Single Radial Complement Fixation Test for Assaying Antibody to Influenza Virus Type-Specific Antigens

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An immunodiffusion technique in agarose is described for assay of complement-fixing antibodies against the type-specific soluble antigen of influenza virus. Under the test conditions, positive human serum produced a definite unlysed zone around the well, and the annulus area showed a high correlation with the antibody level in a conventional complement fixation test with log serum titer. This paper also describes the use of this method as a diagnostic procedure for the assay of antibodies against soluble antigens of influenza A and B viruses in paired human sera collected from persons infected with the virus or who received ether-split vaccine. This method appears to be more sensitive and gives more consistent results for serodiagnosis of infection cases than do the hemagglutination inhibition, neuraminidase inhibition, and complement fixation tests. Our results suggest that the single radial complement fixation test can provide a simple and reliable method for serodiagnosis of influenza virus infection.

It is well known that the envelope antigens of influenza virus, hemagglutinin (HA), and neuraminidase (NA), are strain-specific antigens and that the internal antigens of the virions, the so-called soluble (s) antigens, are type-specific (14). As for antibodies against envelope antigens, inhibition tests for each biological activity have been employed, that is, hemagglutination inhibition (HI) and neuraminidase inhibition (NAI) tests. On the other hand, the complement fixation (CF) test is commonly employed to assay antibody against the internal virion antigens in most clinical virus laboratories (7).

At present in Japan, split vaccine of influenza virus is given annually to school children from kindergarten to senior high school age (1). Antibodies against both HA and NA antigens, but not against internal antigen, nucleoprotein (NP), have been reported to rise after split influenza virus vaccination (15). While it is necessary to titrate antibody levels against s antigen to distinguish natural infection from vaccine response, the standard CF test remains less popular for serodiagnosis than the HI test because it is somewhat more complex and time consuming. In previous publications, the immuno-double-diffusion and the single radial immunodiffusion tests against NP of influenza A virus have been reported (12, 16). Other authors have suggested modifying the principle of CF for the immunodiffusion technique, using agarose containing influenza virus s antigen, complement, sheep erythrocytes (SRBC), and hemolysin (2, 3). In this communication we describe use of the single radial complement fixation (SRCF) test to assay influenza virus s antigen (modified by Sato et al. [10]) as a useful and simple method for assaying complement-fixing antibodies to the type-specific antigens of influenza virus in paired human sera collected from subjects who became infected and who received vaccine.

MATERIALS AND METHODS

Specimens for serodiagnosis. A total of 101 paired sera were collected from persons infected with influenza viruses. Definite infection with influenza virus was confirmed by viral isolation from pharyngeal swabs in all cases. Forty-four persons were identified as being infected with influenza A virus closely related to A/Kumamoto/37/79(H1N1) in 1980 to 1981, and the remaining 57 cases were infected with influenza B virus related to B/Singapore/222/79 in 1981 to 1982. The mean intervals between acute- and convalescent-phase sera collections were 23.4 days for influenza A virus infection in 1980 to 1981 and 17.4 days for influenza B virus in 1981 to 1982. The sera were stored at −20°C until assayed.

Vaccination with influenza virus. The vaccine employed was an ether-split product prepared by Saikin Kagaku Institute Company, Ltd., Sendai, Japan. The vaccine used in 1982 contained 250 chicken cell agglutination U per ml of A/Kumamoto/37/79(H1N1), 300 chicken cell agglutination U per ml of A/Niigata/102/81(H3N2), and 150 chicken cell agglutination U per ml of B/Singapore/222/79. For the analysis of antibody responses after vaccination, sera from a total of 35 12- to 13-year-old school children were employed. Two doses (0.5 ml) of the vaccine were given subcutaneously, separated by a 10-day interval. Serum
was collected from each child before vaccination and 7 weeks after the second immunization.

**Serodiagnosis for influenza virus infection.** A standard microtiter technique for the HI test was employed, and the following HA antigens were used: A/Kumamoto/37/79(H1N1), A/Bangkok/1/79(H3N2), A/Tohoku University/1/81(H1N1), B/Singapore/222/79, and B/Tohoku University/63/82. Anti-NA antibodies in titers were determined by a chemical NAI technique with a fetuin substrate, using the following antigens: A/equine/Prague/1/56(Heq1)-A/UISR/90/77(N1), A/equine/Prague/1/56(Heq1)-A/Texas/1/77(N2), and B/Tohoku Univ./63/82. The B/Tohoku Univ./63/82 strain was pretreated with 0.1% Triton X-100 to prevent the steric hindrance effect from HI antibodies (15). Conventional CF tests against the s antigens of influenza A and B viruses were also employed (16).

**Virus isolation.** Virus isolation was carried out using Madin-Darby canine kidney cell cultures with 5 μg of trypsin per ml. The inoculated cultures were incubated at 35°C for 72 h, and isolates were detected by positive hemagglutination with chicken and guinea pig erythrocytes after three passages. All the isolates were serologically identified by using the HI and NAI tests against antiserum to reference strains of influenza A and B viruses. The antisera were kindly provided by Alan P. Kendal, Centers for Disease Control, Atlanta, Ga.

**SRCF test.** The SRCF test plates were prepared by Denka Institute of Biological Sciences, Ltd., Tokyo, Japan. The technique was based on the original method described by Haahem (2, 3), except for two modifications. (i) The SRCF test plate contained hemolysin-coated SRBC and s antigen of influenza A or B viruses in agarose. Guinea pig serum was omitted from the agarose plate. (ii) Instead of adding complement into the agarose, a gelatin film containing guinea pig serum, bovine albumin, and glycerin was set onto the agarose test plate after the wells were filled with serum specimens (4, 10).

The agarose gel was prepared in Veronal buffer solution (VBS), pH 7.2, to give a final concentration of 1.0%. The type-specific s antigens of influenza A and B viruses were obtained from disrupted chorioallantoic membranes of embryonated chicken eggs infected by the allantoic route with A/Kumamoto/37/79(H1N1) or B/Singapore/222/79 strains according to the standard procedures (13). The s antigen preparations were diluted in VBS to give a final concentration of 5.6 antigenic U, determined by standard CF test, in the agarose. A 2% suspension of SRBC was sensitized with the optimal concentration of hemolysin, as determined by the standard CF test, and added to the agarose to give a final concentration of 0.75% SRBC.

The s antigen and hemolysin-coated SRBC were mixed into molten agarose gel kept in a water bath at 40°C. After mixing well, the agarose was poured onto a plastic plate and then stored at 4°C. The complement film was prepared by mixing diluted guinea pig serum, 1.5% gelatin, 1.0% bovine albumin, and 0.5% glycerin in VBS. The mixture was layered onto a transparent plastic film and then lyophilized. The guinea pig serum was diluted to give a final complement activity of 15 50% hemolytic complement U per ml in the mixture.

Ten microliters of heat-inactivated test sera was added to wells of SRCF plates. After the gelatin film containing complement was set onto the plates, they were incubated at 4°C for 16 h. Then plates were transferred to 37°C and incubated for an additional 2 h. Positive reactions appeared as a zone of unlysed cells around the well. Since most of the complement was fixed by the target antigen-antibody complex (the type-specific antibody and s antigen), it could not cause lysis of indicator SRBC. In the case of negative reactions, however, hemolysin-coated SRBC were completely lysed by the unfixed complement (see Fig. 1). Antibody levels were first represented as the square of the diameter (in millimeters) of the unlysed zone and then antibody U per milliliter were calculated as: antibody U = [(unlysed annulus area of test serum)/(unlysed annulus area of standard antiserum)] x (antibody U of standard antiserum).

As in the standard CF test, controls for serum anticomplementary activity and antibody against normal tissue antigen were included in the SRCF test plate. For the anticomplementary control, s antigen was omitted and replaced with VBS. Normal noninfected s antigen diluted 1:2 was added in place of infected s antigen for the assay of antibody against normal tissue antigen.

**Antisera.** To confirm the specificity of the SRCF test

FIG. 1. The SRCF test plate against s antigen of influenza A virus. (a) Test plate with human sera containing antibody against infected s antigen of influenza A virus. (b) Test plate in which normal noninfected s antigen was added instead of infected s antigen for the assay of antibody against normal tissue antigens. (c) Test plate from which s antigen was omitted, for the assay of the serum anticomplementary activity. The left wells contained acute phase serum and the right wells were convalescent serum. The arrow indicates the edge of unlysed zone.
plate, we used antisera prepared in goats against purified NP, membrane protein (MP), HA, and NA derived from the following strains: NP from A/Scotland/840/74(H3N2), MP from the recombinant of A/NWS/34(H1N1)-A/equine/Prague/1/56(Neq1), HA(H1) from A/FM/1(47/H1N1), NA(N1) from the recombinant of A/equine/Prague/1/56(Heq1)-A/USSR/90/77(N1), NP and MP from B/Lee/1940, and HA and NA from B/Hong Kong/8/73. These antisera were kindly donated by Robert G. Webster, St. Jude Children's Research Hospital, Memphis, Tenn., through the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Viral polypeptides were recovered from s antigen solution by immunoprecipitation, using hyperimmune antisera to disrupted virions and Sepharose-linked Staphylococcus aureus protein A, as described by Kessler (5). The samples, containing bovine serum albumin as an internal standard, were layered on 10% polyacrylamide gels. At the end of electrophoresis, the gels were stained with Coomassie brilliant blue and then scanned at a wavelength of 590 nm (6).

RESULTS

Reproducibility and specificity of the SRCF test. Table 1 shows the standard deviations and the coefficients of variation for five different human sera. The coefficients of variation obtained for 10 titrations ranged from 1.5 to 4.9% and standard deviations were from 3.0 to 9.8% of their respective mean SRCF units. In this study, an increase in SRCF units of more than 30% was considered statistically significant. This represented a difference in antibody potency of approximately 1 log₂ step in CF titration and was a threefold or greater improvement over the standard CF test.

The specificity of the SRCF test plate was confirmed by using antisera to each viral structural polypeptide antigen, HA, NA, NP, and MP. The SRCF test plate containing s antigen derived from A/Kumamoto/37/79(H1N1) showed a positive reaction with the antiserum against NP purified from A/Scotland/840/74(H3N2), but was negative with the antisera against envelope antigens, HA(H1) and NA(N1) and MP of influenza A virus (Fig. 2). The same results were also obtained when the SRCF test plate containing s antigen derived from B/Singapore/222/79 was tested. The polypeptide profile of s antigen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the original s antigen showing 32 antigenic U by the standard CF titration contained approximately 280 µg of NP and 270 µg of MP per ml, in addition to proteins of host cell origin (data not shown).

Correlation between CF and SRCF tests. A total of 120 human sera were employed to compare SRCF units and CF titers against s antigen of influenza B virus. A good linear relationship was obtained between CF titers and SRCF units for the sera, with a correlation coefficient of 0.89 (Fig. 3). Also, the regression line was calculated as $Y = 10.4 \times \log(X) + 1.5$, where $Y$ is the SRCF units and $X$ is the CF titer. On the other hand, the correlation coefficients between SRCF units and HI titers against B/Singapore/222/79 and NA(N1) titers against B/Tohoku Univ./63/82 were 0.69 and 0.12, respectively. These results indicate that the SRCF units demonstrated were highly specific for the internal s antigen of influenza virus rather than for envelope antigens HA and NA.

Distribution of SRCF units in paired sera collected from persons with confirmed influenza infections. Fifty-seven paired sera taken from persons from whom influenza B virus was isolated and 44 paired sera taken from those yielding H1N1 influenza A virus were examined by using the SRCF test against each s antigen of influenza virus. Figure 4 shows the distribution of SRCF units in acute- and convalescent-

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**TABLE 1. Reproducibility of SRCF test with single serum**

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Mean SRCF U</th>
<th>SD</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7.4</td>
<td>0.37</td>
<td>4.9</td>
</tr>
<tr>
<td>II</td>
<td>10.4</td>
<td>0.25</td>
<td>2.4</td>
</tr>
<tr>
<td>III</td>
<td>15.2</td>
<td>0.50</td>
<td>3.3</td>
</tr>
<tr>
<td>IV</td>
<td>18.7</td>
<td>0.33</td>
<td>1.7</td>
</tr>
<tr>
<td>V</td>
<td>19.3</td>
<td>0.29</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Mean readings from 10 trials.
phase sera. In the case of influenza B virus infections, almost all the acute-phase sera contained fewer than 12 U of SRF, whereas the convalescent-phase sera showed over 12 U of SRF. Based on this cutoff, only 5 sera of the 114 test specimens were exceptional, with two acute-phase sera showing over 12 U and 3 convalescent-phase sera showing fewer than 12 U. On the other hand, in the case of influenza A virus infections, the difference between acute- and convalescent-phase sera antibody levels was unclear. SRF units did not distinguish convalescent- from acute-phase sera (Fig. 4). This trend was also observed in CF titrations.

FIG. 3. Correlation between SRF test antibody units and standard CF titers against s antigen of influenza B virus for 120 human sera.

FIG. 4. Distribution of SRF antibody units and CF titers in acute- and convalescent-phase human sera. Dotted bars, Acute-phase sera; cross-hatched bars, convalescent-phase sera. (a) CF antibody titers in sera collected from persons who became infected with influenza B virus. (b) SRF antibody units in sera collected from persons who became infected with influenza B virus. (c) As (a), but from persons who became infected with influenza A virus. (d) As (b), but from persons who became infected with influenza A virus.
Serodiagnosis of virus infection by various tests. The results of serological tests on persons infected with either influenza A or B viruses are summarized in Table 2. In influenza B virus infections, significant antibody rises against s antigen were observed with 93% of the paired sera in the SRCF test, whereas the CF test detected significant titer rises in only 86% of the subjects. Also, the HI and NAI tests revealed significant antibody rises in 79 and 68% of the subjects, respectively. All of the patients showed significant antibody rises in either SRCF tests or HI tests, or both. In influenza A virus infections, the SRCF test detected significant antibody rises in 73% of the cases. HI, NAI, and CF positive test results were 68, 71, and 64%, respectively. In influenza B virus infections, the ratios of antibody levels between acute- and convalescent-phase sera titrated by SRCF ranged from 1.1 to 7.2 and the mean ratio was 2.5. The ratios ranged from 1.0 to 2.1 with a mean of 1.4 in influenza A virus infections (data not shown). It is noteworthy that the SRCF test detected the highest incidence of antibody rises in both influenza A and B infections, and that only 52% of influenza A and 58% of influenza B patients showed significant antibody rises against the full set of HA, NA, and s antigens.

SRCF antibody response after influenza virus vaccination. Figure 5 shows antibody responses against s antigens of influenza A and B viruses determined by the SRCF test after administration of split influenza virus vaccine containing A/Kumamoto/37/79(H1N1), A/Niigata/102/81(H3N2), and B/Singapore/222/79. The SRCF units against s antigen of influenza B virus did not show any significant antibody changes in any of the vaccinees, although postvaccination antibody levels rose slightly in some cases. In contrast, the antibody levels against influenza A virus s antigen rose significantly in 7 of 35 individuals. According to the epidemiological records from the beginning of January 1983, the Hong Kong variant (H3N2) was endemic in Sendai City where the school employed in our study is located (personal communication from Miyagi Prefectural Institute of Health). Some of the persons who showed significant antibody rises against influenza A virus s antigen in the SRCF test complained of fevers after vaccination. However, we could not conclusively determine whether or not they became infected with the Hong Kong variant, since they showed significant antibody rises in both HI and NAI tests against recent isolates of Hong Kong virus and vaccine strains along with other vaccinees.

![Graphs showing antibody response](http://jcm.asm.org/)
DISCUSSION

In Japan there is a difficult situation with regard to serodiagnosis of influenza virus infection. That is because almost all school children annually receive split vaccines of influenza A and B viruses, and thus, their antibody levels against envelope antigens HA and NA rise (15). Under these conditions, it is sometimes difficult to make a definite serodiagnosis when only the HI test is employed. Therefore, we believe it is necessary to estimate antibody against the internal type-specific antigens of influenza virus. The studies reported in this communication were initiated to establish an easy and sensitive method for quantifying antibody levels against the type-specific antigen of influenza virus. While there have been reports on estimate antibodies against the internal antigens of influenza virus, NP and MP, by single radial immunodiffusion and immuno-double-diffusion (9, 12, 16), the conventional CF test against s antigen is the most widely used for this purpose in clinical virus laboratories.

The SRCF test was first described by Haah-heim (2, 3). However, the original technique had the following problems: (i) instability of the test plate containing the antigen and complement, and (ii) a complex technique for the preparation of the second overlay containing sensitized SRBC. The modified SRCF test described here provided a simple method for the quantification of antibodies against the type-specific antigens of influenza virus. Also, our results with the specific antisera to each viral polypeptide antigen (HA, NA, NP, and MP) revealed that the test plate was reactive only against anti-NP antiserum. We confirmed that the s antigen contained an almost equal amount of MP antigen; however, it is likely that MP antigen has poor antigenicity in humans and weak activity in CF when compared with NP antigen. Also, Oxford et al. (9) reported that it was necessary to reduce the viral concentration to 20 μg/ml for anti-MP antibody assay by single radial immunodiffusion test, whereas a concentration of 150 μg/ml could be used for anti-NP antibody assay. As for test plate stability, it has been reported that test plates containing antigen and sensitized SRBC and gelatin films containing complement are stable at 4°C for 3 months (8).

It became apparent through our studies that the SRCF test was more sensitive and reliable than the conventional CF test, especially for detecting lower levels of antibody. This method gave excellent results during two seasons of influenza virus outbreaks, which included influenza A virus H1N1 in 1980 and 1981 and influenza B virus in 1981 and 1982. In serodiagnosis of cases from whom influenza viruses were isolated, the SRCF test was the most sensitive in detecting significant antibody rises as compared with the HI, NAI, and CF tests. It is well recognized that it becomes difficult to choose an appropriate antigen for the HI or NAI tests when an antigenic shift or drift occurs. Also, although the isolate recovered during the epidemic would be the best antigen for the tests, viral isolation always lags behind the clinical manifestations of an influenza outbreak. The SRCF test has the advantage of being able to estimate the antibody response against the type-specific antigen of the virus and is unaffected by antigenic drift or shift of the envelope antigens. Although it was demonstrated that a small antigenic difference exists between the NPs of influenza A viruses isolated from different species (birds, pigs, horses, and humans) (11), the SRCF test plate containing s antigens derived from H3N2 virus and H1N1 virus showed the same antibody units against antiserum to NP and also in human sera (personal communication from Denka Institute of Biological Sciences, Ltd.).

Antibody responses to influenza virus vaccination of school children detected by the SRCF test differed somewhat from those described in our previous reports. Most students did not show any significant antibody rises against s antigen of influenza B virus, but 7 out of 35 vaccinees showed 1.3-fold or greater rises in antibody levels against influenza A virus between pre- and postvaccination sera. Our previous studies did not demonstrate any changes in the antibody levels against influenza A virus NP by single radial immunodiffusion (15, 16). Oxford et al. (9) reported that the influenza virus vaccine used in England could elicit antibody rises against NP in vaccinees, but not against MP, and that the vaccine preparations contained NP. Unfortunately, in this study we could not ascertain whether the vaccine presently used in Japan contained NP or MP as antigenically active forms or whether such internal components in the vaccine could evoke antibody responses in vaccinees. With this in mind, it becomes necessary to analyze the vaccine itself and also the antibody responses to vaccination on a larger scale.

In summary, the SRCF test described here provides a simple technique for serodiagnosis of influenza virus infections, and is not affected by antigenic drifts or shifts as are HA and NA. The principles of this method are also applicable to other viral, bacterial, fungal, and mycoplasmal infections. A more detailed study of antibody response to various antigens is presently under way.

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LITERATURE CITED