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Automated Procedure for Measuring Antigenicity of Extracted and Intact Influenza Virus

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An automated serum-blocking (S-B) technique was developed in an attempt to find an in vitro test for the determination of the antigenicity of extracted influenza vaccines. The S-B test depends on the ability of an antigen to combine with (or block) specific (in this case, hemagglutination-inhibiting) antibodies. After mixing the test virus with a constant amount of specific antiserum and hemagglutinating virus in an Auto Analyzer, chicken erythrocytes were pumped into the system and the mixture was incubated by passing through coils. The hemagglutinated cells were removed and the residual cells were lysed. The optical density was read and recorded automatically. The S-B test was much more reproducible than the chicken cell agglutination (CCA) test. There was good correlation between the S-B and CCA titers of intact influenza virus, but not of ether-extracted influenza virus. The CCA titer of influenza strains of type A was reduced significantly during ether-extraction. The S-B titers indicated that there was no significant loss in specific antigenicity when influenza strains of types A and B were extracted with ether and Tween-80 according to the described procedure. The S-B test seemed to be a true measurement of the total antigens present in influenza vaccines.

In 1953, Hoyle observed that the infectivity of influenza viruses could be destroyed by ether and that this was owing to disruption of the virus with release of viral hemagglutinins (11). Several workers subsequently confirmed his results and utilized this principle for the preparation of purified influenza vaccines. Ether (16), lipid solvents (17), or detergents (13, 14) were used to separate hemagglutinating subunits from the nucleoprotein "soluble" antigen and from lipids in the viral envelope of influenza and other myxoviruses.

It is conventional to standardize intact influenza virus vaccines on the basis of hemagglutinin titer. Choppin and Stoeckenius (3), however, found that the hemagglutinating activity of ether-extracted virus can be detected only if the virus fragments are "divalent" or "multivalent." Other workers showed that the presence of hemagglutinins is not essential for the immunogenic activity of an influenza virus preparation (5, 10, 12, 18). They disrupted several strains of influenza by the use of lipid solvents or detergents, or both, removed any residual hemagglutinins, and found that the purified material was still immunogenic in animals.

Several workers have attempted to develop an in vitro test which would offer a better measurement of potency for vaccine standardization than do tests which measure only hemagglutinin concentration. Eckert (5) used the blocking antigen (BA) test for this purpose. The BA test measures the ability of an antigen to bind (or block) specific hemagglutination-inhibiting (HI) antibodies. We have found the BA test as described by Eckert to be simple, but it lacks reproducibility because the end point can be read only as positive or negative, with no intermediate points. Hobson (10) used another version of the BA test, which he referred to as a "serum-blocking test." In his procedure, serial dilutions of the unknown antigen and antiserum were tested against each other. In our hands, the test was fairly reproducible, but it was very time-consuming and required large volumes of reagents.

We have adopted the same principle to develop an automated serum-blocking (S-B) test using the Auto Analyzer (Technicon Controls, Inc., Ardsley, N.Y.). This report describes the technique, its reproducibility, and the correlation of results obtained with the S-B test and the conventional chicken cell agglutination (CCA) test (15).

MATERIALS AND METHODS

Virus suspensions. The A/PR-8/34, A_1/Ann Arbor/1/57, A_2/Jap/170/62, A_2/Taiwan/1/64, B/Massachusetts/3/66, and B/Maryland/1/59 strains of

influenza virus grown in eggs were used. Virus dilutions were made in 0.01 M phosphate-buffered isotonic saline (PBS; *p*H 7.2) containing 6 drops/liter of Tween-80.

Virus extraction. Extracted preparations of the strains were used. Partially purified, concentrated, monovalent preparations were further purified by gel-filtration and then extracted with Tween-80 and ether as described by Brandon et al. (2).

Chicken erythrocytes. Blood was obtained either by cardiac puncture or from the wing vein and mixed with an equal volume of Alsever's solution. The erythrocytes from a 10-chicken blood pool were filtered through gauze, washed three to four times with PBS, and packed by low-speed centrifugation. The packed cells were held in the refrigerator for a maximum of 10 days and the quantity required for a given day's test was removed and prepared as a 0.6% suspension in PBS. The suspension was kept cold during the test by placing it in an ice bath.

Monospecific hyperimmune serum. A 1-ml amount of an emulsion (1:1) of a particular influenza virus strain and Freund's complete adjuvant was injected subcutaneously into the neck region of a rabbit. One week later, 1.0 ml of the same emulsion was injected intraperitoneally. In the third week, 0.5 ml of the unadjuvanted viral preparation was injected intravenously. The animal was bled for 7 days after the last injection, and weekly thereafter. This schedule routinely gave high-titered monospecific antiserum. Each serum sample was checked for cross-reactivity with the other viral strains; with two exceptions, cross-reactivity was very low. The exceptions were cross-reactivities between the two Asian strains, A_2/Jap/170/62 and A_2/Taiwan/1/64, and between the two type B strains, B/Massachusetts/3/66 and B/Maryland/1/59. This cross-reactivity was expected because of the similar serological structure of the strains involved.

Lysing agent. A 1.0% sodium hydroxide solution was used to lyse the chicken erythrocytes.

Auto Analyzer. Illustrated diagrammatically in Fig. 1, the apparatus is basically similar, with a few important modifications, to the system described previously by Hebeka et al. (7) for the assay of hemagglutination titer of influenza virus. The large sampler was used, substituting 2-ml plastic cups for standard test tubes. The sampling arm was adjusted to sample through two separate lines (plastic tubing) at the same time; one led to the cups on the sampler and the other to a bottle containing the hemagglutinating virus. A third line was connected to the sampling arm to introduce PBS to the system when virus was not being sampled, thus separating one virus sample from the other. The automated test consisted of three main parts; (i) titration of the hemagglutinating (HA) virus, (ii) titration of the specific antiserum, and (iii) measurement of S-B activity.

Titration of the HA virus. Twofold dilutions of a virus concentrate of the same strain as the material to be tested were placed in sampler cups. The other two sampling lines introduced PBS only during this reaction. The virus was sampled for 40 sec and followed by PBS for 95 sec. The virus or PBS was then combined with chilled erythrocytes introduced as a continuous stream, and the mixture was allowed to react in the mixing coils. The erythrocyte suspension was kept in continuous, mild agitation by a magnetic stirrer. After passage through the mixing and time-delay coils, the agglutinated erythrocytes, clearly visible to the naked eye, settled to the bottom of the fluid stream and were removed through a series of decanting T-tubes. Sodium hydroxide was then pumped into the flowing liquid and combined with the remaining nonagglutinated erythrocytes. The erythrocytes were lysed and the liberated hemoglobin was read colorimetrically and recorded automatically as percent transmittance (% T; Fig. 2).

Titration of the specific antiserum. The antisera were first treated to remove the nonspecific inhibitors. Sera to be used for the titration of viruses of the A, A_1, and B types were first heated at 56 C for 30 min

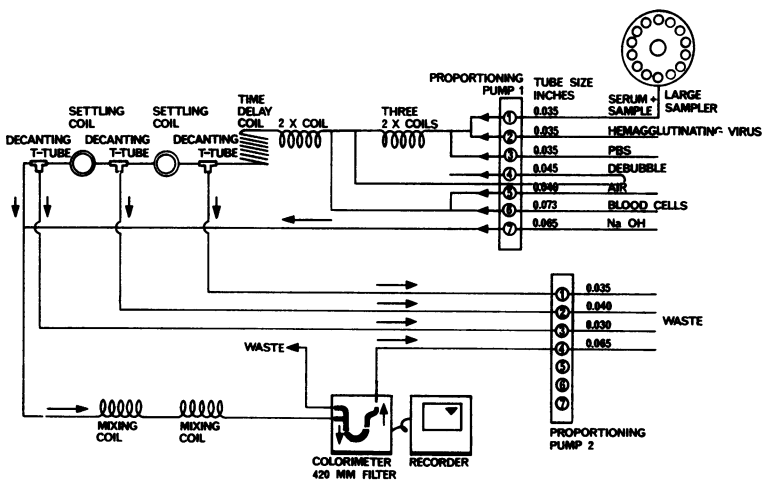

FIG. 1. *Flow diagram of the system used for the automated serum-blocking test.*

and then treated overnight with receptor-destroying enzyme at 37 C. For titration of type A_2 viruses, trypsin-containing sera were inactivated at 56 C for 30 min and then treated with M/90 KIO_4 at room temperature for 15 min. After addition of glycerine to a final concentration of 0.3%, the sera were used for titration.

Twofold dilutions of a treated monospecific antiserum were mixed with an equal volume of PBS and placed in the sampler cups. At sampling time, the serum dilution and the appropriate HA virus dilution (determined in the first part) were introduced. The reagents were mixed and incubated by passing through three double-length glass coils (about 25 min of incubation). Chicken erythrocytes were introduced and the reaction proceeded as described above (Fig. 2). The appropriate virus and serum dilutions were determined (as explained later) and used in the S-B test.

Measurement of S-B activity. Twofold dilutions of the virus sample to be assayed (intact or ether-extracted) were mixed with an equal volume of the appropriate dilution of serum determined in the second part of the test. The mixture was incubated at room temperature for 45 min and then placed in the sampler. This was the only manual part of the test. The test sample serum mixture was sampled simultaneously with the appropriate dilution of HA virus. The reaction then followed the exact procedure described above. Figure 3 represents an example of the data obtained with the S-B test.

A viral suspension of known CCA titer (average of five determinations) was used as a standard to correct for day-to-day variation. At least three points (that fell on a straight line) from the curves of the standard and the unknown were submitted to computer analysis. From the readings and the slope of the curves, the CCA value of the unknown material as related to the standard was determined.

Several readings of the same sample were analyzed to determine the geometric mean, standard error, and 95% confidence limits.

RESULTS

Titration of HA virus. A sigmoidal-type curve was obtained with serial dilutions of the HA virus (Fig. 2). The dilution that gave the highest reading and fell on the straight part of the curve (in Fig. 2, 1:8) was used in the subsequent reactions. It

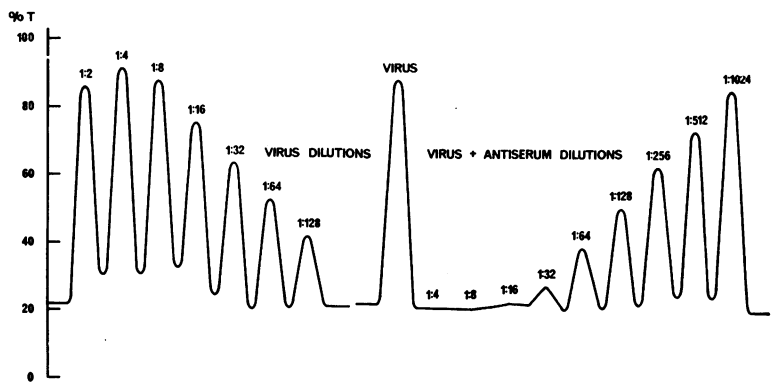

FIG. 2. *Titration of hemagglutinating virus (left) and its specific antiserum (right).*

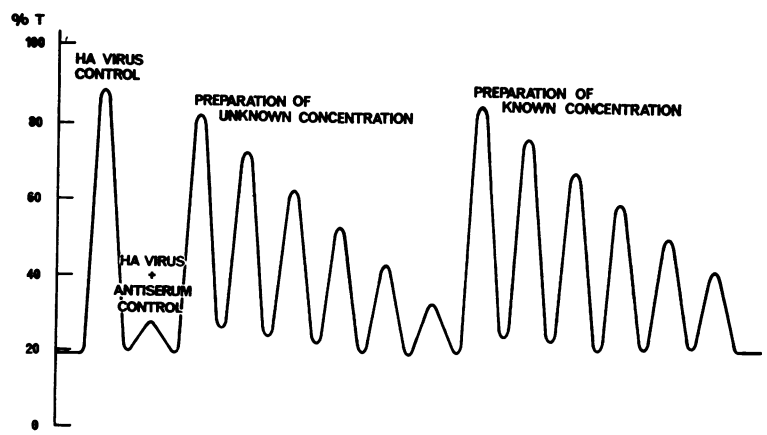

FIG. 3. *Serum-blocking test with hemagglutinating (HA) virus and serum controls.*

TABLE 1. *Reproducibility of the automated S-B test*

Strain	Sample	No. of tests	GM	SEM	95% CL
A/PR-8/34	Intact	29	2,005	5.3	1,806–2,227
	Extracted	18	5,893	6.3	5,192–6,688
A_1/Ann Arbor/1/57	Intact	28	1,902	2.6	1,808–2,002
	Intact	4	973	4.2	595–1,059
	Extracted	11	7,336	6.2	6,483–8,300
A_2/Taiwan/1/64	Intact	16	1,884	2.9	1,830–1,938
	Intact	6	631	4.6	575–691
	Extracted	11	1,328	5.2	1,197–1,472
	Extracted	6	1,498	5.0	1,356–1,655
A_2/Jap/170/62	Intact	10	1,631	2.8	1,542–1,724
	Extracted	8	2,103	5.3	1,890–2,340
B/Massachusetts/3/66	Intact	53	2,125	1.1	2,079–2,171
	Intact	12	3,018	5.7	708–890
	Extracted	4	214	1.8	206–222
	Extracted	4	978	4.5	894–1,070
	Extracted	16	5,840	6.4	5,141–6,633
B/Maryland/1/59	Intact	12	3,018	3.0	2,840–3,206
	Extracted	6	2,652	4.0	2,448–2,874

was observed that strains A_2/Jap/170/62 and A_2/Taiwan/1/64, which had high CCA titers, gave low HA titer by the Auto Analyzer. This problem was solved by immersing the time-delay coil in an ice bath when assaying the Asian strains.

Titration of the specific antiserum. A serum titration as recorded by the Auto Analyzer is represented in Fig. 2. The first peak is due to the HA virus only; i.e., PBS, but no serum, has been placed in the sampler cup. With increasing dilution of serum, the percentage transmission of light increases. The serum dilution that gives the first peak is used in the S-B test. The appropriate dilution of the antiserum is 1:32 (Fig. 2). Parts one and two of the test are done only once for each set of reagents. The same dilutions can be used from day to day.

Measurement of S-B activity. The results of the third part of the test were further divided into (i) reproducibility of the technique and (ii) correlation between S-B and CCA titers.

Reproducibility of the technique. Several preparations of different influenza virus strains were assayed for the S-B titer, expressed in terms of CCA units. Intact and ether-extracted viral preparations were tested on the same day or on successive days. The geometric mean (GM), standard error of mean SEM, and 95% confidence limits (CL) of the mean are presented in Table 1. The SEM is expressed as percentage of GM. It is clear from these results that the test is fairly reproducible with SEM between 1.1 and 6.4. The reproducibility does not seem to change with the different strains or with the kind of antigen (intact or extracted).

TABLE 2. *Correlation between CCA and S-B titers of unextracted influenza preparations*

Strain	CCA titer	S-B titer	Ratio of CCA to S-B
A/PR-8/34	1,612	2,005	0.80
A/Ann Arbor/1/57	1,722	1,902	0.91
	935	971	0.96
A_2/Taiwan/1/64	1,815	1,884	0.96
	600	631	0.95
A_2/Jap/170/62	1,555	1,631	0.95
B/Massachusetts/3/66	1,785	2,125	0.84
	1,808	1,726	1.05
	2,623	2,847	0.92
	2,207	2,027	1.09
	832	794	1.05
B/Maryland/1/59	2,821	3,018	0.93

Correlation between S-B and CCA titers. The CCA titers (average of between two and five estimations) and the S-B titers of the same sam-

TABLE 3. *Correlation between CCA and S-B titers of ether-extracted influenza preparations*

Strain	CCA Titer		Per cent recovery of CCA	S-B titer	Ratio of CCA (extracted) to S-B
	Before extraction	After extraction			
A/PR-8/34	3,342	2,692	81	4,596	0.59
A_1/Ann Arbor/1/57	4,317	3,151	73	6,309	0.50
A_2/Taiwan/1/64	2,993	928	31	1,959	0.47
	1,655	1,056	64	1,498	0.70
A_2/Jap/170/62	1,873	881	47	2,103	0.42
B/Massachusetts/3/66	6,131	6,438	105	5,839	1.10
	220	209	95	214	0.98
	950	942	99	978	0.96
B/Maryland/1/59	2,510	2,292	91	2,651	0.86

ples are presented in Tables 2 and 3. The correlation between CCA and S-B titers of intact influenza virus preparations (Table 2) is good. The ratio of CCA to S-B titers varied between 0.8 and 1.05, with 1.0 being perfect correlation. The correlation does not vary significantly from one strain to another.

The CCA and S-B titers of the ether-extracted viral preparations are summarized in Table 3. A much greater loss of CCA after extraction is apparent for the Asian strains than for the B strains, which showed little or no change in CCA titer after extraction. With the exception of B/Massachusetts/3/66 and B/Maryland/1/59, the S-B titer was significantly higher than the CCA titer. The correlation between CCA and S-B titers of the different preparations of B/Massachusetts/3/66 and B/Maryland/1/59 is excellent. This is true for both the intact and ether-extracted preparations.

DISCUSSION

Immediately after Hirst (9) described the agglutination of erythrocytes by influenza virus, many investigators recognized the potential utility of this technique for estimating virus concentration, and they developed a number of different methods for this purpose. The determination of CCA activity by influenza virus, as described by Miller and Stanley (15), has been widely accepted and used as a routine, in vitro test for the determination of the concentration of influenza virus in a vaccine.

It has been shown recently that immunogenicity of ether-extracted influenza virus preparations is not dependent on the hemagglutinin concentration (5, 10, 12, 18). Not infrequently, the hemagglutinin concentration of intact influenza virus vaccines shows little correlation with antigenicity. Hennessy and Davenport (8) immunized humans with monovalent A_2/Jap/305/57 or A_2/Jap/170/62 influenza vaccines and determined the antibody response. Although each of the vaccines contained 200 CCA units per ml, the immunogenicity of the A_2/Jap/170/62 strain was higher than that of the A_2/Jap/305/57 strain.

The test reported here uses the hemagglutination and HI systems as indicators only. The concentration of a vaccine is determined by the degree of its reactivity with monospecific influenza antiserum. The vaccine is incubated with a standard amount of antiserum after which the HA or challenge virus is added. The higher the concentration of virus in the vaccine tested, the more antibody is bound and the less the amount of antibody left to react with the HA virus. The concentration of residual HA antigen is determined in a system similar to that described by Hebeka et al. (7). As reported previously, the Asian strains A_2/Jap/170/62 and A_2/Taiwan/1/64 have low avidity for chicken erythrocytes in the automated system (7). The low HA readings could have been owing to elution of the virus during incubation in the Auto Analyzer; therefore, we immersed the time delay coil in an ice bath when the Asian strains were being tested. This improved the HA titer of the A_2 strains significantly, but not that of the B strains which have been reported to have high avidity for chicken erythrocytes in the automated system (7).

The reproducibility of the conventional CCA test is very low (4, 6). We have found the automated S-B test to be much more reproducible

than the CCA test, with the SEM varying between 1.1 and 6.4.

Several investigators reported a decrease in CCA titer after extracting influenza virus preparations with ether or detergents, or both. Under the conditions used for extracting the samples reported in Table 3, the recovery of CCA units varied between 31 and 105%. These same samples, however, tested with the automated S-B test, showed no reduction in antigen content. This can be explained by the fact that ether extraction causes a disruption of the viral envelope to progressively smaller fragments. Concomitant with the decrease in the size of the fragments, there is a decrease in the strength of binding of the fragments to erythrocytes (3). The affinity of the smaller fragments for erythrocytes falls below the level necessary for agglutination. The CCA test, therefore, detects the presence of the large fragments in the ether-extracted preparation, whereas the S-B test detects the total antigens present. This can be confirmed by the excellent correlation between the CCA and S-B tests for estimating the concentration of unextracted viral preparations (Table 2). In these preparations, all viral particles should be intact or in large fragments and, therefore, can be detected by both the CCA and S-B test.

In recent reports, Webster and Laver (19) and Brandon et al. (1) compared the immunogenicity of intact influenza virus and its ether-isolated hemagglutinins in animals and humans, respectively. Both groups of investigators prepared their vaccines by diluting the ether-treated vaccine to the same extent as the standard virus preparation without regard to its postether CCA titer. This was done because the hemagglutinins titer after treatment could not be considered to be a measure of virus content (19). The antibody response to both vaccines was equal. The percentage of recovery after ether-extraction, according to the S-B test, was about 100% (Table 3). Consequently, diluting the extracted vaccines to the same extent as the intact virus preparations should give vaccines of equal antigenicity.

The human response to intact and extracted vaccines of known S-B titers will be studied.

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