Evaluation of the Single Radial Hemolysis Test for Measuring Hemagglutinin- and Neuraminidase-Specific Antibodies to H3N2 Influenza Strains and Antibodies to Influenza B

KHOSROW FARROHI,¹ FAKHRISSADAT K. FARROHI,¹ GARY R. NOBLE, HAROLD S. KAYE, AND ALAN P. KENDAL*

World Health Organization Collaborating Center for Influenza, Respiratory Virology Branch, Virology Division, Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia 30333

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Antibodies to the H3 hemagglutinin of influenza A virus could be specifically measured by single radial hemolysis (SRH) when test antigens were recombinant viruses containing the relevant H3 hemagglutinin antigen and irrelevant Neq1 neuraminidase of A/equine/Prague/1/56 virus. Antibodies to influenza B virus could also be measured by the SRH technique. Antibody rises to influenza A or B virus measured by SRH agreed with results of hemagglutination inhibition (HI) tests for about 80% of the sera tested, including sera from volunteers receiving killed influenza vaccine and sera from patients naturally infected with influenza. Correlation between antibody titers measured by SRH and HI was also good. Antibodies to the N2 neuraminidase of influenza A virus could be specifically measured by SRH when test antigens were recombinant viruses containing the relevant N2 neuraminidase antigen and irrelevant Heq1 hemagglutinin of A/equine/Prague/1/56 virus. The SRH test for neuraminidase antibodies was more strain specific than was the SRH test for hemagglutinin antibodies. Probably for this reason, agreement between neuraminidase antibody determinations in human sera by the SRH test and by the neuraminidase inhibition test was poorer than agreement between the SRH test for hemagglutinin antibodies and the HI test.

Antibodies to influenza virus nucleoprotein, hemagglutinin, and neuraminidase antigens may be measured by standardized complement fixation (CF) (17), hemagglutination inhibition (HI) (13), and neuraminidase inhibition (NI) (1) tests, respectively. These procedures have, however, certain recognized disadvantages, including the relatively low sensitivity of the CF test, the potential interference by NA antibody and nonantibody serum inhibitors in the HI test, and the complexity of the NI test which limits the number of samples that can be assayed in a given time.

Alternative procedures have therefore been suggested. These include: the single radial diffusion (SRD) test (14) which may measure antibodies to any of the nucleoprotein, HA, and NA viral antigens as well as the viral matrix protein antigen (11); and the HI (9) and enhanced hemagglutination inhibition (NHI) (7) tests, and a passive hemagglutination test (5), which

have been used for measuring neuraminidase antibodies. Each of these newer tests has its problems. The single radial diffusion test requires large amounts of purified virus, and the NHI and passive hemagglutination techniques require specialized biological reagents which introduce the possibility for errors due to the variable performance of these reagents (4).

Recently another procedure, single radial hemolysis (SRH), has been reported to successfully measure antibodies to influenza A hemagglutinins (11), without sera having to be treated to destroy nonspecific inhibitors. The test was reported to be sensitive and did not require large amounts of virus antigen. Initial results were obtained in a study with wild-type influenza A viruses used as antigens. It has since been reported, however, that neuraminidase antibodies can also react in the SRH procedure (2).

In this report we describe the evaluation of the SRH test for its correlation with the HI test under circumstances where reaction of neuraminidase antibodies in the SRH test was elimi-

¹ Visiting Scientists from Tehran University, Basic Medical Science, Department of Microbiology and Immunology, Tehran, Iran.

nated by using antigenic recombinant viruses. Additionally, we examined the SRH test for its ability to measure influenza B antibodies, so that the potential value of the test could be extended to include serodiagnosis of infections with all viruses of the influenza virus genus.

At the same time, we evaluated the SRH test for measurement of neuraminidase antibodies in human serum, with the specific objective of determining whether antigenic drift of human influenza A neuraminidase would affect the reliability of the test. We did this because the test had been reported to be insensitive to neuraminidase antibodies other than those highly specific for the virus strain used as test antigen (2).

MATERIALS AND METHODS

Viruses. Recombinant influenza A viruses used in this study were originally obtained from Robert G. Webster. Influenza B/Hong Kong/5/72 used was a high-growth-yielding strain (BX 1) originally supplied to us by Merrell-National Laboratories, Swiftwater, Pa. Wild-type influenza A viruses were from the reference collection of this laboratory. Inactivated virus antigens were prepared by adding 0.1% beta-propiolactone (BPL) to allantoic fluid containing 0.025 M disodium monohydrogen phosphate and then incubating the mixture at 32°C for 2 h. The fluid pH was adjusted to 7.3 with 7% sodium bicarbonate solution when necessary.

Preparation of SRH plates. The procedure was based on that described by Russell et al. (13). Packed sheep erythrocytes were mixed with an equal volume of 5×10^{-4} M potassium periodate in phosphate-buffered saline (PBS), pH 7.2, and reacted at room temperature for 10 min with gentle mixing. Virus-containing allantoic fluid was then added to the cells in such a proportion that each 0.2 ml of erythrocyte-periodate mixture was reacted with 1,000 hemagglutinating units of virus. After 15 min, during which the mixture was gently shaken, the treated cells were washed three times in PBS and made up to a final 50% suspension. For each plate, 0.1 ml of treated cells (influenza A viruses) was mixed at about 42°C with 2.8 ml of a 1% solution of Indubiose A37 (L'Industrie Biologique Francaise) in PBS and 0.1 ml of guinea pig serum. Gels were poured into immunodiffusion plates and allowed to set; wells with diameters of 2 mm were then punched. The gels were stored at 4°C until used, within 1 to 3 days of preparation. Preliminary tests established that similar results were obtained with active or BPL-inactivated virus. Since inactivated antigens are preferable for use in diagnostic laboratories, all subsequent tests were conducted with BPL-treated virus. As described in Results, clearer zones of hemolysis were found for influenza B virus when gels contained 60% of the erythrocyte concentration used with influenza A viruses.

Performance of the SRH test. Preliminary studies demonstrated that with undiluted human or animal convalescent sera, 1- μ l volumes gave satisfactory zone areas in most SRH tests. Larger volumes

produced zone areas so extensive that they seriously limited the number of sera that might be tested on a single plate. Therefore, in all subsequent studies 1- μ l volumes of serum were dispensed into wells, using 5- μ l disposable capillary pipettes calibrated in 1- μ l increments. Plates were placed in a moist atmosphere at 4°C overnight and then transferred to 35°C for 4 h. Diameters of hemolysis zones were determined with the aid of a 7 $\times$ magnifying measurer and converted to areas, correcting for the area of the well. A ratio of 1.5-fold between areas of any two hemolysis zones was considered as indicating a significant difference in antibody concentrations of the sera tested. This ratio of areas corresponded to a difference in diameter of about 0.5 mm for small (3.5 mm) zones, or 1 mm for large (6 mm) zones, which in practice represented the smallest increase in size that could be objectively measured when comparing an acute-phase (or prevaccination) serum with its corresponding convalescent-phase (or postvaccination) serum.

Conventional serological tests. HI tests, with sera treated with receptor destroying enzyme (Center for Disease Control reference reagent), and CF tests were performed by standard procedures (3, 17). NI tests were conducted with fetuin substrate in a manner similar to that described by Kendal et al. (8) but with twice the previous fetuin concentration and 3.2-fold or 4-fold serum dilutions.

Animal sera. Ferrets were infected intranasally with 1 ml of undiluted allantoic fluid and bled out 12 to 14 days later.

Human sera. Human sera were from two sources. Prevaccination and postvaccination sera were from adult volunteers immunized in September 1975, with a 1974 to 1975 formulation killed whole virus vaccine (Merck, Sharp and Dohme "Fluvac"), containing 700 chick cell agglutinin (CCA) units of A/Port Chalmers/1/73(H3N2) and 500 CCA units of B/Hong Kong/5/72. Vaccine was administered subcutaneously, and postvaccination sera were collected 3 weeks later. Serum pairs from persons naturally infected with influenza virus or other viruses had been submitted for diagnosis to the Respiratory Virology Branch from 1973 to 1975. Such sera were from patients with a clinical upper respiratory infection. Bleeding intervals were not uniform, but each convalescent-phase serum was collected at least 3 weeks after the acute-phase serum. All sera had been stored frozen at -20°C. No preservatives were added.

RESULTS

(i) **SRH for influenza A hemagglutinin antibodies, measured with recombinant virus antigens.** To determine whether SRH effectively measured hemagglutinin antibodies under conditions where activity by neuraminidase antibodies was excluded, we prepared SRH plates with recombinant antigen A/Port Chalmers/1/73(H3)-equine/Prague/1/56(Neq1) and tested with animal sera and human sera lacking antibodies to the Neq1 neuraminidase. Figure 1A illustrates the dose-response relationship when serum from a ferret infected with A/

FIG. 1. Detection of influenza hemagglutinin antibodies by SRH: dose-response relationship. SRH plates contained sheep erythrocytes coated with recombinant virus A/Port Chalmers/1/73(H3)-equine/Prague/1/56(Neq1). Hemolysis areas produced by dilutions of (A) serum from a ferret infected with A/Port Chalmers/1/73(H3N2) virus; and (B) convalescent sera from two people naturally infected with A/Port Chalmers/1/73-like virus.

Port Chalmers/1/73 was tested at various dilutions. This test demonstrated that at high antibody concentrations a linear relationship existed between the area of hemolysis produced and the serum dilution. As the antibody concentration decreased, however, this relationship changed. At serum dilutions producing hemolysis areas smaller than about 15 mm², the sensitivity of the SRH test to serum dilution was much less than at serum dilutions producing hemolysis areas of greater than about 15 mm². A similar effect was observed with other animal sera (not shown) and also with convalescent-phase human sera from patients infected with A/Port Chalmers/1/73-like virus (Fig. 1B). When animal or human sera having "high" activities (hemolysis area >20 to 90 mm² in the SRH test) were tested on three different days with gels prepared from different batches of erythrocytes, the standard error of results in the SRH test was 1 to 4%; this degree of reproducibility is similar to that obtained by Russell et al. (13).

(ii) SRH for influenza B hemagglutinin antibodies. Since influenza B neuraminidases fall

within one antigenic subgroup (6, 12), it was not possible to use an influenza B recombinant virus with an irrelevant neuraminidase. SRH plates were therefore prepared with the representative B/Hong Kong/5/72 whole virus antigen. Plates were tested initially with ferret serum to the virus B/Hong Kong/7/75, which is closely related to B/Hong Kong/5/72. The clearest patterns of hemolysis were obtained by preparing SRH plates with only about one-half of the erythrocyte concentration of that in plates made with influenza A viruses. Under these conditions, the ferret serum produced readily measurable clear zones of hemolysis, with a similar dose-response relationship to that shown for influenza A virus reactions (see Fig. 1). Additional analysis of the measurement of influenza B antibodies in human sera by the SRH technique is presented below.

(iii) Evaluation of the SRH test for measurement of influenza hemagglutinin antibody rises in human sera. Paired sera selected for testing were from influenza vaccine recipients and from persons naturally infected with influenza viruses. Vaccinees had been immunized in 1975 with a vaccine containing A/Port Chalmers/1/73 killed virus and thus might be expected to produce antibodies to the prior A/Hong Kong/8/68 strain as well as to the A/Port Chalmers/1/73 strain (10). Therefore, HI and SRH tests were conducted with both A/Hong Kong/8/68 and A/Port Chalmers/1/73 antigens. In all cases the antigens used in the SRH test were recombinant viruses, thus excluding any effects of neuraminidase antibodies. Table 1 shows that in 67% of the cases examined there was a total agreement between the HI and SRH results for the frequencies of antibody rises to both A/Hong Kong/8/68 and A/Port Chalmers/1/73 antigens. Furthermore, in 57 cases where an HI rise to A/Port Chalmers/1/73 was found, 52 (91%) also showed an SRH rise to A/Port Chalmers and, including A/Hong Kong/8/68 antigen, added little to the ability of either test to measure overall rates of seroconversion in response to the A/Port Chalmers vaccine. In a few instances where the HI test did not demonstrate a fourfold antibody rise, we observed an experimentally significant rise in titer obtained by the SRH technique. These rises, however, were of low magnitude, implying that there is no fundamental difference between the sensitivities of the two tests when detecting rises in levels of hemagglutinin antibodies.

Antibody response to the influenza B component of the vaccine was also compared by HI and SRH tests. The overall agreement between SRH and HI in measuring the seroconversion rate to influenza B in the vaccinees was 83%

TABLE 1. H3 hemagglutinin antibody rises in 75 recipients of vaccine containing killed A/Port Chalmers/1/73 virus: comparison of hemagglutination inhibition (HI) and single radial hemolysis (SRH) tests

Antibody rise by SRH ^a	Antibody rise by HI ^b			
	A/Port Chalmers/1/73 and A/Hong Kong/8/68	A/Port Chalmers/1/73 only	A/Hong Kong/1/68 only	Neither virus
A/Port Chalmers/1/73 and A/Hong Kong/8/68	35 ^c (47)	10 (13)	3 (4)	2 (3)
A/Port Chalmers/1/73 only	2 (3)	5 (7)	0 (0)	1 (1)
A/Hong Kong/1/68 only	0 (0)	2 (3)	0 (0)	2 (3)
Neither virus	1 (1)	2 (3)	0 (0)	10 (13)

^a A 1.5-fold or greater increase in area of clear hemolysis zone (or from 0 to ≥4 mm²). Antigens used to coat erythrocytes contained neuraminidase Neq1.
^b A fourfold or greater rise in HI titer (or from <10 to ≥20). Antigen used in the HI test contained hemagglutinin and neuraminidase antigen of the designated strain.
^c Number (percentage) of individuals with indicated response(s). Results underlined indicate agreement between HI and SRH tests.

(Table 2). As an additional assessment of the correlation between HI and SRH tests for measuring hemagglutinin antibodies in this study, we performed a correlation analysis comparing HI titer values and SRH hemolysis zone areas obtained for antibody to each of the three hemagglutinins, A/Hong Kong/8/68, A/Port Chalmers/1/73, and B/Hong Kong/5/72. For each homologous set of results we obtained a correlation coefficient of from 0.79 to 0.90 (Table 3).

To further determine the value of the SRH procedure, we compared it with the HI test for serodiagnosis of influenza infections. Paired sera received by the Respiratory Virology Branch at the Center for Disease Control for serodiagnosis during the years 1973 to 1975 were examined by HI and SRH tests for antibodies to the A/Port Chalmers/1/73 and B/Hong Kong/5/72 viruses. These two strains were representative of viruses responsible for influenza epidemics during the period. Fifty serum pairs were examined, 13 pairs with a positive serodiagnosis of infection with a respiratory virus other than influenza, and 37 pairs with a positive serodiagnosis by HI and/or CF of influenza infection. Table 4 demonstrates that of 30 cases with a positive influenza infection diagnosed by HI, 24 (80%) were correctly diagnosed by the SRH test. Similarly, there was an agreement of about 80% in the influenza-negative diagnosis by the two tests. This 80% agreement between SRH and HI tests is consistent with results described above for measuring seroconversion in vaccine recipients. For those patients where evidence of influenza infection depended solely on an antibody rise to the soluble antigen of the virus, however, agreement between the CF results and the SRH results was poor. This is not surprising since the SRH test with erythrocytes

TABLE 2. Hemagglutinin antibody rise in 75 recipients of vaccine containing killed B/Hong Kong/5/72 virus: comparison of hemagglutination inhibition (HI) and single radial hemolysis (SRH) tests

Antibody response ^a	No. (%)
Rise by HI and SRH	47 (63)
Rise by HI only	7 (9)
Rise by SRH only	6 (8)
No rise by HI or SRH	15 (20)

^a Antibody response is fourfold or greater rise in titer (or <10 to >20) by HI test; and 1.5-fold or greater increase in area of clear hemolytic zone (or area increase from 0 to ≤4 mm²) by SRH. Results underlined indicate agreement between HI and SRH tests.

TABLE 3. Correlation between hemagglutination inhibition (HI) titer and single radial hemolysis (SRH) area obtained with sera of 75 volunteers vaccinated^a with killed influenza virus

Serum	Virus	Correlation coefficient ^b
Prevaccination	A/Hong Kong/8/68	0.80
Postvaccination	A/Hong Kong/8/68	0.79
Prevaccination	A/Port Chalmers/1/73	0.90
Postvaccination	A/Port Chalmers/1/73	0.79
Prevaccination	B/Hong Kong/5/72	0.80
Postvaccination	B/Hong Kong/5/72	0.79

^a Seventy-five vaccinees each received 700 CCA units of A/Port Chalmers/1/73 and 500 CCA units of B/Hong Kong/5/72 viruses.
^b Correlation of SRH area with HI titer. HI test performed with standard virus; SRH tests performed with standard virus for influenza B, and recombinant virus containing neuraminidase Neq1 for influenza A strains.

TABLE 4. Comparison of single radial hemolysis (SRH) to hemagglutination inhibition (HI) and complement fixation (CF) tests in the serodiagnosis of influenza infections

SRH serodiagnosis	Conventional serodiagnosis ^a		
	Influenza positive		Influenza negative ^b
	HI	CF	
Influenza positive ^c	24	2	2
Influenza negative	6	5	11

^a Conventional serodiagnosis was by tests of acute- and convalescent-phase serum pairs received from 1973 to 1975. Diagnosis of influenza infection by HI = fourfold or greater increase in HI titer (or from <10 to >20) with or without a CF titer rise with A/Port Chalmers/1/73 (H3N2) or B/Hong Kong/5/72 used as antigens. Diagnosis of influenza infection by CF = fourfold or greater increase in complement-fixing titer to influenza A or influenza B soluble antigen, in the absence of an HI antibody rise.

^b Negative by CF and HI.

^c A 1.5-fold or greater increase in area of clear hemolytic zone (or from 0 to ≥ 4 mm²) with red erythrocytes coated with either A/Port Chalmers/1/73 (H3)-equine/Prague/56 (Neq1) or B/Hong Kong/5/72 inactivated viruses used.

coated with whole virus used as indicator would not be expected to detect antibodies to the soluble antigen of the virus.

(iv) SRH for influenza A neuraminidase antibodies, measured with recombinant virus antigens. For these tests, erythrocytes were coated with recombinant antigens containing the hemagglutinin of A/equine/Prague/1/56 (Heq1) and tested with animal sera to whole virus or with human sera, thus excluding any reaction by hemagglutinin antibodies. Preliminary studies with ferret serum as the source of antibody showed that reducing the concentration of erythrocytes incorporated into the agar increased the areas of hemolysis produced by the neuraminidase antibodies in ferret serum, but at the expense of the distinctness of the zones. Increasing the amount of virus used to coat the cells had little effect on the test results. Therefore, in further tests we used gels prepared with erythrocyte and virus concentrations similar to those used for measuring hemagglutinin antibodies. When cells coated with recombinant viruses containing the neuraminidases of A/Port Chalmers/1/73 or A/Hong Kong/8/68 were tested with ferret sera to the homologous wild-type virus, clear hemolysis zones were produced with a dose-response relationship similar to that shown for hemagglutinin antibodies in Fig. 1 and 2. When cells

FIG. 2. Detection of influenza hemagglutinin antibodies by SRH: homologous and heterologous reactions. Hemolysis areas produced by (A) ferret sera to A/Hong Kong/8/68(H3N2) virus; or (B) ferret sera to A/Port Chalmers/1/73(H3N2) virus tested with cells coated with A/Hong Kong/8/68-equine/Prague/56 (—) and A/Port Chalmers/1/73-equine/Prague/56 (---).

coated with the recombinant virus containing A/Port Chalmers/1/73 neuraminidase were tested with ferret serum to A/Hong Kong/8/68 virus, however, only partial hemolysis was observed, and the hemolytic areas were very difficult to measure accurately (Fig. 3). A similar observation was made for the reciprocal test of A/Port Chalmers/1/73 serum against erythrocytes coated with recombinant containing A/Hong Kong/8/68 neuraminidase. This result confirmed the findings by Callow and Beare (2) and suggested that problems might arise in applying the SRH test to the measurement of neuraminidase antibodies in human sera. In human sera, populations of homologous and heterologous neuraminidase antibodies, which are present in varying proportions, might cause varying degrees of hemolysis against any one antigen.

Therefore, the human sera, obtained before and after immunization with the vaccine containing 700 CCA units of A/Port Chalmers/1/73 virus, were tested for neuraminidase antibodies by SRH and NI procedures. In each case, two recombinant viruses containing A/Hong Kong/68 and A/Port Chalmers neuraminidases were used as antigens. In the SRH test, we measured only distinct hemolytic areas; therefore, we scored as negative the sera producing a narrow ring of hemolysis as the circumference of a nonhemolyzed zone, and sera producing only indistinct and not clearly measurable zones.

Overall agreement between the SRH and NI tests in measuring seroconversions to the two neuraminidases was 43% (Table 5). Of 40 indi-

viduals with a significant antibody rise to A/Port Chalmers/1/73 as measured by the NI test, only 24 (60%) demonstrated a significant antibody rise to A/Port Chalmers/73 as measured by SRH. Twenty-four (69%) of 35 sera showing a rise in titer to A/Port Chalmers/1/73 neuraminidase by SRH also showed a rise by NI. Analysis of the titers obtained in the two tests showed a correlation of between 0.34 and 0.60 (Table 6). The results indicate that neuraminidase antibody determinations in human sera by

NI and SRH tests agree less well with each other than do results of hemagglutinin antibody determinations by HI and SRH tests. We do not believe that this results from the way in which rises are scored in the two tests (i.e., 1.5-fold increase in area for SRH and titer rise from <10 to >10 in NI). Only two serum pairs were scored as showing a rise by NI when the titer of the second serum was between 10 and 32, and no SRH rise was observed.

DISCUSSION

In the present study of SRH with influenza A(H3N2) viruses, recombinant virus strains containing irrelevant hemagglutinin or neuraminidase antigens of A/equine/Prague/1/56 virus were used, so that H3 hemagglutinin antibodies could be measured without interference by N2 neuraminidase reactions, and vice versa. We considered this necessary because Callow

FIG. 3. Detection of influenza neuraminidase antibodies by SRH: homologous and heterologous reactions. SRH plates contained sheep erythrocytes coated with recombinant viruses A/equine/Prague/1/56(Heq1)-Hong Kong/1/68(N2) or A/equine/Prague/1/56(Heq1)-Port Chalmers/1/73(N2). Hemolysis areas produced by ferret serum to A/Hong Kong/8/68(H3N2) virus (rows 1 and 3) and A/Port Chalmers/1/73(H3N2) virus (rows 2 and 4) tested with cells coated with A/equine/Prague/56-Hong Kong/1/68 (top slide) and A/equine/Prague/1/56-Port Chalmers/1/73 (bottom slide). Serum dilutions from right to left: undiluted, 2, 4, 8, 16.

TABLE 6. Correlation coefficients between neuraminidase inhibition (NI) titer and single radial hemolysis (SRH) area obtained with sera of volunteers vaccinated^a with killed influenza virus

Serum	Virus	Correlation coefficient ^b
Prevaccination	A/Hong Kong/1/68	0.51
Postvaccination	A/Hong Kong/1/68	0.53
Prevaccination	A/Port Chalmers/1/73	0.69
Postvaccination	A/Port Chalmers/1/73	0.34

^a Vaccinees received 700 CCA units of A/Port Chalmers/1/73 and 500 CCA units of B/Hong Kong/5/72 viruses.

^b NI and SRH tests performed with recombinant viruses containing the hemagglutinin Heq1.

TABLE 5. Neuraminidase antibody rises in 75 recipients of vaccine containing killed A/Port Chalmers/1/73 virus: comparison of neuraminidase inhibition (NI) and single radial hemolysis (SRH) tests

Antibody rise by SRH ^a	Antibody rise by NI ^b			
	A/Port Chalmers/1/73 and A/Hong Kong/8/68	A/Port Chalmers/1/73 only	A/Hong Kong/1/68 only	Neither virus
A/Port Chalmers/1/73 and A/Hong Kong/8/68	<u>5^c (7)</u>	10 (13)	2 (3)	0 (0)
A/Port Chalmers/1/73 only	3 (4)	<u>6 (8)</u>	1 (1)	8 (11)
A/Hong Kong/1/68 only	2 (3)	<u>2 (3)</u>	<u>0 (0)</u>	0 (0)
Neither virus	3 (4)	9 (12)	3 (4)	<u>21 (28)</u>

^a A 1.5-fold or greater increase in area of clear hemolytic zone (or from 0 to ≥ 4 mm²). Antigens used to coat erythrocytes contained hemagglutinin Heq1.

^b A threefold or greater increase in NI titer (or from <10 to ≥ 10). Antigens used in the NI test contained hemagglutinin Heq1.

^c Number (percentage) of individuals with indicated response(s). Results underlined indicate agreement between NI and SRH tests.

and Beare observed that neuraminidase antibodies may react in the SRH test (2).

In the absence of any possible reaction by neuraminidase antibodies, we found that the SRH test reproducibly and sensitively measured antibodies to H3 hemagglutinins. Provided hemolysis zones produced were greater than about 15 mm² under the conditions used, a linear relationship was observed between hemolysis area and serum dilution. Deviation from this linear relationship at low antibody activities (high serum dilutions) was reproducibly observed. This, however, had little detrimental effect on a practical application of the test as evidenced by the 80 to 90% agreement between SRH and HI test results, when we measured frequencies of antibody rises after vaccination with killed virus or natural influenza infections. The results we obtained with antigenic recombinant viruses are very similar to those Russell et al. (13) obtained with wild-type viruses, and therefore suggest that in most circumstances it may be unnecessary to use the antigenic recombinant viruses in SRH tests for measuring influenza hemagglutinin antibodies. In situations such as the one occurring in 1968, however, when influenza hemagglutinin underwent a marked antigenic shift without a corresponding change in the neuraminidase (16), use of nonrecombinant viruses in the SRH test applied to studies with human sera might well affect the validity of hemagglutinin antibody measurement.

Since hemagglutinin and neuraminidase antigens of influenza B viruses show less dramatic variability than the corresponding antigens of influenza A viruses and since influenza B recombinant viruses with irrelevant neuraminidase are not routinely prepared for serological studies, we used wild-type virus as antigen for measuring influenza B antibodies in the SRH test. Although a minor variation in technique appeared advantageous (see Results), we found that the SRH test could also be used to measure antibodies to influenza B in animal or human sera. This has not been previously reported. The degree of correlation between HI and SRH test results was as good for influenza B virus as for influenza A, so that the SRH test appears quite suitable as a diagnostic procedure for detecting influenza antibody rises. SRH might also be used for antibody prevalence studies. Interpretation of results is complicated, however, by the possibility that the relationship between HI titer and the hemolytic area obtained with any serum may vary with different virus antigens. In the present study for example, we observed that human sera with HI

titers of 40 for any one of the three viruses A/Hong Kong/8/68, A/Port Chalmers/1/73, or B/Hong Kong/5/72 produced average hemolytic zones of 17, 10, and 21 mm² with the corresponding antigen in the SRH test.

Time-saving by use of SRH instead of HI cannot be assured, since plates are not available commercially, and since plates must be used within about 1 week after preparation because of the lability of the complement components. Furthermore, the SRH test as described is unlikely to detect antibodies to the influenza ribonucleoprotein (S) antigen (Table 4), so a type-specific CF test, in addition to the SRH test, would have to be used for maximum sensitivity in detecting antibody rises after infection. (Such is also the case when HI tests are used to diagnose infection.) This may be particularly important when influenza B infections, which can often be diagnosed only by a rise in titer of antibody to type B ribonucleoprotein (unpublished observation), are being detected.

Although in our study the SRH test performed well in measuring hemagglutinin antibodies, results for measuring neuraminidase antibodies were not so encouraging. Neuraminidase antibodies in ferret sera reacted well in the SRH test, but only for homologous antigen-antibody systems. With systems where heterologous reactions of cross-reactive antigen and ferret sera were tested, hemolysis was usually incomplete, as Callow and Beare (2) also noted. The poor agreement between SRH and NI measurement of neuraminidase antibodies in human sera probably results from the variability of the SRH test in many instances to measure heterologous antibody present in human sera, although that antibody did react in the NI test. There are several possible reasons why Callow and Beare (2) observed a fairly good agreement between SRH and NI measurements of neuraminidase antibody in human sera, and we did not: (i) Callow and Beare measured neuraminidase inhibition at a single serum dilution in their NI test, whereas we determined titers corresponding to the serum dilution producing a 50% inhibition of activity. (ii) Volunteers studied by Callow and Beare were immunized with A/Hong Kong/68 virus and tested with antigen containing the Hong Kong virus neuraminidase. In our study volunteers in 1975 had received the H3N2 antigenic variant A/Port Chalmers/1/73 virus so there was a greater probability that heterologous antibody responses would occur than in the earlier study. (iii) Callow and Beare's volunteers were infected with live virus vaccine, in contrast to the killed virus vaccine in our study. This differ-

ence might also affect the degree of homospecificity in the antibody responses in the two studies.

Therefore, we do not believe that there is any fundamental difference between the results of our study measuring neuraminidase antibodies by SRH and the results of Callow and Beare (2). Rather, our results relate to a human population that has been exposed to a wide variety of cross-reacting N2 neuraminidase antigens, including those of the 1972 and 1973 isolates which have drifted significantly from the neuraminidase of the A/Hong Kong/68 virus (15). Since antigenic drift of neuraminidases is expected to be a common occurrence with future influenza viruses, and on the basis of our observations, it would seem that the SRH test should be used with caution for measuring neuraminidase antibodies in human sera.

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