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Immunogenicity and Antigenicity of Human Coronaviruses 229E and OC43

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The immunogenicity and antigenicity of human coronaviruses 229E and OC43 were studied by quantitative immunoelectrophoresis. Three distinct antigens were recognized in both coronavirus strains when Triton X-100-solubilized whole virus was tested by two-dimensional immunoelectrophoresis against homologous rabbit antiserum. No antigens cross-reacted between strains, but the electrophoretic patterns against homologous antiserum were highly similar in that both strains had one electrophoretically fast antigen, one of intermediate mobility, and one of slow mobility. Immunization of rabbits with 10^9 plaque-forming units of virus was required for production of antiserum which recognized the three antigens; lesser amounts gave rise to antisera which recognized only one or two components. Precipitin lines excised from two-dimensional immunoelectropherograms were used successfully as immunogens to prepare monospecific antiserum to each of the antigens of OC43 and 229E. Monospecific antiserum to the slow component of 229E neutralized 229E only, and monospecific antiserum to the slow component of OC43 both neutralized and inhibited hemagglutination of OC43 virus. Human convalescent sera which possessed both complement-fixing and neutralizing antibody also recognized the slow-moving component.

Human coronaviruses have been identified as etiological agents of acute upper respiratory tract infections (11, 15, 18, 19, 28). Their role in disease of the lower respiratory tract (12) and other illnesses (6) is undefined. This gap in knowledge is related to two major problems in research of human coronaviruses: great difficulties in primary virus isolation and inadequate cell culture systems for mass virus propagation. Consequently, serological test procedures to detect infections are lacking for most human strains. Potent antisera are also needed for antigenic analysis of known serotypes. Variable success has been reported in the preparation of hyperimmune animal antisera (4, 13, 14, 20) and, therefore, the antigenic structure of human coronaviruses is little understood. We studied the growth characteristics of prototypes 229E and OC43 in human cell lines (23) and were able to obtain high virus titers (10^9 plaque-forming units [PFU]/ml). This report describes both the antigenic composition of coronaviruses 229E and OC43 and their immunogenicity for rabbits.

MATERIALS AND METHODS

Cell lines and virus strains. The source and cultivation of the human diploid fetal tonsil (FT) and human heteroploid rhabdomyosarcoma (RD) cells were described previously (23). Human coronaviruses 229E/WI-38/P_{14} (15) and OC43/BSC-1/P_5 (18) were received from A. Z. Kapikian, National Institutes of Health, Bethesda, Md. Both virus strains were selected for rapid growth in FT and RD cells and then plaque purified six times in both cell lines.

Plaque assay. Plaque assay was performed in RD cells as described previously (23). Neutral red staining was used to visualize plaques.

Serological test antigens. Both virus strains were grown at 33°C on RD cell monolayers prepared in roller bottles (1,585-cm^2 growth area). The multiplicity of infection was 10 PFU/cell, and virus adsorption was carried out at 23°C for 1 h. Maintenance medium was Eagle minimal essential medium supplemented with nonessential amino acids, 1% heat-inactivated fetal calf serum, and 20 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.0, at 33°C. Supernatant fluids were collected 42 h after infection, cellular debris was removed by centrifugation in a type GS-3 rotor in a Sorvall RC2-B Superspeed centrifuge at 4,000 × *g* for 30 min at 4°C, polyethylene glycol 6000 was added at a concentration of 7.5% (wt/vol), and the mixture was incubated for 6 h at 4°C. The precipitate was collected by centrifugation in a type GS-3 rotor at 13,200 × *g* for 40 min at 4°C, and the pellet was suspended in buffer (0.02 M HEPES-0.15 M NaCl-0.005 M disodium ethylenediaminetetraacetic acid), pH 7.0, and subjected to ultrasonic treatment for 2 min in a Bransonic model 12 ultrasonic cleaner to break up aggregates. Viral suspensions were concentrated by centrifuging through a column of 15% sucrose (10 ml) onto a cushion of 55% sucrose in buffer (4 ml) at 79,000 × *g* for 3 h at 4°C in

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an SW25.1 rotor. Concentrates at the 15/55% sucrose interface were pooled and purified by rate zonal centrifugation on 28 ml of a 15 to 45% linear sucrose gradient in an SW25.1 rotor at 73,000 × g for 2 h. Fractions of 1 ml were collected by bottom puncture and assayed for infectivity by plaque assay. The major virus-containing fractions were pooled and concentrated onto a 1-ml (1.20 g/ml) cushion of Renografin (methylglucamine diatrizoate, 76% for injection; E. R. Squibb & Sons, New York) by centrifugation. Concentrates were purified by isopycnic banding on preformed Renografin density gradients (1.10 to 1.25 g/ml) in a type 65 fixed-angle rotor at 176,000 × g for 18 h at 4°C. Virus bands were collected by aspiration, pooled, diluted with buffer, and pelleted. Pellets were soaked in buffer for 4 h and then suspended by ultrasonic treatment. Viral infectivity was determined by plaque assay (23), and protein concentrations were measured by the method of Lowry et al. (17).

Immunogens. Immunogens were grown in RD cells by using 2% agamma calf serum instead of whole serum. Growth conditions and purification methods were the same as described for preparation of serological test antigens. Crude immunogens consisted of sucrose step-gradient concentrates. Virus preparations additionally purified by rate zonal and isopycnic centrifugation were used as purified immunogens. Immunogens were standardized by PFU per milliliter, and protein concentrations were measured.

Preparation of antisera. Antisera to whole virus were prepared in female New Zealand white rabbits by the method of Cooney and Kenny (7), which uses an initial intramuscular (i.m.) injection of 2 ml of immunogen with incomplete Freund adjuvant followed by a series of intravenous (i.v.) injections (0.1 to 0.5 ml) without adjuvant. The total immunogen dose was 3×10^7 to 3×10^{10} PFU per rabbit. Antisera were also prepared in rabbits by subcutaneous (s.c.) injection of immunogens emulsified in an equal volume of complete Freund adjuvant at three separate sites. Forty injections were given in the inguinal region (20 injections per side), and 20 injections were given in the nuchal region of the neck. After 21 days, rabbits were injected i.m. with 1 ml of immunogen with incomplete adjuvant. Bleedings were done 10 days later.

Antisera to each unique precipitin line were prepared in rabbits by injection of excised precipitin lines from two-dimensional electropherograms (5). These antisera were termed monospecific antisera because they were induced by an immune complex obtained from a single and well-separated precipitin line (1, 5). The antiserum concentration in the second-dimension gel was adjusted so that the precipitin line to be excised was clearly separated from other precipitates. Slides were washed for 48 h in 0.005 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)–0.15 M NaCl, pH 7.0, with frequent buffer changes before excision of the precipitin lines. The antibody-antigen complexes in agarose were suspended in saline and emulsified in an equal volume of complete or incomplete Freund adjuvant for s.c. or i.m. injection into rabbits as described above. Each rabbit was immunized with pooled precipitates from 24 electropherograms; the amount of Triton X-100-solubilized protein was 60 to 80 μg on each slide. A more quantitative determination was not feasible due to the limited amount of available material in the immunoprecipitates.

Serological tests. Complement fixation (CF), hemagglutination inhibition (HI), and neutralization (NT) tests were performed in microtiter plates (24). Two units of complement, 8 U of antigen, and overnight fixation at 4°C were used in the CF test. Four units of antigen and a final concentration of 0.26% chicken erythrocytes were used in the HI test. In the NT test, 300 50% tissue culture infective doses of virus contained in a volume of 0.025 ml was mixed with 0.025 ml of twofold serum dilutions, and the mixture was incubated at room temperature for 30 min. A volume of 0.05 ml of minimal essential medium-5% calf serum, containing 16,000 FT cells, was added to each well. Plates were incubated at 33°C in a 2.5% CO_2 atmosphere. Microscope readings and staining with crystal violet were carried out when virus controls indicated the presence of 100 50% tissue culture infective doses.

Human sera. Paired sera (acute collected at as close to onset as possible, and convalescent collected 25 to 58 days later) obtained from patients with pneumonia from an intensive study of the etiology of pneumonia (9) were tested for antibody titers to OC43 virus (CF, NT, and HI) and 229E virus (CF and NT). Twenty patients showed fourfold or greater antibody rises to OC43 virus (usually by two or more tests), whereas only four patients showed fourfold antibody rises to 229E virus.

Determination of neutralization rate constants. Equal volumes of diluted serum and virus (10^5 PFU/ml) were mixed and incubated at 37°C in a water bath. Virus controls were treated similarly. At several time intervals, samples were taken from the serum-virus mixture and virus control, diluted 1:100 in ice-cold medium to stop the reaction, and tested for unneutralized virus by plaque assay. The neutralization rate constant, K, was calculated by the method of Dulbecco et al. (8).

Two-dimensional immunoelectrophoresis. The method of Thirkill and Kenny (26) was used. Glass slides (4.2 by 4.2 cm) were covered with 3 ml of a 0.6% agarose gel (Bio-Rad Laboratories, Richmond, Calif.) in barbital buffer (pH 8.6; ionic strength 0.05) containing 0.5% Triton X-100 and 0.01% sodium azide. Antigen (60 μg of protein in a 15-μl volume) solubilized with 1% Triton X-100 was electrophoresed in the first dimension at 5.6 V/cm for 1.5 h. Barbital buffer (pH 8.6; ionic strength 0.1) was used in the baths. The second-dimension gel (2 ml) contained 10 to 20% antiserum. Second-dimension electrophoresis was carried out at 2.7 V/cm for 8 h. After electrophoresis, the slides were submerged for 48 h in TES-saline for 24 h in distilled water and then dried at 60°C and stained with Coomassie brilliant blue. A small amount of bovine serum albumin (BSA) was coelectrophoresed during first-dimension electrophoresis, and rabbit BSA antiserum was added to the second-dimension gel to produce a marker used as a standard to calculate the relative electrophoretic mobilities of precipitin peaks (26). Peak suppression, peak enhancement, and absorption of antisera were used as analytical methods to identify virus-specific precipitin peaks (2, 26).

Counterimmunoelectrophoresis. The method described by Kenny et al. (16) was used for detection of antibody. Briefly, glass slides (2.5 by 7.5 cm) were covered with 3 ml of a 0.6% agarose gel in barbital buffer (pH 8.6; ionic strength 0.05) containing 0.05% Triton X-100 and 0.01% sodium azide. Eight wells (two rows of four) 2 mm in diameter were cut and aspirated. Triton X-100-solubilized virus (2 μg of protein in a 2-μl volume) was added to the cathodal wells, and two-fold dilutions of patients' sera (2 μl) were added to the anodal wells. Electrophoresis was carried out for 30 min at 5 V/cm. Barbital buffer (pH 8.6; ionic strength 0.1) was used in the baths. Slides were observed immediately after electrophoresis for precipitin lines and then prepared for staining with Coomassie brilliant blue as described for two-dimensional immunoelectrophoresis.

Virus identification. The identity of strains 229E and OC43 was confirmed by virus neutralization with reference antisera: 229E immune guinea pig antiserum and OC43 immune mouse ascitic fluid received from the Centers for Disease Control, Atlanta, Ga. No cross-reaction was observed by plaque reduction or kinetic neutralization between 229E and OC43 with hyperimmune rabbit antisera. Both virus strains showed typical coronavirus morphology by electron microscopy.

RESULTS

A titration experiment was conducted that used highly purified virus preparations as immunogens (Table 1). All immunogenic doses (3×10^7 to 3×10^{10} PFU/rabbit) elicited antibody response as measured by CF and NT (229E virus) and HI, CF, and NT (OC43 virus) tests. However, at least 10^9 PFU of OC43 virus had to be injected to elicit a strong antibody response as measured by both kinetic neutralization and ability to resolve three antigens in two-dimensional immunoelectrophoresis. In contrast, a 10-fold-higher virus dose was necessary with 229E virus to resolve three virus peaks (one peak was faint), and a 100-fold-higher dose was required to induce antibody and resolving power similar to that induced by 3×10^9 OC43 virus. With both viruses, CF titers, *K* values, and the resolving power of antisera were all directly related. The highest immunogenic dose of purified virus resulted in a weak antibody response to one host cell component with OC43 virus and to two components with 229E virus, whereas partially purified immunogens (sucrose step gradients) induced antibody to three to five host components. A different immunization method was used to test the possible effect of route of immunization on antibody response. Injection s.c. of the maximum virus dose used in previous experiments (3×10^{10} PFU) (using complete Freund adjuvant) resulted in a higher antibody response with OC43 virus. In contrast, the 229E antibody response remained virtually unchanged, although the resolving power of the antisera was improved.

Antigenic composition of coronaviruses 229E and OC43. Three precipitin peaks with electrophoretic mobilities relative to BSA of 0.25, 0.41, and 0.57 were resolved for 229E virus (Fig. 1a). Likewise, three OC43 virus peaks were resolved by rabbit antiserum with electrophoretic mobilities of 0.23, 0.44, and 0.56 (Fig. 1d). These peaks were identified as virus peaks, since peak heights were not increased by addition of host cell material or calf serum to the test sample before electrophoresis in the first dimension, nor were peak heights increased by adsorption of antisera with host cells. Conversely, peak suppression was not observed by addition of antiserum prepared against host cell or calf serum

TABLE 1. *Immunogenicity of coronaviruses 229E and OC43*

Virus	Total PFU	Protein	Method of immunization	Antibody titer[a]			TDIE[b]
				HI	CF	NT	
229E	3.5×10^{10}	3.2×10^{-3}	i.m.,[c] i.v.		320	320 (70)[d]	3[e]
229E	3.5×10^9	3.2×10^{-4}	i.m., i.v.		128	320 (68)	2
229E	3.5×10^8	3.2×10^{-5}	i.m., i.v.		64	128 (48)	2[e]
229E	3.5×10^7	3.2×10^{-6}	i.m., i.v.		16	64 (42)	1
OC43	3.4×10^{10}	2.5×10^{-3}	i.m., i.v.	2,560	1,280	960 (490)	3
OC43	3.4×10^9	2.5×10^{-4}	i.m., i.v.	1,280	1,280	800 (224)	3[e]
OC43	3.4×10^8	2.5×10^{-5}	i.m., i.v.	960	640	640 (206)	2
OC43	3.4×10^7	2.5×10^{-6}	i.m., i.v.	940	64	320 (102)	1
229E3	3.5×10^{10}	3.2×10^{-3}	s.c.[f] i.m.		320	640 (78)	3
OC43	3.4×10^{10}	2.5×10^{-3}	s.c.,[f] i.m.	5,120	7,680	6,400 (1,100)	3

[a] Reciprocal of the highest dilution of serum giving a positive reaction.
[b] Two-dimensional electrophoresis. Data expressed as number of virus-specific precipitin peaks.
[c] With incomplete Freund adjuvant.
[d] Value in parentheses is kinetic neutralization constant.
[e] One peak very faint.
[f] With complete Freund adjuvant.

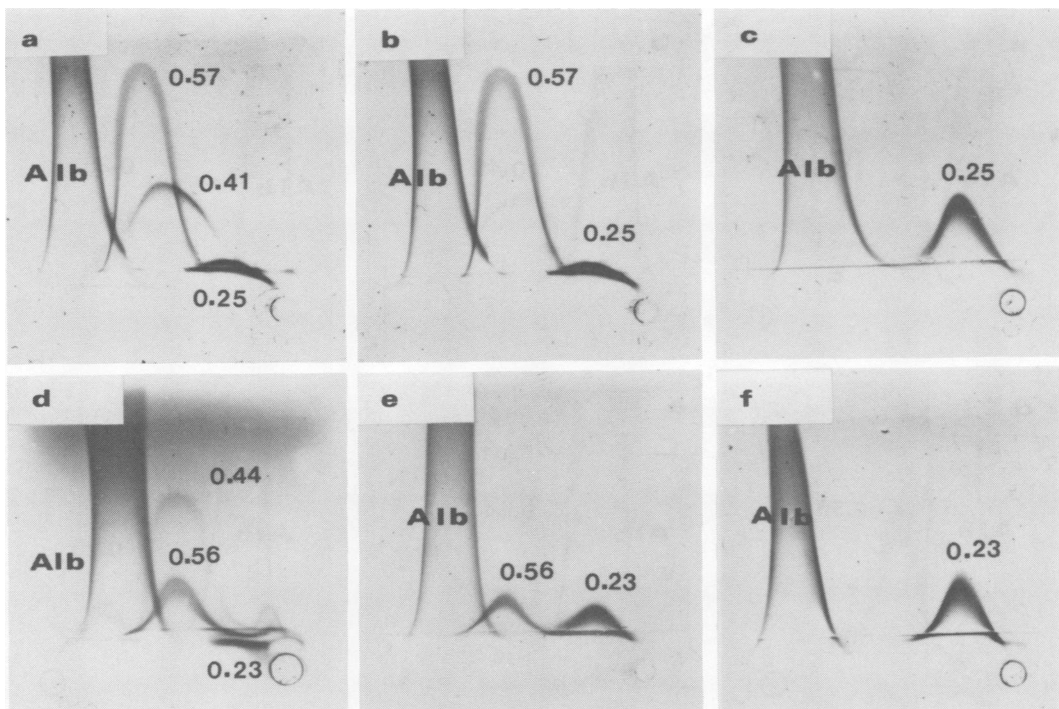

FIG. 1. *Effect of immunogenic dose on antibody response to 229E and OC43 virus antigens. Antisera were prepared in rabbits to* 3×10^{10} *(a),* 3×10^{9} *(b), and* 3×10^{7} *(c) PFU of 229E virus and to* 3×10^{10} *(d),* 3×10^{8} *(e), and* 3×10^{7} *(f) PFU of OC43 virus. Antigen (60 μg of protein of solubilized whole virus) was electrophoresed in the first phase (anode to left). For the second-dimension electrophoresis, gels contained 15% (a) and 20% (b, c) of respective rabbit 229E antiserum and 10% (d) and 20% (e, f) of respective rabbit OC43 antiserum (anode at top). BSA (Alb) was used as standard marker to calculate the relative electrophoretic mobilities of precipitin peaks. (Although animals immunized i.m. showed three peaks in panel a, better photographic resolution was obtained with the antiserum produced by s.c. immunization [see Table 1].)*

to the second-dimension gel. Neither preimmune serum nor antiserum to the mycobacterium (contained in the complete Freund adjuvant) reacted with any antigen preparations. Analysis of antisera derived from the titration experiment by two-dimensional immunoelectrophoresis showed that, for both viruses, the antibody response to a low virus dosage resulted in recognition of only one virus peak, the slowest-migrating antigen (Fig. 1c,f). An increased immunogenic dosage resulted in recognition of a second antigenic component, the fastest-migrating antigen (Fig. 1b,e). Finally, highest immunogenic dosage resulted in recognition of the third antigenic component with intermediate electrophoretic mobility (Fig. 1a,d).

Identification of specific coronavirus antigens. Antisera were prepared to each viral antigen to determine the role of these antigens in viral serological reactions. The antisera prepared in rabbits by injection of excised precipitin lines from two-dimensional electrophorograms were monospecific because each resolved only one peak against solubilized 229E virus (Fig. 2a–c). Similar results were obtained with OC43 virus (Fig. 2d–f). Monospecific antisera were tested for virus NT, CF, and HI activities. Each monospecific antiserum to the three 229E antigens fixed complement, but only antigen 0.25 elicited a neutralizing antibody response (Table 2). All monospecific antisera to OC43 virus exhibited CF activity, but NT and HI antibody titers were found only in antiserum prepared against antigen 0.23 (Table 2). None of the monospecific antibodies or the polyvalent antibodies (prepared against whole virus) cross-reacted with the heterologous virus in any of the serological tests used, showing that the three antigens for each virus were all unique.

Antigens recognized by human sera. We sought to determine which antigens were recognized by humans who had been infected recently by coronaviruses. Sera from six patients with proven fourfold antibody rises and sufficient volume (0.4 ml) for testing were tested as the antiserum in the second phase of two-dimen-

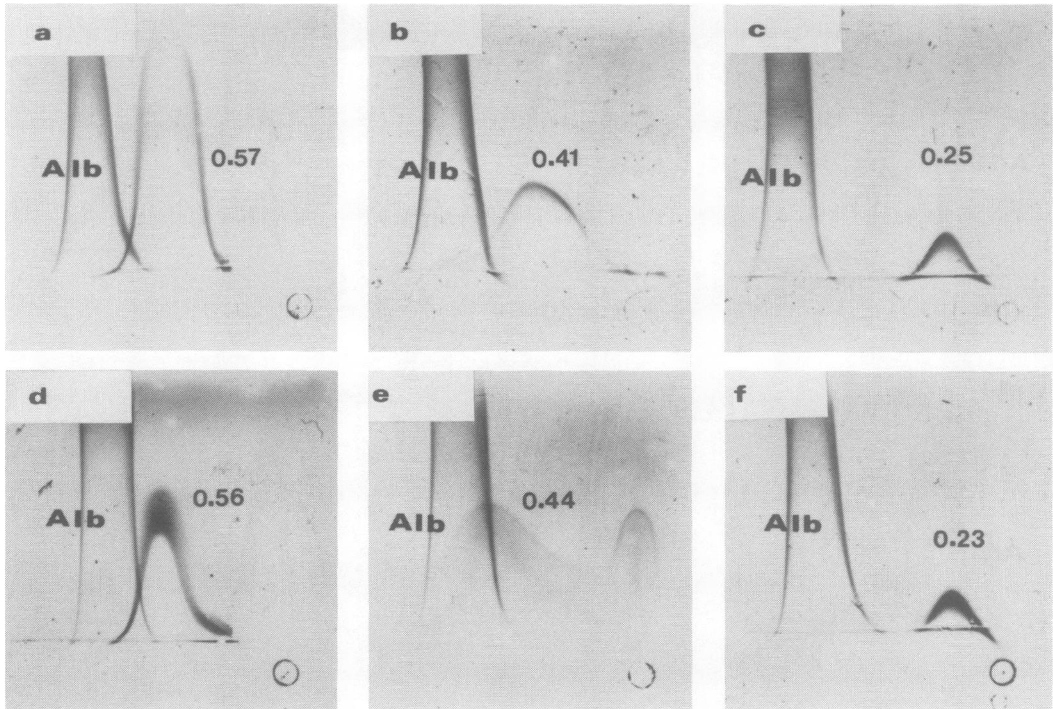

FIG. 2. *Precipitin peaks of 229E and OC43 viruses resolved by monospecific antisera. Solubilized virus (60 μg of protein) was electrophoresed in the first phase. The second-dimension gels contained antisera to peaks 0.57 (a), 0.41 (b), and 0.25 (c) of 229E virus and antisera to peaks 0.56 (d), 0.44 (e), and 0.23 (f) of OC43 virus. Concentration of antibody in the second-phase gel was 20% (a, b, e) or 15% (c, d, f).*

TABLE 2. *Monospecificity of antisera prepared against excised precipitin peaks of 229E and OC43 virus*

Rabbit antiserum	Antibody titer[a]				TDIE[b]
	HI	CF	NT		
229E, whole virus		320	640	(78)[c]	3
229E, peak 0.57		128	<2		1
229E, peak 0.41		64	<2		1
229E, peak 0.25		16	512	(58)	1
OC43, whole virus	5,120	7,680	6,400	(1,100)	3
OC43, peak 0.56	<2	64	<2		1
OC43, peak 0.44	<2	64	<2		1
OC43, peak 0.23	1,024	32	1,024	(362)	1

[a-c] See footnotes *a*, *b*, and *d*, Table 1.

sional immunoelectrophoresis against solubilized whole virus as antigen (Table 3). No precipitin peaks were observed with acute sera, but five of six convalescent sera resolved a single peak with a slow migration rate identical to that of the component identified as the neutralizing antigen by using rabbit antiserum. In one case of OC43 infection, where we had a sufficient volume of serum for additional testing, we were able to demonstrate that the peak recognized by human serum could be suppressed by rabbit antiserum monospecific to OC43 peak 0.23, indicating that the antigen recognized by human serum was the same as the antigen which elicited a neutralizing antibody response in rabbits. One precipitin line was observed by counterimmunoelectrophoresis in each case where a precipitin peak had been observed by two-dimensional immunoelectrophoresis when human serum was used as antibody (in the anodal well) against solubilized virus (in the cathodal well). A precipitin line also was seen in one acute serum (patient 4) which was not detected by two-dimensional immunoelectrophoresis.

DISCUSSION

We investigated the immunogenicity of human coronaviruses OC43 and 229E by using as immunogens and serological test antigens virus grown in human rhabdomyosarcoma cells (23). Previous workers had shown that high antibody titers could be obtained with OC43 virus (4, 13, 14, 20) but that the immune response to 229E virus was weak (13). Injection of 10^{10} PFU of highly purified virions per rabbit yielded not only antiserum with high antibody titers for OC43 virus but also antiserum only slightly contaminated with antibodies to host cell components. In contrast, at least 10-fold more immu-

TABLE 3. *Antigens recognized by sera from pneumonia patients with fourfold antibody rises to coronavirus OC43 or 229E*

Antibody	Patient no.	Serum[a]	Antibody titer[b]			TDIE[c]	CIE[d]
			CF	NT	HI		
OC43	1	A	<4	<4	<4	Neg	Neg
		B	8	16	8	Neg	Neg
	2	A	<4	<4	<4	Neg	Neg
		B	8	32	32	0.23	1
	3	A	4	16	8	Neg	Neg
		B	16	64	32	0.23	1
	4	A	4	32	16	Neg	1
		B	16	128	64	0.23	1
229E	5	A	<4	<4		Neg	Neg
		B	16	32		0.25	1
	6	A	<4	4		Neg	Neg
		B	16	64		0.25	1

[a] Acute (A) and convalescent (B) sera.
[b] Reciprocal of the highest dilution of serum giving a positive reaction.
[c] Two-dimensional immunoelectrophoresis. Data expressed as electrophoretic mobility of precipitin peak relative to BSA. Neg, Negative.
[d] Counterimmunoelectrophoresis.

nogen was required for producing similar-quality antisera to 229E virus as to OC43.

Double immunodiffusion studies (4, 13, 14) showed three to four precipitin lines when OC43 virus was tested against hyperimmune animal serum, but only one line with 229E virus. These studies were compromised not only by the lack of potent antibodies and antigens, but also by the difficulties in differentiating cellular from viral antigens. However, it is generally agreed that animal coronaviruses have three antigens (27). Tevethia and Cunningham (25) showed three distinct antigens for infectious bronchitis virus by using antiserum from infected chickens to avoid problems of nonviral antigens. Similarly, Mengeling (21) showed three specific antigens for hemagglutinating encephalomyelitis virus by using serum from infected pigs. We used two-dimensional immunoelectrophoresis for antigenic analysis because this method is highly sensitive and has high resolution, allowing for differentiation of viral from cellular antigens, and antigen-antibody complexes can be recovered for use as immunogens. We identified three specific virus peaks with mobilities labeled fast (0.57 for 229E virus, 0.56 for OC43 virus relative to BSA), intermediate (0.41, 0.44), and slow (0.25, 0.23). The mobilities of the fastest and slowest antigens were highly reproducible and similar between the two viruses; however, the mobility of the intermediate antigen varied with different antigen preparations, particularly for OC43 virus. Cross-reactions between the two viruses have been observed by some investigators (4, 20) but not by others (22). Not only did we find no evidence for common antigens by two-dimensional immunoelectrophoresis, but also we saw no cross-reactions in plaque reduction or kinetic neutralization. The three antigens detected for each virus were found to be negatively charged at pH 8.6, and we did not find positively charged antigens by the less sensitive technique of ordinary immunoelectrophoresis (data not shown). However, a positively charged component was observed by Bohac and Derbyshire (3) in alkaline extracts of infected pig intestine; however, we do not know whether this component is part of the virion. Hajer and Storz (10) found a fourth antigen in alkaline intestinal extracts from calves, and they showed that this component was not seen in purified virions.

When the antisera obtained from the titration experiment used to assess immunogenicity (Table 1) were tested by two-dimensional immunoelectrophoresis, antibodies to the slow-moving component were found at low immunogenic doses without evidence of antibodies to the other two components. Similarly, the only antigen recognized by human sera was the slow component, suggesting that the human immune response during infection is similar to that of rabbits immunized with 10^7 PFU. Further evidence for the superior immunogenicity of the slow-moving antigen was obtained during preparation of monospecific sera when we observed that excellent and fair immune responses were seen by the slow- and fast-moving antigens, respectively, whereas the response to the antigen of intermediate migration was poor, particularly for 229E virus.

The successful production of antiserum monospecific to each antigen permitted the demonstration that the slow-moving antigen elicited a neutralizing antibody response for 229E virus

and both the neutralizing and HI antibody response for OC43 virus. The monospecific antisera were most important in a definitive distinction of virus and host antigens, since these antisera were free of antibodies to both the host cells and the serum component of the medium. The relative mobilities of peaks detected by monospecific antisera were identical to those resolved by whole virus antiserum, and the height of the specific peaks could be suppressed by their corresponding monospecific antiserum but not by monospecific antisera to any other component on either virus. Accordingly, we have demonstrated that coronaviruses 229E and OC43 each possess three separate and distinct viral antigens, one of which, the antigen with the slowest electrophoretic migration, elicits neutralizing antibody response, is the most immunogenic component, and is the antigen recognized by human serum. Characterization and correlation of these antigens with virion structural components are the topics of a future publication (O. W. Schmidt and G. E. Kenny, submitted for publication).

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