The results of this experiment demonstrate that SK appears to be more closely related to the OC43 antigen than does A59. This observation confirms the results of the neutralization test (Table I) in which this antisera also recognized isolates SK or SD more readily than the mouse virus A59.

Immunoprecipitation of coronavirus SK or SD S^{35} methionine labeled cytoplasmic extracts. Viral specific polypeptides immunoprecipitated from SK-infected and SD-infected 3T3 cells are identical as is shown in Figure 3. The viral specific polypeptides are defined as those precipitated by immune serum from infected cells, but not detected in preimmune sera immunoprecipitations of infected cells or immune sera immunoprecipitation of uninfected cells. Molecular weight estimates relative to VSV markers indicate that coronavirus polypeptides have molecular weights of 180,000 (180K), 90,000 (90K), 50,000 (50K), 24,000 (24K), and 22,000 (22K). An additional polypeptide of 42,000 (42K) is observed in variable amounts in either SK or SD infections. Similar polypeptides are also observed in SK-infected cytoplasmic extracts that are immunoprecipitated with either anti-A59, SD, or OC43 sera (Figure 4). The inverse experiment (Figure 5) confirms that these same polypeptides are immunoprecipitated from A59 infection by

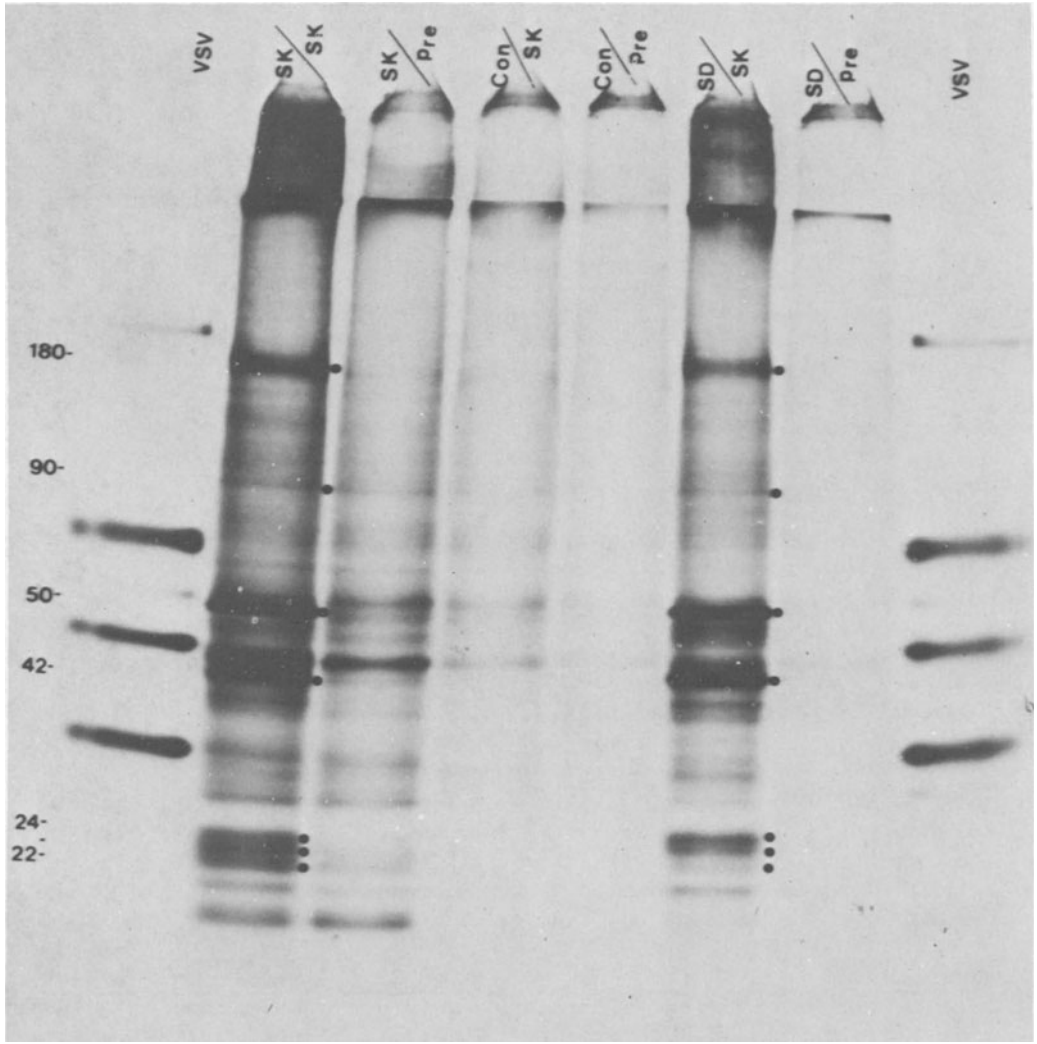

Figure 3: Polyacrylamide gel electrophoresis of S^{35} labeled polypeptides immunoprecipitated from SK or SD infected 17Cl-1 cytoplasmic extracts. Viral polypeptides are identified by black dots and estimated molecular weights given in kilodaltons. SK/SK = SK infected cell extract precipitated by anti-SK serum. SK/pre = SK infected cell extract precipitated with preimmune serum. Con/SK = uninfected cell extract precipitated with anti-SK serum. SD/pre = SD infected cell extract precipitated with preimmune serum. SD/SK = SD infected cell extract precipitated with anti-SK serum.

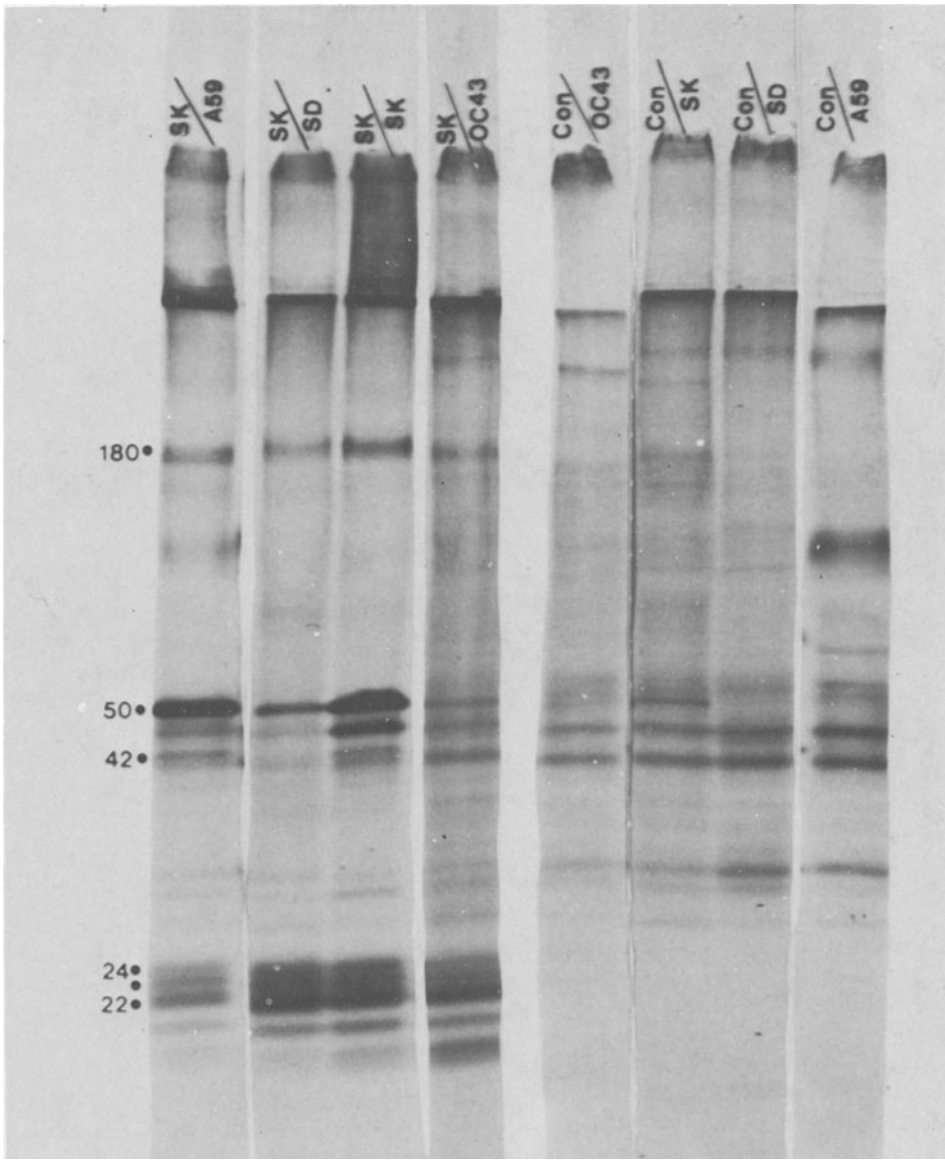

Figure 4: Polyacrylamide gel electrophoresis of S^{35} labeled polypeptides immunoprecipitated from SK infected 17Cl-1 cytoplasmic extracts utilizing homologous and heterologous antisera. SK/SK, SK/A59, SK/SD, SK/OC43 = polypeptides precipitated from SK infected cell extract by antisera directed against SK, A59, SD, and OC43. Con/SK, Con/A59, Con/SD, Con/OC43 = polypeptides precipitated from uninfected cell extract by antisera directed against SK, A59, SD, and OC43.

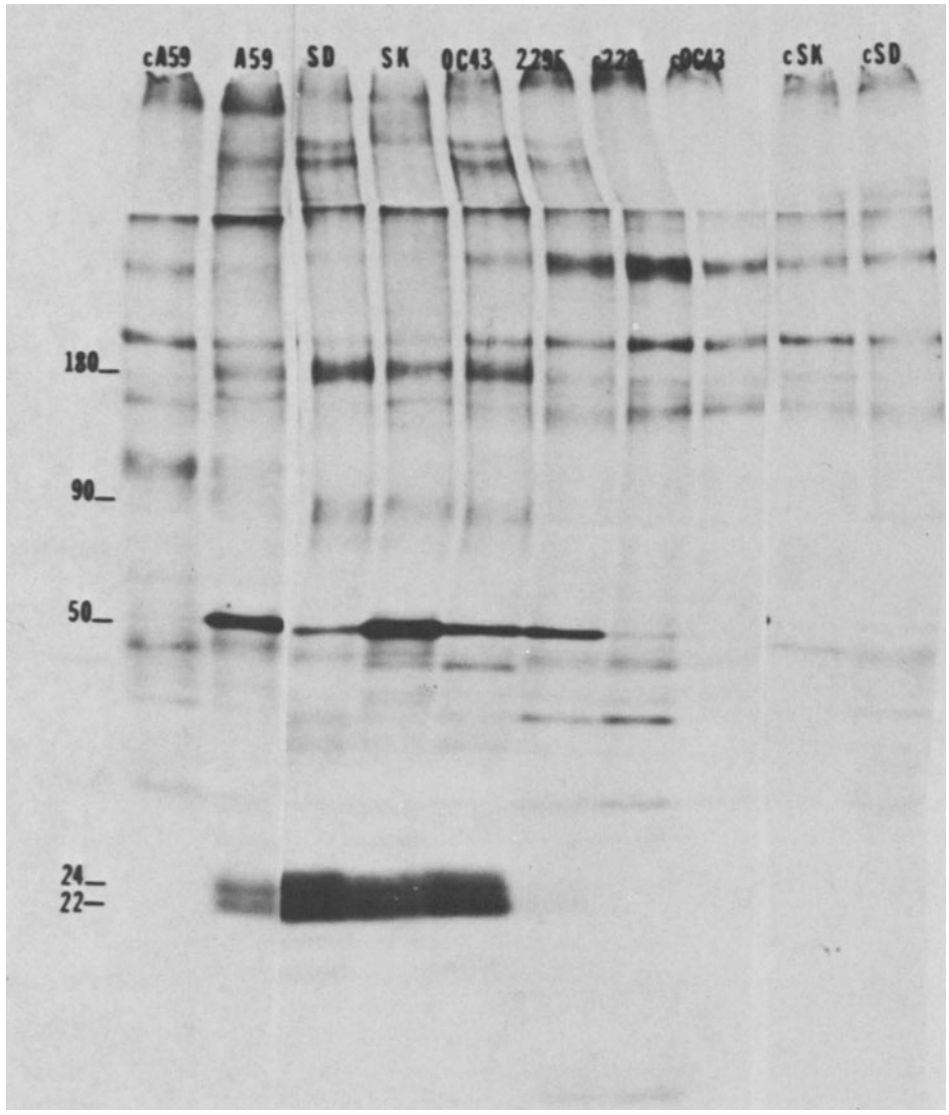

Figure 5: Polyacrylamide gel electrophoresis of S^{35} labeled polypeptides immunoprecipitated from A59 infected 17Cl-1 cytoplasmic extracts utilizing homologous and heterologous antisera. A59, SK, SD, OC43, 229E = polypeptides precipitated from A59 virus infected 3T3 cytoplasmic extract by antisera directed against A59, SK, SD, OC43, or 229E. cA59, cSK, cSD, cOC43 = polypeptides precipitated from uninfected 3T3 cytoplasmic extract by antisera directed against A59, SK, SD, 229E, or OC43.

antisera directed against A59, SD, SK, or OC43. Antiserum directed against human virus 229E recognizes only the 50K protein.

DISCUSSION

Antigenic cross-reactivity between human virus OC43 and various MHV strains has previously been reported by several investigators (2,7,16,17). For these studies cross-reactivity was demonstrated by fluorescent antibody, serum neutralization, or complement fixation tests. Our results suggest that coronaviruses SK and SD also belong in this antigenic group.

Utilizing cross-neutralization values and competitive enzyme linked immunoabsorbent assays, MS isolate SK is more closely related to human virus OC43 than is mouse virus A59. However, the more qualitative assay of immunoprecipitation suggests some degree of homology between OC43, A59, SD, and SK for all of the major component polypeptides. Since human and mouse coronaviruses share antigenic determinants, the identification of the species origin of unknown coronaviruses is not possible on the basis of antigenicity. In summary, we have shown that MS isolates SD and SK differ from human coronaviruses OC43 and 229E and mouse coronaviruses JHM and A59. However, coronaviruses A59, SD, SK, and OC43 all share antigenic determinants.

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