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Comparison of Different Approaches to Measuring Influenza A Virus-Specific Hemagglutination Inhibition Antibodies in the Presence of Serum Inhibitors

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The A/Los Angeles/2/87 (H3N2) (A/LA/2/87) virus is sensitive to inhibitors of hemagglutination present in certain human sera. It was found that the effect of these inhibitors could be removed by treating sera with high-concentration receptor-destroying enzyme or trypsin-periodate or by using inhibitor-resistant viruses in the hemagglutination inhibition (HAI) test. Inhibitor-resistant viruses were not effective for detecting rises in antibody titers in the sera of volunteers infected with the A/LA/2/87 wild-type virus, while rises in antibody titer were readily detected in sera treated with trypsin-periodate and tested against A/LA/2/87 wild-type virus in an HAI test. It is therefore suggested that chemical or enzymatic methods be used to inactivate serum inhibitors and that standard virus be used in the HAI test for the currently circulating H3N2 viruses.

The hemagglutination inhibition (HAI) test, which detects subtype-specific antibodies against hemagglutinin (HA), is commonly used for serologic diagnosis of influenza virus infection and for determination of susceptibility to influenza virus in epidemiologic and vaccine studies. Naturally occurring inhibitors of hemagglutination by influenza viruses present in the sera of humans and animals were first described by Francis in 1947 (7). These substances act like antibody in an HAI test by interacting with the influenza HA and thus preventing agglutination of erythrocytes (RBCs) by the virus. Three known classes of inhibitors, designated alpha, beta, and gamma, have been detected in human sera (9). The properties and nature of these inhibitors have been described elsewhere (3, 9, 12).

It is desirable to remove the activity of these inhibitors of hemagglutination from serum specimens because they make it difficult to use the HAI test to accurately assess HA-specific antibody in sera. A variety of techniques to inactivate serum inhibitors have been developed. These include treatment with heat, receptor-destroying enzyme (RDE) of *Vibrio cholerae*, trypsin, periodate, or a combination of these factors (6). Each treatment method inactivates one or more classes of inhibitors (8, 12). The effects of various methods of inactivating inhibitors in sera of humans and several animal species have been compared, but no single treatment method was found to be effective against all virus strains tested (2). Another approach to measurement of serum HAI antibodies in the presence of inhibitors is to use an inhibitor-resistant influenza virus strain in the HAI test (6). This approach has not been described for detection of HAI antibody to H3N2 viruses, either because inhibitors reactive with H3 HA were readily removed by the standard heat and RDE treatment or because earlier H3N2 strains were not sensitive to inhibitors. In 1962, Alexandrova analyzed isolates of influenza A2 (H2N2) viruses recovered during annual epidemics in Leningrad and found that over 4 years, there was a gradual increase in isolation of inhibitor-sensitive strains (1). Recently emerging H3N2 viruses, specifically A/Los Angeles/2/87 (A/LA/2/87), appear to be sensitive to inhibitors that persist in heat and RDE-treated sera. In screening sera for the presence of antibodies to A/LA/2/87 HA, we found a low rate of seronegativity despite heat and RDE treatment of sera. In addition, sera collected prior to 1968, which should lack antibody to H3 HA, were reactive with A/LA/2/87 virus despite heat and RDE treatment. We therefore sought ways to measure antibodies in sera containing inhibitors. The availability of well-characterized inhibitor-resistant mutant viruses derived from the A/LA/2/87 virus (12) enabled us to compare the various methods for measurement of HAI antibodies in human sera containing inhibitors to this virus.

Inhibitor-resistant viruses were previously isolated in 11-day-old embryonated eggs by limiting dilution following the incubation of the A/LA/2/87 (H3N2) parent inhibitor-sensitive virus with an equal volume of undiluted animal serum and selection of mutant viruses that lost reactivity to the inhibitor present in the serum used for selection (12). The inhibitor-resistant strains were designated as follows: (i) A/LA.HS for virus isolated in the presence of horse serum, (b) A/LA.PS for virus isolated in the presence of pig serum, (c) A/LA.RS for virus isolated in the presence of rabbit serum, (d) A/LA.HSPS.BB for a mutant virus isolated from an A/LA.HS strain after exposure to pig serum, and (e) A/LA.HSPSRS for virus isolated from A/LA/2/87 first exposed simultaneously to horse and pig serum and subsequently exposed to rabbit serum. The mutations identified in the HA of each of these strains are summarized in Table 1 (12).

Four methods were used to inactivate serum inhibitors: (i) incubation at 56°C for 30 min, (b) RDE treatment, (c) periodate treatment, and (d) a combination of trypsin and

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TABLE 1. Characteristics of inhibitor-resistant mutants

Virus	Serum that virus was isolated in presence of	Site of amino acid substitution[a]			No. of MAbs reactive in HAI test[a,b]	Avg log_2-fold rise in HAI antibody titer[b,c]
		Position	WT	Mutant		
A/LA/2/87	None	None	NA	NA	9	2.6
A/LA.HS	Horse	128	Asn	Thr	7	1.6
		145	Asn	Lys		
		155	Tyr	His		
A/LA.RS	Rabbit	220	Arg	Gly	7	1.4
		246	Asn	Asp		
A/LA.PS	Pig	186	Ser	Ile	5	0.2
A/LA.HSPS.BB	Horse, then pig	128	Asn	Thr	2	0
		137	Tyr	Asp		
		145	Asn	Lys		
		155	Tyr	His		
		186	Ser	Ile		
A/LA.HSPSRS	Horse and pig, then rabbit	145	Asn	Asp	2	0
		186	Ser	Ile		
		248	Thr	Ile		

[a] Data previously published (4). WT, wild type; NA, not applicable.
[b] Pearson's correlation, $r = 0.94$; $P < 0.01$. MAbs, monoclonal antibodies.
[c] Rise in titer (log_2) was the log_2 mean of the ratios of postinfection to preinfection titers of data from Table 3.

periodate treatments. The procedures used for treatment with RDE, periodate, and combined trypsin and periodate were as described previously (6). Serial twofold dilutions of the treated sera, from 1:10 to 1:1,280, were tested in a standard HAI test with 4 HA units of virus and a 0.5% suspension of chick RBCs in phosphate-buffered saline (6). The HAI titer was the reciprocal of the highest dilution of serum that completely inhibited agglutination of RBCs.

The first group of human sera tested in this study included serum samples from 10 adults which were collected and stored in a serum bank prior to 1968, the year that H3N2 viruses first appeared in the community (10). These sera were selected from a larger group because they exhibited two properties: (i) they lacked HAI antibody to the A/Hong Kong/X-31/68 (H3N2) virus, confirming that they lacked antibody to the H3 HA; and (2) they had HAI activity against the A/LA/2/87 inhibitor-resistant parent virus, presumably because of the presence of inhibitors of hemagglutination in the sera. Approximately 67% of these sera tested contained inhibitors. Each of the 10 serum samples selected from this group were tested for HAI activity separately against the parent A/LA/2/87 virus as well as against each of the five inhibitor-resistant strains. The sera were tested untreated or after treatment by one of the four methods described above (Table 2). The method of treatment of sera and the particular virus strain used in the test each influenced the levels of HAI activity of the sera. When the parent virus (A/LA/2/87) was used as the antigen, we found that trypsin-periodate treatment was most effective in inactivating serum inhibitors. The titers obtained in sera from each of the 10 subjects following this treatment were in the range (≤1:10) that would be interpreted as seronegative. The use of any of the five inhibitor-resistant viruses combined with RDE, periodate, or trypsin-periodate treatment of sera also resulted in a marked decrease in the level of HAI activity. An RDE preparation from Takeda Industries, Osaka, Japan, was more effective in removing inhibitors than the RDE from Whittaker MA Bioproducts, Walkersville, Md., at comparable units of enzymatic activity (data not presented). Increasing the concentration of the RDE from Whittaker MA Bioproducts fivefold also resulted in complete inactivation of inhibitors. These results suggested that trypsin-periodate treatment of serum, treatment with a high concentration of RDE, or the use of inhibitor-resistant viruses in the HAI test could be used when HAI antibodies are measured.

We then compared the abilities of the parent and inhibitor-resistant mutant viruses to detect rises in HAI antibody titer for the parent and inhibitor-resistant viruses by testing trypsin-periodate treated pre- and postinfection serum samples obtained from 16 healthy adult volunteers who were experimentally infected with A/LA/2/87 wild-type virus as part of a vaccine development program (4a). These subjects were shown to be infected by isolation of virus from nasal washings and/or by a rise in serum antibody titer measured

TABLE 2. HAI titers in sera collected prior to 1968 and tested against A/LA/2/87 wild-type or inhibitor-resistant viruses

Treatment of serum[a]	Mean ± SE log_2 HAI titer in serum using the indicated virus[b]					
	A/LA/2/87	A/LA.HS	A/LA.PS	A/LA.RS	A/LA.HSPSRS	A/LA.HSPS.BB
Heat	6.8 ± 0.2	5.7 ± 0.2	5.7 ± 0.2	4.9 ± 0.6	6.9 ± 0.2	≤2.3
RDE-heat	3.7 ± 0.2	3.5 ± 0.3	≤2.3	3.0 ± 0.2	2.7 ± 0.2	≤2.3
Periodate	3.3 ± 0.7	3.7 ± 0.7	≤2.3	3.3 ± 0.5	≤2.3	≤2.3
Trypsin-periodate	2.4 ± 0.1	2.8 ± 0.4	≤2.3	2.4 ± 0.1	≤2.3	≤2.3

[a] Additional treatment of sera with RDE, periodate, or trypsin-periodate significantly lowered HAI titers from values obtained following heat treatment alone ($P < 0.001$). Use of inhibitor-resistant viruses also significantly influenced the level of HAI activity compared with that measured against A/LA/2/87 wild-type virus ($P < 0.001$).
[b] HAI titers for 10 serum samples lacking specific antibodies to the H3 HA. Titers of ≤1:10 were assigned a value of 1:5. Titers were determined after treatment with procedures that inactivate inhibitors of HA.

TABLE 3. Measurement of pre- and postinfection HAI antibodies in trypsin-periodate-treated sera using A/LA/2/87 wild-type or inhibitor-resistant mutant viruses

Parameter	Mean ± SE $\log_2$ HAI titer serum using the indicated virus[a]					
	A/LA/2/87	A/LA.HS	A/LA.PS	A/LA.RS	A/LA.HSPS.BB	A/LA.HSPSRS
Preinfection serum collection	2.9 ± 0.3	3.1 ± 0.3	≤2.3	3.1 ± 0.2	≤2.3	≤2.3
Postinfection serum collection	5.5 ± 0.3	4.7 ± 0.3	2.5 ± 0.2	4.5 ± 0.3	≤2.3	≤2.3
Rise in titer[b]	2.6 ± 0.4	1.6 ± 0.4	0.2 ± 0.2	1.4 ± 0.6	0	0
% with ≥4-fold rise	94	50	62	50	0	0

[a] HAI titers of 16 serum samples from volunteers infected with the A/LA/2/87 virus. Titers of ≤1:10 were assigned a value of 1:5 for calculation of mean titers.
[b] Rise in titer ($\log_2$) was the $\log_2$ mean of the ratios of postinfection to preinfection titers.

by kinetic enzyme-linked immunosorbent assay (13). The geometric mean HAI antibody titers, the mean rise in titer, and the frequency of HAI titer rises are presented in Table 3. The mean postinfection titer and mean rise in titer detected in HAI tests using the A/LA/2/87 parent virus were greater than those detected in assays using the inhibitor-resistant viruses. The A/LA/2/87 parent virus also detected more fourfold rises than any of the inhibitor-resistant viruses. The A/LA.HSPS.BB and A/LA.HSPSRS viruses failed to detect any titer rises. Similar results were obtained with RDE-treated sera.

The $\log_2$-fold rise in titer against the individual inhibitor-resistant viruses correlated with the frequency with which a panel of nine monoclonal antibodies reacted with the inhibitor-resistant mutants (Table 1). These results indicated that an HAI assay using the A/LA/2/87 inhibitor-sensitive parent virus and enzymatically or chemically treated sera was the most sensitive method for measurement of HAI antibodies and detection of titer rises.

The findings in the present study indicated that the inhibitor-resistant mutant viruses were not efficient for detecting rises in HAI antibody titer. There are several possible explanations for the lack of sensitivity of the inhibitor-resistant viruses in detecting HAI titer rises in the volunteers infected with the A/LA/2/87 virus. First, the mutations in the viruses that resulted in the inhibitor-resistant phenotype occurred in or near the antibody-binding regions of the HA, thereby altering the antigenicity of the HA and rendering them less capable of detecting HA antibodies. The mutations in the inhibitor-resistant strains have been identified (Table 1) and are localized to the area of the HA molecule in and around the receptor-binding site (12). Several antigenic changes in the HA were noted when these viruses were tested against a panel of anti-HA monoclonal antibodies, and it is possible that amino acid substitutions in and around the HA receptor-binding site could alter binding between antibodies and HA directly or indirectly by changing the conformation of the HA (12). There was a strong inverse correlation (r = 0.94) between the loss of reactivity with anti-HA monoclonal antibodies and a rise in antibody titer detected in infected volunteers when the inhibitor-resistant mutants were used. This suggests that altered antigenicity of the HA in the inhibitor-resistant viruses contributed significantly to the inability of the HA to detect a rise in antibody titer in infected volunteers. However, it is still somewhat surprising that the inhibitor-resistant strains were also not efficient in detecting a polyclonal HAI antibody response. The naturally occurring A/Eng/42/72 virus had substitutions in each of the five antigenic sites of the H3 HA but was only four- to eightfold different from the original H3 prototype strain A/NT/68 in an HAI assay (4). The inhibitor-resistant strain A/LA.PS used in our study appears to show a difference in the HAI test of a similar magnitude despite having sustained only one amino acid substitution (Table 1). While the amino acid substitutions in the inhibitor-resistant strains could have had a global effect on the HA structure, this seems unlikely in view of the data on the crystal structure of a receptor-binding mutant HA, which suggests that the structural changes are of a local nature (15).

Second, it is possible that the antigenic site-specific responses of the volunteers infected with the A/LA/2/87 wild-type virus were highly restricted and were localized to the sites bearing amino acid changes in the HA of mutant viruses. Such a restricted response in each of 16 individuals seems unlikely, especially for a secondary antibody response, which would be expected to be broad (5, 14). A third possibility is that the mutations in the HA of the inhibitor-resistant viruses resulted in an altered affinity of binding of the virus HA to the chicken RBCs used in the HAI test. Because antibodies and RBCs compete for binding to the virus HA, an increased affinity between virus HA and RBCs could result in a lower measured titer of antibody. Although our data do not address these three separate possibilities, they do reveal the limitations to the use of the A/LA/2/87 inhibitor-resistant strains in HAI assays. It is possible that inhibitor-resistant mutants with changes in sites other than the antibody-binding site, for example, a mutation in the pocket on the distal tip of the HA molecule (11), would not have such limitations.

The choice of the ideal method for treating sera to inactivate inhibitors depends on the nature of inhibitor present and the virus. The results of this study indicate that the inhibitors present in our subjects were not beta inhibitors, because heating alone did not inactivate them. The ability of trypsin-periodate and RDE to inactivate them suggests that they belong to either the alpha or the gamma class of inhibitors. We can conclude from this study that combined chemical or enzymatic methods are effective for inactivating the widely prevalent inhibitors against currently circulating H3 HA. The use of inhibitor-resistant virus strains as a source of antigen in HAI tests is not recommended for these H3N2 viruses because of their decreased ability to detect antibodies specific to the HA.

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