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# STUDY OF THE AVIDITY OF ANTI-INFLUENZA ANTIBODIES IN SERA OF CONVALESCENTS OR SUBJECTS VACCINATED WITH A LIVE INFLUENZA VIRUS VACCINE

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*Summary.* — Secondary influenza infection and vaccination with live influenza vaccines result in a significant increase of the specific avidity of anti-influenza antibodies. The avidity of convalescent sera or sera obtained from human subjects 3 or 4 weeks after revaccination was higher than that of healthy human sera, as evidenced by more stable complex between antibodies and inhibitor-resistant strain of influenza A2 virus and also by the higher rate of antibody — virus reaction. A comparative study of the level of specific avidity of the convalescent sera obtained from persons of different age groups showed that small children, who were primarily affected by influenza in 1965, developed less avid antibodies than adults or older children. This provides an extra evidence that the secondary infection contributes to the enhancement of the avidity of anti-influenza antibodies. Sera collected from the population at different epidemiological periods were found to posses different avidity. Sera examined after the drop of the outbreak were most active in the virus — serum reaction, while those collected before the onset of the epidemics showed minimal activity.

## *Introduction*

Secondary infection of man or animals with influenza virus results in an increased avidity of anti-influenza antibodies (Aleksandrova *et al.*, 1964). As shown by haemagglutination-inhibition (HI) tests, adult human sera studied after influenza epidemics in 1962 contained more avid antibodies than convalescent sera examined in 1957. The reaction between the latter sera and the inhibitor-resistant strain of influenza virus was rather slow and the neutral complex formed was unstable and readily dissociated under the effect of hen erythrocytes. Antibodies in these sera of low avidity could be detected in HI tests only in the presence of human 0-group erythrocytes. The convalescent sera collected during influenza A2 epidemis in 1962 more readily reacted with the virus and formed a more stable neutral complex which was unaffected by hen erythrocytes.

To make more precise decision concerning the influence of repeated influenza infections or vaccination of man with a live influenza virus vaccine

on the specific properties of anti-influenza antibodies, sera collected from the healthy population of Leningrad in November—December, 1964 were studied in terms of their antibody activity. The data thus obtained were compared with the results of serological examination of blood specimens obtained from human subjects recovered from influenza in 1965 or re-vaccinated with the live influenza A2 virus vaccine. In addition, a comparative study of the avidity of sera collected both before and after the influenza A2 outbreak in Leningrad in 1965 was carried out.

## *Materials and Methods*

*Human sera.* The following groups of sera were examined:

a) sera from healthy adult subjects, collected in December, 1964 or March, 1965;

b) paired sera obtained from patients of different ages recovered from influenza A2 in January—February, 1965; and

c) sera from adult volunteers vaccinated with the live influenza A2 virus vaccine. These sera were kindly supplied by the Donors' Department of the Leningrad Pasteur Institute of Epidemiology and Microbiology.

The sera were stored at —14° C and heated at 58° C for 30 minutes just before testing.

*Virus.* The inhibitor-resistant A2/Singapore/57 strain of virus was stored lyophilized and refreshed by passages in 11- or 12-day-old chick embryos.

*HI tests* were carried out on plastic plates. To 0.2 ml of the serum dilutions, equal volumes of virus were added. The virus-serum mixture was incubated at 4° C for 18 hours and then a 0.75% suspension of hen or human 0-group erythrocytes was added in 0.4 ml amounts. In some of the experiments the incubation period of the virus with the serum was shortened to 10 minutes at room temperature or to 40 minutes at 40° C.

*The avidity of serum anti-influenza antibodies* was examined by the rate of the reaction between the serum and the inhibitor-resistant A2/Singapore/57 strain of the virus and also by the stability of the neutral complex formed. The stability of the virus-antibody complex was evaluated by the following methods:

1) Comparison of the results obtained in HI tests with hen or human 0-group erythrocytes;

2) quantitative titrations of the virus in developing chick embryos after its adsorption from the hyperneutral complex by hen or human erythrocytes (for details see Smorodintsev *et al.*, 1958; Aleksandrova, 1960; Aleksandrova *et al.*, 1964), and

3) reactivation of heated sera after addition of cofactor (nonspecific thermolabile antibody stimulator) as measured in HI tests performed by the method of Shuratov *et al.* (1967). The cofactor was obtained from native guinea pig serum. The first step of the experiment involved selection of the cofactor dose which would provide the highest antibody activity in the heated reference sera and would not inhibit the virus. Heated human test-sera were then diluted both in saline and in the normal native serum containing cofactor and diluted to a required concentration. Virus dilutions containing 4 haemagglutinating units (HAU) were added in 0.2 ml amounts to the serum dilutions. After incubation of the mixture at 40° C for 40 minutes, 0.4 ml of a 0.75% suspension of hen erythrocytes was added. The coefficient of reactivation was calculated by the increase in the serum antibody titre in the presence of cofactor.

The rate of immune response was determined by comparing the results of HI tests using incubation of the test sera and A2/Singapore/57 virus for 10 minutes or 18 hours in the presence of human 0-group erythrocytes.

## *Results*

Table 1 summarizes the results obtained in HI tests with human adult sera and hen or human 0-group erythrocytes. Serological examination of healthy subjects or influenza cases during the first 5 days after the onset of the disease showed that serum antibody titres measured by HI tests were 12 times lower with hen erythrocytes than with human erythrocytes.

**Table 1. Examination of sera from healthy subjects, convalescents and adult volunteers vaccinated with live influenza virus vaccine in HI tests with hen or human erythrocytes**

| Sera from | No. of sera | Geometric mean antibody titre with erythrocytes | | Coefficient of decrease with hen erythrocytes |
|---|---|---|---|---|
| | | hen | human | |
| Healthy subjects | 50 | 7.0 | 84.0 | 12.0 |
| Patients (5 days after the onset of the disease) | 40 | 6.5 | 79.0 | 12.1 |
| Convalescents | 44 | 42.0 | 182.00 | 4.3 |
| Live influenza virus vaccine recipients | 40 | 8.6 | 79.0 | 9.0 |

Antibody titres of sera from vaccinees obtained in HI tests with human erythrocytes were 9 times higher than with hen erythrocytes. Convalescent sera were most active in HI tests and differed from healthy human sera not only in higher antibody levels determined by titration with human erythrocytes but also in an increased activity in the presence of hen erythrocytes. The increased activity of the sera from vaccinees and, in particular,

**Table 2. Serological examination of healthy subjects or convalescents in HI tests with hen or human erythrocytes**

| Antibody titre in HI tests with hen erythrocytes | Antibody titres in HI tests with human erythrocytes in sera from | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | healthy subjects | | | | | convalescents | | | | |
| | 40 | 80 | 160 | 320 | Total No. of sera | 40 | 80 | 160 | 320 | Total No. of sera |
| 0 | 6* | 10 | 1 | 0 | 17 | 0* | 0 | 0 | 0 | 0 |
| 10 | 1 | 7 | 3 | 0 | 11 | 4 | 0 | 3 | 0 | 7 |
| 20 | 2 | 11 | 1 | 1 | 15 | 0 | 4 | 2 | 1 | 7 |
| 40 | 0 | 2 | 3 | 1 | 6 | 0 | 1 | 4 | 9 | 14 |
| 80 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 8 | 9 |
| 160 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 3 |
| 320 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 4 |
| Total No. of sera | 9 | 30 | 8 | 3 | 50 | 4 | 5 | 11 | 24 | 44 |

* Number of sera with the indicated antibody titre as measured in HI tests with hen or human erythrocytes.

of covalescent sera as evidenced by HI tests with hen erythrocytes indicates an enhanced avidity of anti-influenza antibodies in these sera.

Table 2 summarizes the results of HI tests on individual adult sera with hen and human 0-group erythrocytes. To find out the differences in the

avidity of antibodies, only those sera which contained comparable or mean antibody titres (1 : 40—1 : 320) as measured by HI tests with hen erythrocytes were selected for the analysis.

Most of the healthy human sera examined one month prior to the development of influenza epidemic in Leningrad appeared to be non-avid.

**Table 3. Amount of influenza A2/Singapore/57 virus adsorbed by hen or human erythrocytes from hypernueutral complexes with sera from healthy adults or convalescents**

| Grroups of subjects | Serum No. | Amount of virus (log $ID_{50}$) adsorbed onto the indicated erythrocytes | |
|---|---|---|---|
| | | hen | human |
| Healthy | 1 | 3.5 | 1.5 |
| | 2 | 4.5 | 2.5 |
| | 3 | 3.5 | 0.5 |
| | 4 | 2.5 | 1.5 |
| | 5 | 2.5 | 0 |
| Convalescents | 1 | 0.5 | 0 |
| | 2 | 0 | 0 |
| | 3 | 0 | 0 |
| | 4 | 0.5 | 0 |
| | 5 | 0.5 | 0 |
| Vaccinated with live influenza virus vaccine | 1 | 2.5 | 1.5 |
| | 2 | 1.5 | 0.5 |
| | 3 | 2.0 | 1.0 |
| | 4 | 2.0 | 1.0 |
| | 5 | 3.0 | 1.0 |
| Saline | — | 6.0 | 6.0 |

Accordingly, antibody titres measured by HI tests with hen erythrocytes were 8—32-fold lower than those obtained with human erythrocytes. No similar HI titres were found among these sera, with hen or human erythrocytes, and only in some of them antibody titres obtained with hen erythrocytes were 2 times lower than with human erythrocytes.

Convalescent sera exhibited a higher activity in HI tests. Among these, similar antibody titres with both hen and human erythrocytes were detected in 5 specimens, and the coefficient of decrease with hen erythrocytes dropped to 2 in 4 sera.

One of the most precise methods used to examine specific antibody avidity consists in evaluation of the stability of the neutral complex by quantitative titrations on the virus adsorbed by hen erythrocytes from the hyperneutral mixtures with the test serum. With this aim, the serum containing 16—32 neutralizing units was mixed with 1 HAU of the virus. The serum-virus mixture was incubated for 18 hours, and then a 1% suspension of hen erythrocytes was added. The sedimented erythrocytes were washed with

cold saline to remove antibodies and the virus was titrated in developing chick embryos.

Table 3 presents data on qualitative differences between the sera tested. The adsorption of the virus from the mixture with healthy human sera was rather intensive, and the amount of the virus adsorbed onto hen erythrocytes

**Table 4. The rate of immune response between A2/Singapore/57 virus and sera from healthy subjects or recipients of live influenza virus vaccine**

| Sera from | Serum No. | HI titres at the indicated duration of incubation of the virus with the serum | | Coefficient of titre increase during 18 hours' incubation |
|---|---|---|---|---|
| | | 10 minutes | 18 hours | |
| Healthy subjects | 1 | 10 | 80 | 8 |
| | 2 | 10 | 80 | 8 |
| | 3 | 20 | 160 | 8 |
| | 4 | 10 | 160 | 16 |
| | 5 | 10 | 160 | 16 |
| | 6 | 10 | 160 | 16 |
| | 7 | 10 | 40 | 4 |
| | 8 | 10 | 160 | 16 |
| | 9 | 10 | 40 | 4 |
| | 10 | 10 | 40 | 4 |
| Vaccinees | 1 | 40 | 160 | 4 |
| | 2 | 80 | 160 | 2 |
| | 3 | 10 | 160 | 16 |
| | 4 | 10 | 80 | 8 |
| | 5 | 20 | 160 | 8 |
| | 6 | 40 | 80 | 2 |
| | 7 | 40 | 160 | 4 |
| | 8 | 40 | 80 | 2 |
| | 9 | 10 | 40 | 4 |
| | 10 | 20 | 80 | 4 |
| Convalescents | 1 | 80 | 320 | 4 |
| | 2 | 160 | 640 | 4 |
| | 3 | 40 | 320 | 8 |
| | 4 | 80 | 320 | 4 |
| | 5 | 20 | 80 | 4 |
| | 6 | 320 | 640 | 2 |
| | 7 | 80 | 640 | 8 |
| | 8 | 320 | 640 | 2 |
| | 9 | 2560 | 5120 | 2 |
| | 10 | 80 | 160 | 2 |

correlated with the figures obtained in control titrations of the virus using saline. The amount of the virus adsorbed by hen erythrocytes from the mixture with convalescent or vaccine recipient sera was significantly lower. Thus, both adsorption and HI tests with hen or human erythrocytes suggested an increased avidity of anti-influenza antibodies in adults as a result of

previous influenza infection or vaccination with a live influenza virus vaccine.

The differences in the avidity of these groups of sera were also substantiated by the rate of their interaction with influenza A2/Singapore/57 virus. The more active antibodies in the sera of vaccinees or convalescents more readily reacted with the virus than those contained in healthy human sera (Table 4).

**Table 5. Intensity of reactivation of individual adult sera under the effect of cofactor as evaluated in HI tests with hen erythrocytes**

| Sera from | Serum No. | HI antibody titres with hen (I) or human (II) erythrocytes and serum diluted | | | Coefficient of reactivation the presence of cofactor |
|---|---|---|---|---|---|
| | | I | | II | |
| | | in saline | in normal native serum containing cofactor | in saline | |
| Healthy subjects | 1 | 10 | 20 | 40 | 2 |
| | 2 | 10 | 20 | 40 | 2 |
| | 3 | 10 | 40 | 40 | 4 |
| | 4 | 10 | 80 | 80 | 8 |
| | 5 | 10 | 40 | 80 | 4 |
| Vaccinees | 1 | 40 | 80 | 80 | 2 |
| | 2 | 20 | 80 | 80 | 4 |
| | 3 | 10 | 80 | 80 | 8 |
| | 4 | 20 | 160 | 160 | 8 |
| | 5 | 20 | 160 | 80 | 8 |
| Convalescents | 1 | 40 | 160 | 80 | 4 |
| | 2 | 80 | 320 | 80 | 4 |
| | 3 | 40 | 160 | 160 | 4 |
| | 4 | 80 | 320 | 160 | 4 |
| | 5 | 80 | 80 | 160 | 1 |

The next step in our investigations was to study reactivation in the presence of cofactor of the sera obtained from the indicated groups of adults and heated at 56 C for 30 minutes.

An ability of normal native sera to increase the activity of heated immune sera was first demonstrated by Styk *et al.* (1958) in experiments on strains of influenza A2 virus. Investigations by A. A. Smorodintsev and others have shown cofactor to produce an effect not only on this type of the virus but also on different influenza virus serotypes and on Sendai, vaccinia, respiratory-syncytial and other viruses as well (Smorodintsev and Yabrov, 1963; Shuratov *et al.*, 1967 etc.).

Cofactor produces the most marked reactivation effect on influenza A2 virus strains characterized by low sensitivity to antibodies and poor reaction with the sera in the presence of hen erythrocytes. The experiments with these viruses showed that the degree of reactivation of heated immune sera depends also on the properties of anti-influenza antibodies, that is their specific avidity. The sera lacking avidity became more active in the presence of cofactor, while the activity of hyperimmune sera in HI tests with hen erythrocytes was almost unaffected by addition of

cofactor. These data were obtained in the study of specific properties of sera from albino mice vaccinated once or twice.

Estimation of the intensity of reactivation of heated sera under the effect of cofactor was used in this laboratory as a method to characterize anti-influenza antibodies in human adult sera. For this purpose the test sera were diluted both in native guinea pig sera and also in saline, and hen erythrocytes were added after incubation of the serum dilutions with influenza A2/Singapore/57 virus for 40 minutes at 37° C. The findings thus obtained were then compared with the results of antibody titrations in HI tests carried out under optimal conditions with human 0-group erythrocytes.

**Table 6. Summary of reactivation findings obtained in HI tests with hen erytrocytes and human adult sera in the presence of cofactor**

| Sera from | No. of sera | HI antibody titres with hen (I) or human (II) erythrocytes and serum diluted | | | Coefficient of reactivation in the presence of cofactor |
|---|---|---|---|---|---|
| | | I | | II | |
| | | in saline | in normal guinea pig serum | in saline at 4° C for 18 hr | |
| Healthy subjects | 44 | 2.7 | 22.6 | 49.0 | 8.3 |
| Vaccinnees | 47 | 9.2 | 60.0 | 79.0 | 6.5 |
| Convalescents | 46 | 34.0 | 158.0 | 169.0 | 4.6 |

It appeared that addition of cofactor was less essential for the convalescent sera possessing high avidity than for the sera from vaccinees and especially from healthy subjects; the activity of the latter sera was significantly increased under the effect of cofactor. Tables 5 and 6 present comparative data obtained in HI tests with hen erythrocytes in the presence of cofactor and those obtained by antibody titrations under optimal conditions with human 0-group erythrocytes. No difference was found in the effectiveness of both methods when testing the sera of convalescents or vaccinees, whereas healthy human sera showed better titration results when tested in the presence of human erythrocytes. This fact can be explained by an extremely low avidity of these serum antibodies forming with the virus a highly labile

**Table 7. Results of serological examination in HI tests with hen or human erythrocytes of human subjects of different age who recovered from influenza A2 epidemic in 1965**

| Age of the subjects examined (years) | No. of sera | Geometric mean antibody titres with erythrocytes | | Multiplicity of decrease with hen erythrocytes |
|---|---|---|---|---|
| | | hen | human | |
| 0—2 | 29 | 2.5 | 112.0 | 44.8 |
| 3—6 | 25 | 10.6 | 52.0 | 4.9 |
| 7—16 | 26 | 169.0 | 479.0 | 2.8 |
| ⩾17 | 44 | 42.0 | 182.0 | 4.3 |

complex readily dissociated by addition of hen erythrocytes even in the presence of cofactor.

Thus, a qualitative analysis of adult sera showed significantly lower avidity of antibodies in sera of healthy men compared to that of antibodies in sera of convalescents or recipients of a live influenza virus vaccine.

The increase in the specific avidity of anti-influenza antibody was followed in different age groups (Table 7). As shown by our previous investigations,

**Table 8. Results of examination in HI test with hen or human 0 erythrocytes of sera obtained from the Leningrad population**

| Group No. | Date of serum sampling | No. of sera | Geometric mean antibody titres with erythrocytes | | Multiplicity of decrease with hen erythrocytes |
|---|---|---|---|---|---|
| | | | hen | human | |
| 1 | December, 1964 | 65 | 0.8 | 14.9 | 18.6 |
| 2 | March, 1965 | 100 | 3.5 | 37.0 | 10.5 |
| 3 | December, 1965 | 100 | 2.5 | 34.0 | 13.6 |
| 4 | May, 1966 | 100 | 2.3 | 30.0 | 13.0 |

a regular increase in the serum avidity was observed in the process of subsequent contacts of men and animals with influenza virus (Aleksandrova *et al.*, 1964). It was established that small children primarily infected with influenza developed antibodies of low avidity. Serological examination of children from 1 to 2 years of age, who recovered from influenza during epidemics in 1965, showed that the general properties of their antibodies were similar to those of antibodies usually formed by children of this age group: they namely did not react with the inhibitor-resistant S2/Singapore/57 strain in HI tests in the presence of hen erythrocytes and exhibited a maximal coefficient of decrease in titres (44.8) compared to HI tests with human erythrocytes. Convalescent sera obtained from older children recovered from the second attack of influenza in 1962 behaved in HI tests like human adult sera and showed minimal differences in HI antibody titres with hen or human erythrocytes (coefficient 4.9).

These results illustrate the influence of the secondary infection on changes in the specific properties of anti-influenza antibodies.

In addition to these studies, comparative investigations were carried out on the level of specific avidity of antibodies in sera collected from the Leningrad population at different times during the 1964—1967 period (Table 8). It appeared that sera collected before the influenza outbreak in 1965 contained minimal titres of antibodies to A2/Singapore/57 virus and did not react with the virus in the presence of hen erythrocytes. The sera obtained after termination of influenza A2 epidemics (group No. 2) were most active in HI tests. In this case not only the level of anti-influenza antibodies but also their specific avidity was increased, resulting in higher antibody titres as measured in HI tests with hen erythrocytes. Both the

level of anti-influenza antibodies and their specific avidity in sera examined one or one and a half years after the influenza epidemic later decreased and dropped to minimal values by December, 1966, that is in the period preceding the next influenza A2 outbreak in January, 1967.

## *Discussion*

The present investigations were carried out to obtain additional information on the effect of primary influenza infection or vaccination with a live influenza virus vaccine on the specific properties of anti-influenza antibodies. With this aim in view, antibody activity in the sera of individuals recovered from influenza A2 infection during epidemics in 1965 was studied, and the findings obtained were compared with the results of examination of sera from healthy subjects, collected in November — December, 1964, or of the sera of adults vaccinated with the live influenza A2 virus vaccine.

Antibody avidity was estimated by the rate of immune reaction between the inhibitor-resistant A2/Singapore/57 virus and the serum as also by the stability of the virus-antibody complex formed. The stability of the virus-serum complex was examined by comparing the results of HI tests with hen and human erythrocytes and of quantitative titrations of the virus adsorbed by hen erythrocytes from a hyperneutral complex with serum. These methods are based on competition between the virus neutralization by antibodies and its adsorption by hen erythrocytes, the latter being the most sensitive method to evaluate the stability of influenza A2 virus-serum complex. In case the linkage between the reacting components is not sufficiently strong, erythrocytes adsorb the virus onto their surfaces due to their ability to react with the virus more readily than non-avid antibodies. Furthermore, antibodies inactivated by heating were reactivated in the presence of cofactor.

The examination of sera collected from the Leningrad population before the onset of the influenza outbreak has shown their poor avidity towards the inhibitor-resistant virus. They slowly reacted with the virus and formed an unstable virus-serum complex. Serum antibodies formed by the subjects revaccinated with the live influenza virus vaccine and especially convalescent serum antibodies appeared to react more actively with the virus. These antibodies more readily reacted with the virus and formed a more stable neutral complex than those obtained from sera of healthy nonvaccinated subjects.

Qualitative differences between antibodies in sera from vaccine recepients and convalescents were also substantiated by the results of reactivation in the presence of cofactor. The activity of human sera from healthy subjects was significantly increased under the effect of cofactor, while sera from vaccinees and especially sera from convalescents were essentially unaffected.

Thus, it appeared that convalescent serum anti-influenza antibodies possessed a higher specific avidity than those contained in healthy human sera.

Changes in the level of the specific avidity were traced in different age groups. Sera obtained from children under 1 year of age, unlike the sera from older children and adults, were found to possess a low avidity in HI tests with hen erythrocytes. Children of this age group, who were born in 1963—1964, had only one contact with influenza virus during 1965 epidemics. This fact provides an extra evidence of the significance of secondary infection in the enhancement of the specific avidity of sera from persons of different age groups.

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