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Quantitation of Antibody to Non-Hemagglutinating Viruses by Single Radial Hemolysis: Serological Test for Human Coronaviruses

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A single radial hemolysis test was developed for quantitation of specific antibody to non-hemagglutinating viruses. With the human coronaviruses as models, this test utilizes the binding properties of the chromic cation to attach viruses to glutaraldehyde-treated sheep erythrocytes. The most satisfactory system consisted of stabilizing washed sheep erythrocytes with 0.0073% glutaraldehyde for 15 min at 23°C, binding a high concentration of virus to a 25% erythrocyte suspension with 0.0016% chromic chloride for 20 min at 23°C, stopping the reaction with phosphate-saline, and finally mixing the treated, rewashed cells with complement and agarose at 45°C to prepare a slide gel. The gel mix, which was dispensed in plastic plates (23 by 73 mm) in 3-ml volumes, consisted of 1% agarose, 0.1% sodium azide, 5% reconstituted complement, and 0.82% treated cells. Wells 2 mm in diameter were loaded with 5 μl of antiserum, incubated for 18 h at 4°C for diffusion of antiserum and fixation of complement, and then incubated for 8 to 24 h at 37°C for development of hemolysis zones. The diameter of a zone was linearly related to antibody concentration, as determined by conventional serological tests. This single radial hemolysis test was applicable to human and animal coronaviruses and to selected serotypes of the adenovirus, picornavirus, rhabdovirus, and rotavirus groups.

The single radial hemolysis (SRH) test was first described for the detection of antibodies to immunoglobulin G (12, 40) and other immunological problems (6, 17, 18, 29, 34) and, subsequently, as a test for antibody to toxoplasma (19). It has been further applied as an antibody test for chlamydia (28) and for such hemagglutinating viruses as rubella (10, 37, 38), bovine parainfluenza (33), influenza (2, 35, 36), and mumps (9). These tests depend upon the spontaneous attachment of the virus to erythrocytes (RBCs), and they have proven to be sensitive, reproducible, and readily applicable to serosurveys.

We now describe an SRH test for non-hemagglutinating viruses, based upon their chemical attachment to RBCs. The test is much needed for diagnostic and epidemiological studies of the human coronaviruses, because existing serological tests are unreliable or insensitive. We used coronavirus strain OC-43 as the principal model in developing the test, because it (i) is relatively stable and can be grown to high titers in suckling mouse brain (21, 31) and (ii) is the only human coronavirus that hemagglutinates (21), which provided a convenient assay system in preliminary experiments. To show the general application of the SRH test for antibodies to non-hemagglutinating viruses, it was applied to human coronavirus 229E, the mouse hepatitis virus coronaviruses, coxsackievirus A-9, rabies virus, and human infantile gastroenteritis virus.

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MATERIALS AND METHODS

Viruses. Coronavirus strain OC-43 was used at mouse brain passage 14 (21, 23), with a titer of $10^{4.22}$ mean lethal doses/0.025 ml in 3-day-old suckling mice and a hemagglutination titer of 1:2,560 with chicken RBCs (39). Hemagglutination tests with 0.5% chicken erythrocytes (with incubation at 23°C) and hemagglutination inhibition (HI) tests with human and animal antisera to OC-43 were performed in 0.01 M phosphate-buffered saline diluent as described (16). Strain 229E, a human coronavirus antigenically distinct from OC-43 (1, 13, 30, 32), was propagated in human diploid fibroblast (HELF) tissue culture as previously described (14). Its titer in HELF cells was $10^{7.6}$ mean tissue culture infective doses/ml.

Both viruses were clarified at 5,000 × *g* for 20 min and concentrated through a 15% sucrose interface to a 0.75-ml cushion of 65% sucrose for 30 min at 43,200 × *g* in a Spinco SW36 rotor. Sucrose was removed by

passing the concentrate through a 5-cm Bio-Gel P-30 column equilibrated with normal saline (N-saline; 0.85% NaCl), and the preparations were stored in small samples at −70°C. Concentrates of OC-43 were diluted 1:10 in N-saline before use (see Results). Protein concentrations of the viruses as used were 350 μg/ml for OC-43 and 260 μg/ml for 229E, as determined by the method of Lowry et al. (27).

Antisera. Mouse-immune ascitic fluid and guinea pig antiserum to OC-43 were prepared as described (15). Human convalescent OC-43 serum, 2× concentrated, and a guinea pig 229E antiserum were provided by the Viral and Rickettsial Products Branch, Center for Disease Control (CDC), Atlanta. Rabbit antisera to purified OC-43 and 229E were prepared as described (14). Fifteen acute/convalescent serum pairs from children with OC-43 infection (24) and 15 pairs from children with 229E infection (22) were generously provided by H. S. Kaye, CDC. Nine additional serum pairs showing seroconversion to OC-43 and nine to 229E were obtained from our diagnostic service. All human and animal sera were tested for HI (16) and complement-fixing (3) antibodies to OC-43 and for indirect hemagglutination (IHA) antibodies to 229E (25). All sera were inactivated at 56°C for 30 min to destroy complement and heat-labile, nonspecific inhibitors.

SRH test. (i) RBCs. Sheep RBCs were collected in Alsever solution and stored at 4°C for up to 5 weeks. For the test, the blood was filtered through gauze to remove fibrous material and was centrifuged at 1,000 × *g* for 10 min. The buffy coat was removed by aspiration; the RBCs were washed five times in N-saline and finally adjusted to 50% (vol/vol) in N-saline.

(ii) Glutaraldehyde stabilization. Habicht and Miller demonstrated that treatment of RBCs with glutaraldehyde enhanced the cells' uptake of certain antigens (11). Preliminary experiments with our reagents revealed that such treatment markedly enhanced the sensitivity of the test for low-titered human sera. Glutaraldehyde was obtained as a 25% solution in 10-ml ampoules from Electron Microscopy Sciences, Fort Washington, Pa., and was stored at 4°C. The following procedure was used: samples of 0.5 ml of 50% RBC were diluted to 5.0 ml in N-saline and mixed with 0.15 ml of 0.25% glutaraldehyde in N-saline (final concentration, 0.0073% glutaraldehyde) for 15 min at 23°C. The suspensions were then centrifuged, and the treated RBCs were washed once and adjusted to 25% (vol/vol) with N-saline. The suspension was used for viral attachment within 24 h of storage at 4°C.

(iii) Preparation of chromic chloride. The use of cationic chromium to bind proteins to erythrocytes is well known (4, 8, 20, 26), and Goding recently demonstrated that antigens could be more efficiently coupled by "aged" rather than by freshly prepared chromic chloride solutions (7). A 1% solution of chromic chloride ($CrCl_3 \cdot 6H_2O$, Fisher) in N-saline was adjusted to pH 5.0 with 1.0 N NaOH two or three times per week for 8 weeks. (The stock solution, kept at 23°C, was usable for at least 9 months.) Thereafter, immediately before use for viral attachment, its pH was again adjusted and a portion was diluted in N-saline to 0.003%.

(iv) Viral attachment. Samples of 0.4 ml of washed, glutaraldehyde-stabilized RBCs at a 25% concentration were mixed with 1.2 ml of N-saline and 0.2 ml of a suitable concentration of virus in a 15-ml conical centrifuge tube. Two milliliters of 0.003% chromic chloride was added dropwise with a pipette and with constant mixing. The mixture was incubated for 20 min at 23°C, and the reaction was then stopped with 4.0 ml of phosphate-buffered saline. After centrifuging the cells, they were resuspended to a 25% concentration in Veronal-buffered diluent (3). Preferably, the cells were used in the SRH test the same day to avoid any lysis and an accompanying loss in sensitivity.

(v) Slide preparation. A gel mix of 1.0% (wt/vol) agarose (Indubiose A37, L'Industrie Biologique Francaise SA) and 0.1% (wt/vol) sodium azide (Fisher) in Veronal-buffered diluent was prepared by heating to a boil, dispensing in 2.8-ml samples in screw-capped tubes, and storing at 4°C. For preparation of a test plate, a tube of agarose was remelted, equilibrated to 45°C, and mixed with 0.15 ml of reconstituted complement (Flow Laboratories, Rockville, Md.) and 0.1 ml of the virus-coated RBC suspension. The mixture was immediately poured into a plastic immunodiffusion plate (with lid; Hyland no. 085-710, Travenol Laboratories, Cosa Mesa, Calif.) and allowed to gel. (Due to the lability of solubilized complement, the refrigerator "shelf life" of poured slides was about 2 days.) Wells 2 mm in diameter were then punched in the agarose in a "double-row-of-6" configuration.

(vi) Antibody titrations. Each well was filled with 5 μl of serum. The plates were incubated in a humidity chamber at 4°C for 16 to 20 h and then transferred to 37°C. Zones of lysis indicating the presence of specific viral antibody could be observed by as early as 3 to 4 h. Precise measurements of zone diameter were made using a ×7 measuring magnifier with a metric ocular scale (Bausch & Lomb no. 81-34-35) over a background of indirect light after 8 and 24 h of incubation at 37°C. The slides were then stored without further change for up to 3 weeks at 4°C. When a permanent record was needed, slides were photographed over indirect light with a Polaroid camera and type 55 P/N film.

RESULTS

The various factors affecting the specificity and clarity of hemolysis zones with coronavirus OC-43 reagents were systematically explored. Some of these data, all from trials with semipurified OC-43 virus, are shown in Table 1. The chromic chloride concentration used in attaching virus to RBCs appeared to be the most important factor in determining the size and clarity of the zones. At initial concentrations of 0.007% $CrCl_3$ and above, extensive clumping of the RBCs in the agarose layer was apparent, and measurement of zone size was difficult if not impossible. Less clumping occurred at concentrations of 0.005 and 0.003%, and much sharper zones of sizes similar to those noted at higher concentrations were apparent. At

TABLE 1. *Effects of various parameters on the SRH test with coronavirus OC-43 antisera*

Expt	Variable[a]			Hemolysis zone diam (mm)[b]			Appearance of gel[c]	
	Glutaraldehyde stabilization	% RBC for virus attachment	% $CrCl_3$ (as added to reaction mix)	Mouse ascitic fluid	Rabbit	Human convalescent	RBC clumping	Zone clarity
1	−	25	0.05		Unmeasurable		+++	−
	−	50	0.05	8.5, 8.0	7.5, 8.5	3.0, 3.0	+++	±
	−	25	0.01	10.5, 9.5	8.0, 8.0	3.0, 3.0	++	+
	−	50	0.01	9.5, 9.5	9.0, 8.5	0, 0	++	+
	−	25	0.005	10.5, 9.5	8.0, 8.5	3.0, 3.0	+	++
	−	50	0.005	9.5, 10.0	8.5, 9.5	0, 0	+	+
2	−	25	0.005	8.5, 9.5	9.0, 9.5	0, 3.0	+	++
	+	25	0.005	9.5, 9.0	9.5, 10.0	4.0, 3.5	+	++
	−	25	0.001	0, 0	0, 0	3.0, 3.0	−	+
	+	25	0.001	0, 0	3.0, 0	5.0, 5.0	−	+
3	+	25	0.003	8.5, 9.5	9.5, 10.5	3.5, 3.5	−	+++
	+	25	0.005	10.0, 9.5	9.5, 10.5	3.5, 3.5	+	++
	+	25	0.007	8.5, 9.0	9.5, 10.5	3.5, 3.0	++	±

[a] Procedures for RBC preparation and stabilization, $CrCl_3$ preparation, and viral attachment are given in the text. The $CrCl_3$ solutions used were freshly prepared dilutions of the stock, pH 5-adjusted 1% solution. Tests were done in duplicate with OC-43 virus antigen.

[b] Measured after fixation for 18 h at 4°C, followed by incubation for 24 h at 37°C. Diameters are expressed to the nearest 0.5 mm; 0 = ≤2 mm (the well diameter).

[c] RBC clumping ranged from "none" (−) to "extensive" (+++); zone clarity ranged from "no visible zones" (−) to "barely discernible" (±) to complete hemolysis (+++).

0.001%, insufficient virus had attached to allow the formation of measurable zones with two of the antisera (experiment 2). The use of a 0.003% solution (final concentration, 0.0016% $CrCl_3$) was then settled upon for the routine diagnostic test.

An initial RBC suspension of 25% was adopted because at this concentration the test was more sensitive for low-titered human sera (experiment 1). Similarly, stabilizing the RBCs with glutaraldehyde before attaching the virus gave more consistent results among different sheep and different lots of complement than were obtained with unstabilized cells; hence, the use of glutaraldehyde was considered essential.

Uptake of ^{32}P-labeled OC-43 to sheep RBCs at various chromic chloride concentrations. The attachment of OC-43 to glutaraldehyde-stabilized sheep RBCs in the presence of varying concentrations of chromic chloride was quantitated with radiolabeled virus. Unlabeled OC-43 semipurified concentrates were diluted 1:10 in N-saline and mixed with 1/10 volume of ^{32}P-labeled OC-43, which was purified as described elsewhere (39). The virus mixture was then mixed with N-saline, 25% stabilized RBC, and varying concentrations of chromic chloride as described in Materials and Methods. The attachment reactions were incubated at 23°C for 20 min and stopped with phosphate-buffered saline. The mixtures were centrifuged at 1,000 × *g* for 10 min. The supernatant fluids were then sampled (0.5 ml) for counting the ^{32}P-labeled virus in scintillation fluid as described (14). The percentage of uptake of radiolabeled virus, determined by comparison with the control containing no chromic chloride, is shown in Fig. 1. At a starting concentration of 0.003% (shown above to produce distinct zones of lysis with no clumping), 74% of the label was taken up by the RBCs. At higher concentrations, a relatively small increase in uptake occurred, showing as a plateau from about 0.006% onward. At a concentration of 0.001%, only 39% of the label was associated with the cells, a level shown above to be too small for zone formation.

Relationship between zone size and specific antibody concentration for OC-43 and 229E antisera. Twofold dilution series of rabbit antisera to OC-43 and 229E were made with normal rabbit serum as diluent. Samples of each dilution were added to replicate wells in plates containing RBCs sensitized against the homologous virus under optimal conditions (i.e., 25% glutaraldehyde-stabilized RBCs treated with 0.003% chromic chloride). The zones obtained after fixation at 4°C for 16 h and incubation at 37°C for 24 h were measured and plotted as diameter versus dilution (Fig. 2). The linear

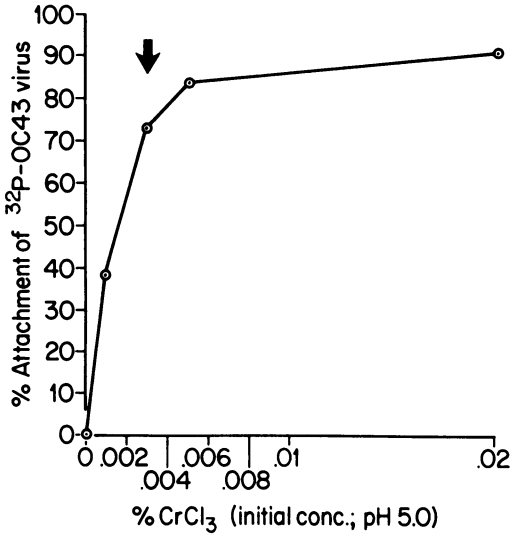

FIG. 1. *Attachment of ^{32}P-labeled OC-43 virus to sheep RBCs with various concentrations of $CrCl_3$ at pH 5.0.*

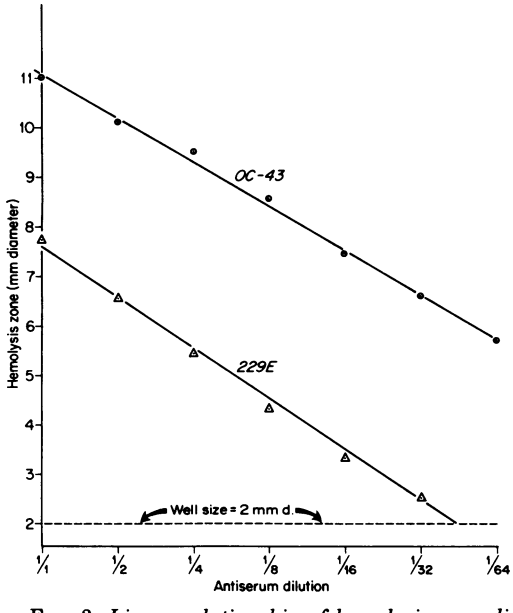

FIG. 2. *Linear relationship of hemolysis zone diameter to homologous antiserum dilution for coronaviruses OC-43 and 229E.*

relationship shown for each virus-antibody system supports the test as a quantitative means of determining antibody concentration. The appearance of the zones over indirect light is shown in Fig. 3.

An analysis of variance was then applied to some of the data to determine the limits of error within the SRH system. The SRH antibody titers to OC-43 in (1) mouse immune ascitic fluid, (ii) rabbit antiserum to purified virus, and (iii) a 2×-concentrated human convalescent serum, having HI titers of 2,048, 1,024, and 64, respectively, were determined in duplicate on three occasions under the optimal conditions defined above. The zone diameters were measured after 16 h at 4°C followed by 24 h at 37°C. Variance was calculated for the sera among days (standard deviation [SD] = 0.30), within days (SD = 0.42), and combined (SD = 0.52). The zone diameter measurements, therefore, reflected a composite within-day and day-to-day SD of 0.5 mm (coefficient of variation [CV] = 7%).

Comparative sensitivity of the SRH test and other tests for human coronavirus antibody. The SRH test was then carried out as a diagnostic procedure with 48 acute/convalescent serum pairs in slides with OC-43- and 229E-adsorbed RBCs. Half of the serum pairs had previously demonstrated a ≥fourfold rise in antibody titer to OC-43 by the HI test, but stable or no titer to 229E by the IHA test and stable or no titer to influenza A and B, parainfluenza 1, 2, and 3, mumps, rubeola, respiratory syncytial virus, herpesvirus, adenovirus, psittacosis, and *Mycoplasma pneumoniae* by the complement fixation test. The other 24 serum pairs had previously demonstrated a ≥fourfold rise in 229E antibody titer by the IHA test but stable or no titer to the other antigens in the respiratory battery by the HI or complement fixation test. Additionally, many of the sera had been found serologically negative for coxsackievirus types A9, A16, A21, A24, B1, B2, B3, B4, and B5 and echovirus types 4, 6, 9, 11, 12, and 33 by serum neutralization tests. Thus, the OC-43 seropositive group and the 229E seropositive group were antigenically specific according to our determinations.

The results of the SRH test with these sera

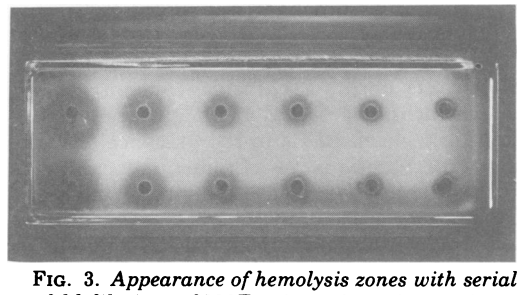

FIG. 3. *Appearance of hemolysis zones with serial twofold dilutions of 229E guinea pig antiserum in an SRH slide with 229E-coupled sheep RBCs. Wells were loaded in duplicate with 5 μl of undiluted serum (left) and 1:2, 1:4, 1:8, 1:16, and 1:32 (right) dilutions in normal guinea pig serum.*

versus both coronavirus antigens are shown in Table 2. For simplicity, the HI titers of OC-43 are grouped into three ranges (<4-fold rise in titer, 4- to 8-fold rise, and ≥16-fold rise), and the increases in hemolysis zone diameter between the S1 and S2 of each pair are indicated across the table. The IHA titers of 229E are similarly grouped. Both sets of sera showed a range of zone diameter increases with their homologous antigen but virtually no change in hemolysis zones with the heterologous virus. Thus, the SRH test for both OC-43 and 229E was at least as sensitive as other serological tests for these viruses, and no serological cross-reaction was observed between them in either direction.

The range in hemolysis zone diameters for both OC-43 and 229E appeared to correlate well with the actual HI or IHA antibody titers of these 96 sera. The zone diameters in the OC-43 SRH test were therefore plotted against OC-43 HI titers (Fig. 4), and the diameters in the 229E SRH test were plotted against 229E IHA titers (Fig. 5). Complement-fixing antibody titers to OC-43 were available for 70 of the sera; these were plotted in Fig. 4 as well. In all cases a linear relationship was observed. These plots can serve as nomographs for converting zone diameters to antibody titer. They also show that in an acute/covalescent serum pair in an OC-43 SRH test, an increase of 2.4 mm in hemolysis zone diameter is equivalent to a fourfold rise in HI antibody titer (i.e., diagnostically positive), and, similarly, in a 229E SRH test an increase of 1.8 mm equals a fourfold rise in 229E IHA titer and is diagnostically positive.

Reference antisera to the human coronaviruses were reciprocally tested in the same manner as the human serum pairs to further document the sensitivity and specificity of the SRH test. The data again indicate the linear relationship between hemolysis zone diameter and serological antibody titer and, as with the human sera, show no evidence of cross-reactions between OC-43 and 229E (Table 3). We also noted that antibodies in sera from all animal sources tested were readily quantitated by the SRH test if the sera were first heat inactivated.

Effect of sequential addition of complement to wells after the addition of antibody. To

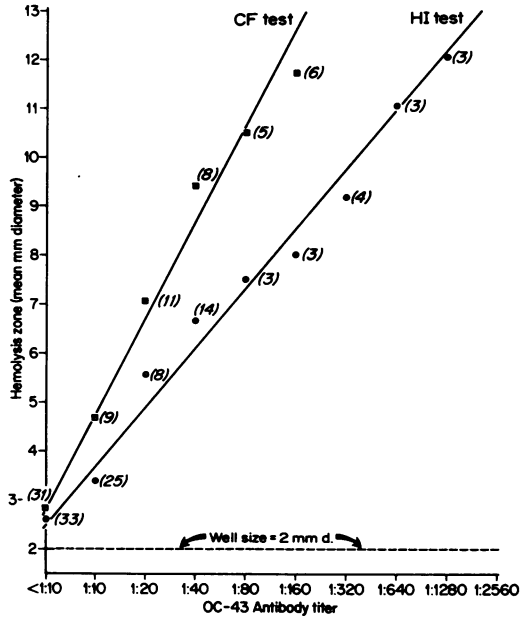

FIG. 4. *Nomograph of hemolysis zone diameter and OC-43 HI or complement-fixing (CF) antibody titers. Number in parentheses after each point is the number of sera averaged for that point.*

TABLE 2. *Reliability and specificity of the SRH test for detecting coronavirus antibody in human sera*

Human sera[a]		No. of serum pairs with indicated increase in hemolysis zone											
		OC-43 RBCs						229E RBCs					
Titer rise	No. of pairs	<1 mm	1 mm	2 mm	3–4 mm	5–6 mm	≥7 mm	<1 mm	1 mm	2 mm	3–4 mm	5–6 mm	≥7 mm
OC-43 (HI)													
<4-fold	24	19	3	2					2	5	7	7	3
4- to 8-fold	14		1	2	5	4	2	13	1				
≥16-fold	10				1	2	7	9	1				
229E (IHA)													
<4-fold	24		1	2	6	6	9						
4- to 8-fold	9	7	1	1					1	2	4	2	
≥16-fold	15	13	1	1					1	3	3	5	3

[a] Acute and convalescent serum pairs bracketing an upper respiratory illness; 24 pairs were "positive" (i.e., ≥fourfold rise in antibody titer between the acute-phase and the convalescent-phase sera) for OC-43 by the HI test but negative for 229E, and 24 pairs were "positive" for 229E by the IHA test but negative for OC-43.

determine whether the SRH test might be more sensitive if complement were added to the wells later instead of added to the agarose when the slide was poured, SRH plates sensitized with 229E virus or with OC-43 virus were set up without incorporated complement. In one test, rabbit anti-229E serum was serially diluted in normal rabbit sera and 5-μl samples were added to the wells. After 18 h at 4°C, 5 μl of reconstituted complement was added to each well, and the plates were incubated at 37°C. Control titration was carried out at the same time with complement incorporated in the agarose cell layer as usual. Very sharp zones appeared in the plate containing sequentially added complement within 2 h at 37°C, but measurements were not made until 7 h. Virus used in sensitizing the cells was of lower titer than usual, and measurable zones were not apparent in the control plate until 20 h, when measurements from both series were taken again. The zones of lysis were sharper in the sequential complement slide than in the incorporated complement slide, and sensitivity appeared the same. However, repeated experiments with both viruses tested against homologous and heterologous antisera and large numbers of human sera showed that the greatest sensitivity and reproducibility were achieved with the complement incorporated in the agarose rather than added later.

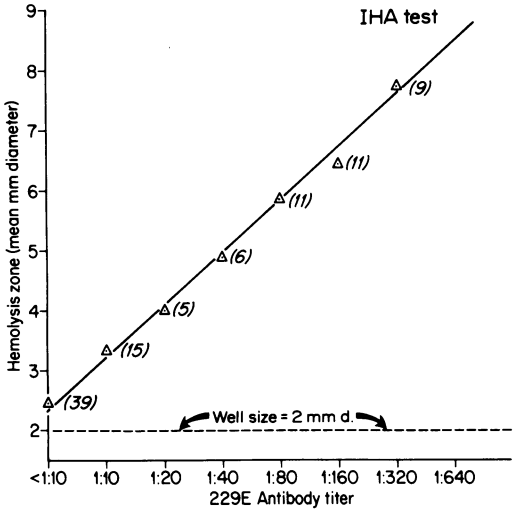

FIG. 5. *Nomograph of hemolysis zone diameter and 229E IHA antibody titer. Number in parentheses indicates the number of sera averaged for that point.*

Application of the SRH test to other non-hemagglutinating viruses. Limited trials with non-hemagglutinating viruses other than coronavirus 229E were carried out to test the general applicability of the SRH test. A Genetron-extracted, clarified stool filtrate containing a high titer of human infantile gastroenteritis virus (recovered from an infant with acute infantile gastroenteritis), as estimated by electron microscopy, was attached without purification to RBCs and tested against the patient's acute/convalescent serum pair. A hemolysis zone increase of 4.8 mm was found in the covalescent serum. Similarly, purified preparations of rabies virus were tested against 1:10 dilutions of homologous rabbit and horse antisera, and the coronavirus, mouse hepatitis virus types 1 and 3, each at $10^{8.5}$ mean tissue culture infective doses/ml after clarification, were

TABLE 3. *Comparison of the SRH test with other serological tests for quantitating specific antibody to coronaviruses*

Serological test[b]	Titer with:[a]				
	OC-43 antiserum			229E antiserum	
	MIAF	Rabbit	Human convalescent	Guinea pig	Rabbit
With OC-43 virus					
HI	2,048	1,024	64	<4	<4
SN	1,024	512	64	<4	<4
CF	128	128	8	<4	<4
SRH	12.1	11.5	7.0	0	0
With 229E virus					
IHA	<4	<4	<4	256	128
SN	<4	<4	<4	256	64
CF	<4	<4	<4	256	32
SRH	0	0	0	7.9	6.7

[a] OC-43 and 229E antisera as in the text. MIAF, Mouse-immune ascitic fluid.

[b] HI, IHA, serum neutralization (SN), and complement fixation (CF) titers listed as dilution factors of the highest dilution showing inhibition according to standard procedures; SRH "titers" listed as mean diameter (in millimeters) of hemolysis zone (average of 14 determinations).

tested against their homologous mouse antisera. Coxsackievirus A9 at $10^{8.3}$ mean tissue culture infective doses/ml, clarified only, was tested against a homologous monkey antiserum. All tests were done by the SRH method described in Materials and Methods. After the prescribed incubation periods, zones of hemolysis were 13.0, 9.8, 7.3, 6.7, and 6.5 mm in diameter, respectively. A clarified culture of adenovirus 5, a typical hemagglutinating adenovirus possessing the mammalian adenovirus group-specific hexon antigen, produced hemolysis-zone diameters of 5.1 mm with adenovirus 5 rabbit antiserum and 6.2 mm with adenovirus 12 rabbit antiserum, revealing hexon-anti-hexon reactions. Although these tests are preliminary, they do suggest that the applicability of the SRH test as described here may not be limited to coronaviruses.

DISCUSSION

The SRH test as previously described (2, 9, 28, 33, 35–38) appeared to be suitable for human coronavirus 229E, a non-hemagglutinating agent of common colds for which convenient serological tests are unavailable. However, efforts to apply these tests to OC-43, a hemagglutinating coronavirus used as a test model, were unsuccessful because the virus could not be attached to the RBCs. Early attempts used periodate-treated sheep and bovine RBCs according to the method of Russell et al. (35) or modification of RBC receptors with trypsin or phospholipase A to allow spontaneous attachment. When these efforts failed to resolve the basic problem of uncoupled virus, the use of glutaraldehyde and $CrCl_3$ was implemented in the SRH test.

The mechanism of action of these components would comprise an interesting study. Glutaraldehyde is known to stabilize the RBC membrane so that subsequent antigen attachment occurs more readily (11). The chromic cation, when inside the RBC membrane, binds irreversibly to the globin moiety of hemoglobin, but its role in attaching antigens to the cell surface is not understood beyond simple charge effects and probable reactions with protein carboxyl groups (7, 8, 20). Even the mechanism by which complement, when bound to an antigen-antibody complex, can disrupt a cell membrane is only understood in general terms (5). Thus, our SRH test might be viewed diagrammatically as in Fig. 6, but little information is available to explain it on the molecular level.

The SRH test for non-hemagglutinating viruses, as described in this report, is shown to be reproducible, sensitive, and specific for quantitating antibody. The data prove the value of the SRH for the human coronaviruses. Further work is in progress to apply the test to strain differentiation among the mouse hepatitis coronaviruses and to provide serological identification of picornavirus infection.

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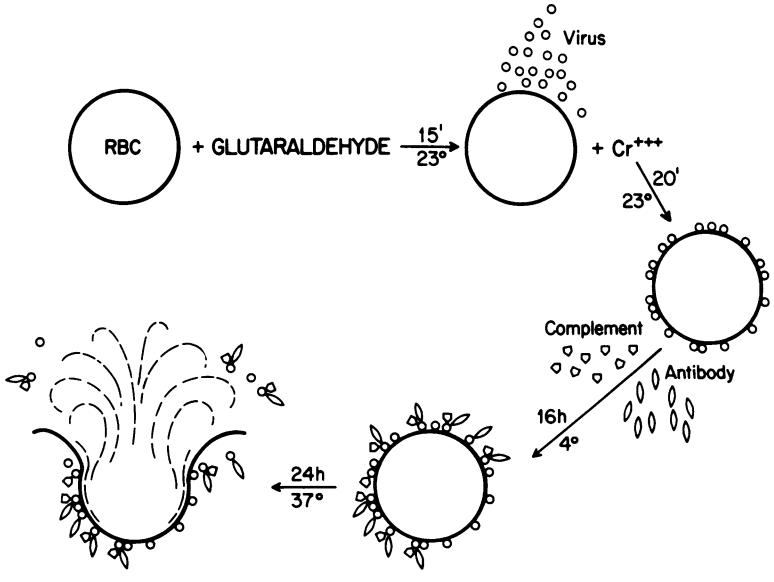

FIG. 6. *Diagrammatic view of the SRH test.*

LITERATURE CITED

1. **Bradburne, A. F.** 1970. Antigenic relationships amongst coronaviruses. Arch. Gesamte Virusforsch. 31:352-364.
2. **Callow, K. A., and A. S. Beare.** 1976. Measurement of antibody to influenza virus neuraminidase by single radial hemolysis in agarose gels. Infect. Immun. 13:1-8.
3. **Casey, H. L.** 1965. Standardized diagnostic complement fixation method and adaptation to micro test. *In* Public Health Service, Monogr. no. 74, Washington, D.C.
4. **Fudenberg, H. H., G. Drews, and A. Nisonoff.** 1964. Serologic demonstration of dual specificity of rabbit bivalent hybrid antibody. J. Exp. Med. 119:151-166.
5. **Gewurz, H.** 1971. The immunologic role of complement, p. 95-103. *In* R. A. Good and D. W. Fisher (ed), Immunobiology, current knowledge of basic concepts in immunology and their clinical applications. Sinauer Associates, Stamford, Conn.
6. **Ghanta, V. K., J. R. McGhee, S. J. Soong, N. M. Hamlin, D. C. Hurst, and R. N. Hiramoto.** 1973. A study of the single radial hemolysis in gel system. III. Quantitation of complement. Immunochemistry 10:645-649.
7. **Goding, J. W.** 1976. The chromic chloride method of coupling antigens to erythrocytes: definition of some important parameters. J. Immunol. Methods 10:61-66.
8. **Gold, E. R., and H. H. Fudenberg.** 1967. Chromic chloride: a coupling reagent for passive hemagglutination reactions. J. Immunol. 99:859-866.
9. **Grillner, L., and J. Blomberg.** 1976. Hemolysis-in-gel and neutralization tests for determination of antibodies to mumps virus. J. Clin. Microbiol. 4:11-15.
10. **Grillner, L., and Ö. Strannegård.** 1976. Evaluation of the hemolysis-in-gel test for the screening of rubella immunity and the demonstration of recent infection. J. Clin. Microbiol. 3:86-90.
11. **Habicht, G. S., and F. Miller.** 1976. Preparation of conjugated erythrocytes for long term use in hemolytic plaque assays, complement fixation studies, or passive hemagglutinations: a comparative study of several methods. J. Immunol. Methods 11:117-127.
12. **Hall, J. M.** 1971. Specificity of antibody formation after intravitreal immunization with bovine gammaglobulin and ovalbumin. I. Primary response. Invest. Ophthalmol. 10:775-783.
13. **Hamre, D., and J. J. Procknow.** 1966. A new virus isolated from the human respiratory tract. Proc. Soc. Exp. Biol. Med. 121:190-193.
14. **Hierholzer, J. C.** 1976. Purification and biophysical properties of human coronavirus 229E. Virology 75:155-165.
15. **Hierholzer, J. C., E. L. Palmer, S. G. Whitfield, H. S. Kaye, and W. R. Dowdle.** 1972. Protein composition of coronavirus OC 43. Virology 48:516-527.
16. **Hierholzer, J. C., M. T. Suggs, and E. C. Hall.** 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. II. Description and statistical evaluation. Appl. Microbiol. 18:824-833.
17. **Hiramoto, R. N., J. R. McGhee, D. C. Hurst, and N. M. Hamlin.** 1970. A simple method for quantitation of hemolytic antibodies. Immunochemistry 7:992-996.
18. **Hiramoto, R. N., J. R. McGhee, D. C. Hurst, and N. M. Hamlin.** 1971. A study of the single radial hemolysis in gel system. I. Factors affecting the model. Immunochemistry 8:355-365.
19. **Jackson, W. B., G. R. O'Connor, and J. M. Hall.** 1974. Plate hemolysin test for the rapid screening of toxoplasma antibodies. Appl. Microbiol. 27:896-900.
20. **Jandl, J. H., and R. L. Simmons.** 1957. The agglutination and sensitization of red cells by metallic cations: interactions between multivalent metals and the red-cell membrane. Br. J. Haematol. 3:19-38.
21. **Kaye, H. S., and W. R. Dowdle.** 1969. Some characteristics of hemagglutination of certain strains of "IBV-like" virus. J. Infect. Dis. 120:576-581.
22. **Kaye, H. S., and W. R. Dowdle.** 1975. Seroepidemiologic survey of coronavirus (strain 229E) infections in a population of children. Am. J. Epidemiol. 101:238-244.
23. **Kaye, H. S., J. C. Hierholzer, and W. R. Dowdle.** 1970. Purification and further characterization of an "IBV-like" virus (coronavirus). Proc. Soc. Exp. Biol. Med. 135:457-463.
24. **Kaye, H. S., H. B. Marsh, and W. R. Dowdle.** 1971. Seroepidemiologic survey of coronavirus (strain OC 43) related infections in a children's population. Am. J. Epidemiol. 94:43-49.
25. **Kaye, H. S., S. B. Ong, and W. R. Dowdle.** 1972. Detection of coronavirus 229E antibody by indirect hemagglutination. Appl. Microbiol. 24:703-707.
26. **Kritzman, J.** 1958. Studies of rheumatoid serum employing a modified Coombs' slide test. J. Lab. Clin. Med. 52:328-334.
27. **Lowrv, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
28. **Lycke, E., and M. Peterson.** 1976. Hemolysis-in-gel test for demonstration of chlamydia antibodies. J. Clin. Microbiol. 4:450-452.
29. **McGhee, J. R., D. C. Hurst, N. M. Hamlin, and R. N. Hiramoto.** 1971. A study of the single radial hemolysis in gel system. II. Application of the model to mouse 19S hemolytic antibodies. Immunochemistry 8:367-373.
30. **McIntosh, K.** 1974. Coronaviruses: a comparative review. Curr. Top. Microbiol. Immunol. 63:85-129.
31. **McIntosh, K., W. B. Becker, and R. M. Chanock.** 1967. Growth in suckling-mouse brain of "IBV-like" viruses from patients with upper respiratory tract disease. Proc. Natl. Acad. Sci. U.S.A. 58:2268-2273.
32. **McIntosh, K., A. Z. Kapikian, K. A. Hardison, J. W. Hartley, and R. M. Chanock.** 1969. Antigenic relationships among the coronaviruses of man and between human and animal coronaviruses. J. Immunol. 102:1109-1118.
33. **Probert, M., and S. M. Russell.** 1975. Measurement of parainfluenza-3 virus antibody by the single radial hemolysis technique. J. Clin. Microbiol. 2:157-161.
34. **Romeyn, J. A., and J. Cook.** 1975. Anti-immunoglobulin analysis by diffusion patterns of inhibition and facilitation of complementary lysis in agar. I. Diffusion-lysis patterns of heterologous guinea pig anti-immunoglobulins. J. Immunol. Methods 6:363-374.
35. **Russell, S. M., D. McCahon, and A. S. Beare.** 1975. A single radial haemolysis technique for the measurement of influenza antibody. J. Gen. Virol. 27:1-10.
36. **Schild, G. C., J. S. Oxford, and J. L. Virelizier.** 1975. Immunity to influenza. Dev. Biol. Stand. 28:253-272.
37. **Skaug, K., I. Ørstavik, and J. C. Ulstrup.** 1975. Application of the passive haemolysis test for the determination of rubella virus antibodies. Acta Pathol. Microbiol. Scand. Sect. B 83:367-372.
38. **Strannegård, Ö, L. Grillner, and I.-M. Lindberg.** 1975. Hemolysis-in-gel test for the demonstration of antibodies to rubella virus. J. Clin. Microbiol. 1:491-494.
39. **Tannock, G. A., and J. C. Hierholzer.** 1977. The RNA of human coronavirus OC-43. Virology 78:257-267.
40. **Weiler, E., E. W. Melletz, and E. Breuninger-Peck.** 1965. Facilitation of immune hemolysis by an interaction between red cell-sensitizing antibody and γ-globulin allotype antibody. Proc. Natl. Acad. Sci. U.S.A. 54:1310-1317.