Identification and Quantitation of the Components of Polyvalent Inactivated Influenza Virus Vaccines by Immunodiffusion

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One of the basic problems in the standardization of inactivated polyvalent influenza virus vaccines has been the determination of the relative potency of the individual strain components. The chicken cell agglutination test measures reliably the total hemagglutinin content of these vaccines. With immunodiffusion techniques, it is now possible to quantitate each strain component of polyvalent vaccines. Routine application of these techniques would serve as an interim procedure to assess antigenic potency of individual strain components of commercial vaccines until improved tests are developed.

Research in the 1940's and 1950's culminated in the development of an inactivated influenza virus vaccine. However, it has only been since the emergence of the Hong Kong variant of A2 influenza in 1968 that reliable laboratory methods for measuring the antigenicity of these vaccines have been available (11). Since then, the standardized chicken cell agglutination (CCA) test, employing a reference vaccine, has allowed reproducible analysis of the total vaccine hemagglutinin content. Although the CCA test has allowed standardization of total hemagglutinin content, it has not been possible to measure the relative content of the several antigenic components in a polyvalent vaccine preparation.

Immunodiffusion (ID) techniques have been shown to be suitable both for the study of influenza virus subunits and the detection of homologous antibody responses in various animal species including man (1–3, 6–10). This report describes the application of ID techniques to analyze the antigenic composition of Formalin-inactivated polyvalent influenza virus vaccines.

MATERIALS AND METHODS

Vaccines. The following inactivated vaccines were employed in this study: (i) National Institutes of Health (NIH) 1967 civilian polyvalent (67CP) and 1971 civilian bivalent (71C) reference vaccines; (ii) lots of commercially prepared influenza vaccines; and (iii) a number of experimental monovalent and polyvalent vaccines of known composition and CCA content. These latter vaccines were made by dilution with 0.01 M phosphate-buffered saline (PBS), pH 7.2, (or, in the case of zonal-purified concentrates, with 0.85% saline containing 0.2% gelatin) and the following Formalin-inactivated influenza virus concentrates: A0/PR/8/34, A1/AA/1/57, and A2/Japan/170/62 monovalent Sharples concentrates; A2/Aichi/2/68 and B/Mass/3/66 monovalent zonal-purified concentrates.

Immune sera. Immune chicken sera were used in these studies. Sera prepared for hemagglutination-inhibition (HI) against A0/PR/8/34, A2/Aichi/2/68, and B/Mass/3/66 were obtained from the Center for Disease Control, Atlanta, Ga. Sera against A1/AA/1/57 were the 17-day-convalvescent sera of White Rock roosters inoculated intravenously with 1.0 ml of experimental vaccine G, containing 1,000 CCA units of A1/AA/1/57 antigen per ml. Nonimmune sera were obtained from un inoculated White Rock roosters.

ID. Double diffusion was performed in either 0.75% Irgarol no. 2 (Colabs) or 0.75% agarose (L'Industrie Biologique Francaise) prepared in distilled water containing various concentrations of NaCl and 0.1% sodium azide. The medium was autoclaved at about 120 C for 7 min, cooled at 60 C, and then 2.5-ml amounts were dispensed into 35-mm-diameter plastic petri dishes (Falcon) which were stored in plastic bags at 4 C until use. Six wells, 4 mm in diameter, evenly positioned around and 9 mm (center to center) from a central 4-mm-diameter well, were cut with a template. Serial twofold dilutions of the reactants were made in the PBS diluent mentioned above. Antigen dilutions were added in duplicate to the outer wells. Precipitin bands were recorded either by visual examination with indirect light or by photography employing
a Polaroid CU 5 Land camera. ID endpoints were expressed as the reciprocal of the highest antigen dilution giving a precipitin reaction on the seventh day. The ID endpoints of the 71C reference vaccine components were converted into CCA units (one ID unit being equivalent to 153.5 CCA units of A2/Aichi/2/68 and 111.5 CCA units of B/Mass/3/66). The relative contribution of each of these antigens to the total CCA content of the various vaccines could then be compared.

Other assay procedures. The CCA test, used to quantitate the hemagglutinating antigen content of the vaccines, was performed by the method of Miller and Stanley (4) as described in an NIH memorandum [Titration of chicken red cell agglutination value (NIH memorandum of 16 September 1946, available from the Division of Biologics Standards, National Institutes of Health, Bethesda, Md. 20014)]. The semiautomatic micro-HI test (5) was employed to measure the antibody content of the sera. When sera were treated with receptor-destroying enzyme (RDE) previously reported methods were used resulting in a 1:4 dilution of sera (12).

RESULTS

Effect of different agar preparations and salt concentration upon precipitin reactions. An example of a typical ID reaction employing whole virus antigen is shown in Fig. 1. Initially, 0.75% Ionagar no. 2 containing 7.5% NaCl was used as the ID medium. Clearer precipitin reactions, however, were obtained employing agarose containing 5.0% NaCl; as the sensitivities of these two media were found to be identical, the latter was ultimately adopted. A very indistinct reaction was obtained with Noble agar (Difco), and salt concentrations less than 5.0% or greater than 10.0% were found to be unsatisfactory with these sera.

Optimal serum dilution for ID precipitation and specificity of reactions. Precipitin reactions could be obtained with undiluted to 1:64 to 1:128 diluted sera; however, by block titration against the homologous vaccine antigens 1:8 to 1:16 serum dilutions were found to be the most sensitive. These dilutions were used subsequentially throughout the study.

The specificity of the untreated immune chicken sera used for ID was shown by HI tests as outlined in Table 1. There were no significant differences between untreated and RDE-treated sera when tested by ID (Table 2). Consequently, untreated sera were generally employed for ID.

Quantitation of antigen content by ID. Table 3 records the quantitative immunoprecipitin reactions as related to the relative CCA antigen content of NIH reference vaccines, lots of commercial vaccines (1971 civilian formulation), and experimental vaccines. There were no reactions between any of the test antigens and either untreated or RDE-treated normal chicken serum.

Vaccines containing A2/Aichi/2/68 and A2/Jap/170/62 were indistinguishable by these means. A vaccine containing a similar quantity of B/Md/1/59 antigen was only moderately reactive against the B/Mass/3/66 immune serum; none of the other antigens reacted with heterologous sera. The commercial vaccine lot 4, containing the A2/Aichi/2/68/X-31 strain, did not cross-react with A0/PR/8/34 serum.

![Fig. 1. Duplicate serial twofold dilutions of vaccine antigen A (Monovalent A2/Aichi/2/68) in outer wells: wells A and B = undiluted; C and D = 1:2 dilution; E and F = 1:4 dilution. Homologous immune chicken serum (1:16 dilution) in central well. Photograph is a 2× enlargement.](image)

### Table 1. Hemagglutination-inhibition of immune chicken sera used for immunodiffusion

| Immune chicken sera | Hemagglutination inhibition* for influenza virus antigens |
|---------------------|----------------------------------------------------------|
|                     | A0/PR/8/34 | A1/AA/157 | A2/Aichi/2/68 | B/Mass/3/66 |
| Normal              | 0          | 0         | 0             | 0           |
| A0/PR/8/34          | 307        | 0         | 0             | 0           |
| A1/AA/157           | 6          | 614       | 0             | 0           |
| A2/Aichi/2/68       | 0          | 0         | 102*          | 0           |
| B/Mass/3/66         | 0          | 0         | 563           | 0           |

*Mean reciprocals of highest dilution of five tests. Zero represents less than 1:4.
*Heterologous reaction with A2/Japan/170/62 antigen was 1:38.
Table 2. Effect of RDE treatment of sera on immunodiffusiona

| Experimental vaccines | Hemagglutinin compositiona | Immunodiffusion with: |
|-----------------------|---------------------------|-----------------------|
|                       | A0  | A1  | A2  | B   | Untreated sera | RDE-treated sera |
| A                     | 0   | 0   | 700 | 0   | 4.0            | 4.0               |
| B                     | 0   | 0   | 600 | 0   | 0.0            | 4.0               |
| C                     | 0   | 0   | 295 | 0   | 2.5            | 2.0               |
| D                     | 0   | 0   | 147 | 0   | 3.5            | 3.0               |
| E                     | 0   | 0   | 441 | 0   | 0.8            | 1.3               |
| F                     | 1,200 | 0  | 0   | 0   | 0.0            | 0.0               |
| G                     | 0   | 1,000 | 0  | 0   | 0.0            | 0.0               |

a Key to abbreviations: A0 = A0/PR/8/34; A1 = A1/AA/1/57; A2 = A2/Aichi/2/68; B = B/Mass/3/66; NC = negative control; RDE = receptor-destroying enzyme.

Based on the results of the 71C reference vaccine, the relative proportions of A2/Aichi/2/68 and B/Mass/3/66 antigens in the bivalent vaccines were determined. The correlation of the theoretical content to the actual determinations of the experimental and commercial vaccines was extremely high. On the basis of these results, commercial vaccine lot no. 4 contained less A2 and more B antigen than the 71C reference and commercial vaccine lots no. 1 to 3. Commercial vaccine lot no. 5 was included in the study because it failed to meet the CCA requirements of the Division of Biologics Standards (DBS). Analysis of these results revealed that it was apparently deficient predominantly in the B components. The sensitivity of the immunoprecipitin test as performed here is in the range of 100 to 150 CCA units per ml.

DISCUSSION

It is generally believed that adequate quantities of the appropriate inactivated influenza virus antigens afford at least some measure of protection against influenza. However, one of the basic problems that has been encountered in the standardization and, therefore, the evaluation of influenza virus vaccines is how to determine the potency of inactivated antigens. The standardized CCA test is both a simple and reliable technique measuring the total hemagglutinin content of these vaccines. However, it previously has not been possible to measure the relative contribution to the total immunizing mass of the several antigenic components of polyvalent vaccines.

In previous studies (3, 7–10) ID techniques have been applied to the study of disrupted influenza A virus subunits. These same workers showed the soluble antigens to be similar for the various influenza A subtypes.

In turkeys, antibody measured by ID with S antigen correlated with the complement-fixation test but not with the HI test (6). Employing Formalin-inactivated antigens, Beard has shown a correlation between the detection of type-specific influenza A antibodies by a similar ID technique and complement fixation in man and horses (2) and the homologous HI tests in birds (1).

Although the immune sera employed in this study undoubtedly contain antibody to a number of surface antigens in addition to the hemagglutinin, the use of a reference vaccine of known strain and hemagglutinin composition has allowed the differentiation in a semiquantitative manner between the influenza subtypes. In this study, lipid solvent split hemagglutinin vaccines were not included as they required a different template and assay system possibly because of a difference in the antigen particle size.

A commercial vaccine which failed to meet DBS standards was examined by these methods and was found to be deficient primarily in the B antigen component. It is probable, therefore, that an error was made during the preparation of the bulk vaccine lot. Conceivably, a vaccine could also fail the CCA test because of a dilutional error which would also be apparent by the ID test. It is hoped that by routine application of these techniques, influenza vaccines released for commercial distribution will be assured not only of adequate total hemagglutinin content but also of adequate potency of each of the component strains.
### Table 3. Relative composition of inactivated influenza virus vaccines

| Vaccines       | Hemagglutinin composition | Actual CCA content | Immunodiffusion | Antigenic composition |
|----------------|---------------------------|--------------------|----------------|----------------------|
|                | A0 | A1 | A2 | B  | Total | A0 | A1 | A2 | B  | Total | A0 | A1 | A2 | B  | Total |
| Experimental  |    |    |    |    |      |    |    |    |    |      |    |    |    |    |      |
| A             | 0  | 0  | 0  | 700| 0  | 700 | 783|    |    |      | 38 | 0  | 0  | 4  | 0  | 4  | 0  | 614 | 0  | 614 |
| B             | 0  | 0  | 0  | 600| 600| 600 | 590|    |    |      | 28 | 0  | 0  | 0  | 0  | 4  | 4  | 0  | 614 | 0  | 491 |
| C             | 0  | 0  | 0  | 392| 296| 687 | 680|    |    |      | 14 | 0  | 0  | 1  | 0  | 2  | 1  | 2  | 322 | 290 | 612 |
| D             | 0  | 0  | 0  | 589| 147| 736 | 703|    |    |      | 11 | 0  | 0  | 0  | 0  | 2  | 9  | 1  | 445 | 112 | 557 |
| E             | 0  | 0  | 0  | 198| 441| 639 | 552|    |    |      | 11 | 0  | 0  | 0  | 0  | 0  | 9  | 3  | 138 | 368 | 506 |
| F             | 1,200| 0 | 0  | 0  | 1,200| 1,198| 6  | 6  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 138 | 368 | 506 |
| G             | 0  | 1,000| 0 | 0  | 1,000| 1,080| 5  | 0  | 4  | 4  | 0  | 0  | 0  | 0  | 0  | 2  | 307 | 190 | 497 |
| Commercial    |    |    |    |    |      |    |    |    |    |      |    |    |    |    |      |
| (Lot no.)     |    |    |    |    |      |    |    |    |    |      |    |    |    |    |      |
| 1             | 0  | 0  | 0  | 800| 600| 1,400| 1,260| 4  | 0  | 0  | 6  | 0  | 6  | 0  | 3  | 921 | 725 | 1,646 |
| 2             | 0  | 0  | 0  | 400| 300| 700 | 959 | 4  | 0  | 0  | 3  | 5  | 4  | 3  | 2  | 537 | 446 | 983 |
| 3             | 0  | 0  | 0  | 400| 300| 1,400| 791 | 4  | 0  | 0  | 2  | 5  | 3  | 0  | 0  | 384 | 335 | 719 |
| 4*            | 0  | 0  | 0  | 800| 600| 1,400| 1,358| 7  | 0  | 0  | 0  | 0  | 4  | 6  | 7  | 706 | 825 | 1,531 |
| 5             | 0  | 0  | 0  | 400| 300| 700 | 476 | 4  | 0  | 0  | 0  | 0  | 2  | 0  | 1  | 307 | 89  | 396 |
| DBS reference |    |    |    |    |      |    |    |    |    |      |    |    |    |    |      |
| 67CP         | 100| 100| 200| 200| 600| 600 | 6  | 1  | 2  | 0  | 1  | 6  | 0  | 3  | 0  | 246 | 335 | 581 |
| 71C*         | 0  | 0  | 400| 300| 700| 700 | 21 | 0 | 0  | 2  | 6  | 7  | 0  | 0  | 0  | 400 | 300 | 700 |

* Key to abbreviations: A0 = A0/PR/8/34; A1 = A1/AA/1/57; A2 = A2/Aichi/2/68; B = B/Mass/3/66; CCA = chicken cell agglutination; DBS = Division of Biologics Standards.

a CCA units per milliliter as determined from the CCA content of concentrates from which the vaccines were prepared.

b Total CCA units per milliliter by actual determination performed on final vaccines; expressed as the mean corrected value of five determinations employing 71C as the reference.

c Immunodiffusion employing immune chicken sera; values expressed as the mean of the reciprocal of the highest antigen dilution giving a precipitin reaction. There were no reactions with normal chicken serum.

d Antigen composition based on the immunodiffusion values related to the 71C reference (see text).

e Vaccine C prepared by mixing equal volumes of A and B; vaccine D by mixing equal volumes of A and C; and vaccine E by mixing equal volumes of B and C.

f Contains A2/Japan/170/62 antigen.

g Contains B/Maryland/1/59 antigen.

h A2 component was the A2/Aichi/2/68/X-31 strain.

i NIH 67 CP (civilian polyvalent) reference vaccine; contains 100 CCA units A0/PR/8/34, 100 A1/AA/1/57, 100 A2/Japan/170/62, 100 A2/Taiwan/1/64, and 200 B/Mass/3/66 per ml.

j NIH 71C (civilian) reference vaccine; contains 400 CCA units of A2/Aichi/2/68 and 300 B/Mass/3/66 per ml.

In our opinion, application of the ID test as described in this report would serve as an interim measure until improved tests are developed. Certainly, the use of standardized monoclonal antibody sera, pretreatment of vaccines, reproducibility, more precise expression of quantitation and other test parameters require additional investigation.

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