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THE CHARACTER OF INTERACTIONS OF ANTIBODIES AND INHIBITORS WITH INFLUENZA VIRUS

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Summary. — Inhibitors and antibody differ substantially from one another in their interaction with influenza virus. As distinct from antibodies, on combination with virus the inhibitors do not impart the complex the property to fix complement. Viral haemagglutinins neutralized with inhibitors retain their ability to subsequent reaction with specific viral antisera both in the complement fixation (CF) test and in the antibody absorption reaction. Conversely, viral haemagglutinins neutralized with antibody lose their ability to subsequent combination with inhibitors. In case of a sufficient level of antibody in the test serum, the inhibitors present in the latter do not interact with virus.

Introduction

The action of viral inhibitors and antibodies on influenza virus is very similar. Both antibodies and inhibitors, when combined with the virion, are capable of neutralizing its adsorbing, haemagglutinating and infectious activities. Serological and electron microscope studies (Rovnova *et al.*, 1963; Kosyakov and Rovnova, 1966) demonstrated that both inhibitors and antibodies are capable of influencing the first phase of infection by breaking the contact of virus with a susceptible cell.

However, the character of the interactions of inhibitors and antibodies with viruses is far from being sufficiently known. It remains obscure whether inhibitors and antibodies become attached to one and the same receptor or whether on the surface of the virion there exist different receptor groups for them. Nor is the question clear as to the time sequence at which antibodies and inhibitors, being simultaneously present in the test serum, do combine with a virus particle. So far only some ideas concerning the problem have been suggested (Belyavin, 1955, 1957; Smorodintsev, 1960; Yeremeyev *et al.*, 1961; Yeremeyev, 1963).

The aim of the present study was an investigation on the character of interactions between antibodies and inhibitors with influenza virus. The problem is not only of theoretical significance since it concerns receptor structure of the surface of the virus particle, but it also is of a considerable practical importance for evaluation of serological reactions employed in the diagnosis of viral infections, so long as antibodies and inhibitors might be simultaneously present in immune sera.

Materials and Methods

Allantoic cultures of two variants of influenza A2 virus, namely the inhibitor-sensitive (IS) strain A2/Sing./57 and the inhibitor-resistant (IR) strain A2/57, were used.

Immune viral antisera were obtained in rabbits according to the schedule described (Rovnova, 1959). Normal sera from rabbits, guinea pigs and horses served as inhibitors. Both native sera and sera heated at 100° C for 10 minutes were used in the tests. Before heating, the sera were diluted 1 : 10, 1 : 20, 1 : 40, and 1 : 100. The technique of the CF, haemagglutination (HA) and haemagglutination inhibition (HI) tests was that routinely employed in our laboratory (Kosyakov and Rovnova, 1965). Viral antigens were used in the form of eluates prepared by adsorption onto and elution from formolized human group O erythrocytes, with HA titres of 1 : 2400 or 1 : 10000. The working dose of the antigen was 0.1 ml in the CF test and 4 or 8 HA units in the HI test.

Results

The data summarized in Table 1 show that positive CF was observed only in experiments in which immune rabbit serum was combined with both IS and IR strains of influenza virus. The same serum deprived of antiviral antibodies by heating at 100° C did not fix complement in the presence of viral antigens. Sera from non-immune animals (rabbits, guinea pigs and horses), containing only inhibitors to the tested viruses, were also incapable of CF. CF was negative with both native and heated normal sera that possessed sufficiently high titres of inhibitors as determined in HI tests.

Table 1. Assay of influenza virus antibodies and inhibitors in the CF test

Serum	HI titre of serum[1])	Serum dilution reciprocals	CF test with A2 virus antigens[2])	
			IR	IS
Rabbit, immune to A2/Sing. virus				
unheated	2560	40	++++	++++
		80	++++	++++
		160	++++	++++
		320	+++	+++
		640	+	+
heated[3])	1280	20—160[4])	—	—
Rabbit, normal				
unheated	640	20— 80	—	—
Guinea pig, normal				
unheated	12800	20—160	—	—
heated	40000	20—160	—	—
Equine, normal				
unheated	5120	20—160	—	—
heated	10240	20—160	—	—

[1]) Reciprocals of the highest serum dilution still inhibiting haemagglutination of 4—8 HA units of A2/Sing. virus.

[2]) +, ++, +++ and ++++: increasing degrees of inhibition of haemolysis of sheep erythrocytes; —: complete haemolysis of erythrocytes.

[3]) Heated at 100° C.

[4]) 20—80 (160) means that the same results were obtained with serum dilutions 1 : 20, 1 : 40, 1 : 80 (and 1 : 160).

These experiments demonstrated that only the interaction of specific antibodies with influenza virus was accompanied by CF. Contrary to antibodies, inhibitors, when combined with virus, were not capable of fixing complement.

In the next series of experiments we studied the serological activity of viral suspension in reaction with antibodies after viral haemagglutinins had been blocked with serum inhibitors.

The following viruses were examined in CF tests against immune serum to A2/Sing. influenza virus: native IS strain A2/Sing. with a HA titre of 1 : 1280; the same virus blocked with inhibitors of rabbit and guinea pig sera, HA being negative; and the IR strain A2/57, the combination of which with inhibitors had no effect on its HA activity.

Table 2. Serological activity of influenza virus in the CF test after blocking of haemagglutinin with inhibitors

Serum dilution reciprocals[1])	Results of CF tests with A2 virus antigens[2])					
	IS			IR		
	Native virus (HA[3]) 1280)	Virus blocked with serum inhibitors from		Native virus (HA 320)	Virus treated with serum inhibitors from	
		rabbits (HA neg.)	guinea pigs (HA neg.)		rabbits (HA 320)	guinea pigs (HA 320)
40	++++	++++	++++	++++	++++	++++
80	++++	++++	++++	++++	++++	++++
160	++++	++++	++++	++++	++++	++++
320	++	++	++	++++	++++	++++
640	+	++	++	++++	++++	++++

[1]) Immune serum to A2/Sing. virus.
[2]) Designations as in Table 1.
[3]) HA titres.

To remove antibodies to host cell antigens before the performance of CF test, the serum was absorbed with formolized tissue of chick embryo chorioallantoic membrane and sheep erythrocytes (Kosyakov and Rovnova, 1965). After absorption, the serum specifically inhibited HA of the two strains of influenza virus.

For blocking haemagglutinins of the IS strain, fractional portions of inhibitors were added to the virus till an excess of inhibitors could be demonstrated in the mixture, the haemagglutinins being no more detectable.

The results of one of the experiments are presented in Table 2. It is evident that complete blocking of viral haemagglutinins of the IS strain A2/Sing. with the inhibitors of rabbit and guinea pig sera (HA negative) did not reduce the ability of the virus to fix complement in the presence of specific antiviral antibodies.

Thus the blocking of viral haemagglutinins with inhibitors of animal sera did not deprive influenza virus of its ability to undergo serological reaction with immune serum and produce a complex able to fix complement.

The ability to react with specific antibody of virus, the haemagglutinin of which is blocked with inhibitors, was studied in absorption experiments.

Immune viral antiserum was absorbed in parallel with three viral suspensions: native A2/Sing. virus (HA titre 1 : 1280); A2/Sing. virus with HA activity partly blocked with rabbit serum inhibitors (HA titre 1 : 160); and the same virus the haemagglutinins of which were completely inhibited with rabbit serum (HA negative). Absorption was followed by serum titration in HI tests with 4 HA units of the IR strain A2/57.

The results of experiments on antibody absorption are summarized in Fig. 1 which shows that the inhibitor-blocked virus absorbed specific antibodies from immune serum with the same success as did native virus taken in an equal amount.

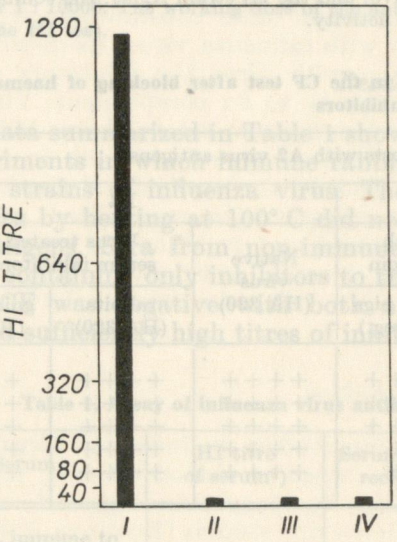

Fig. 1.

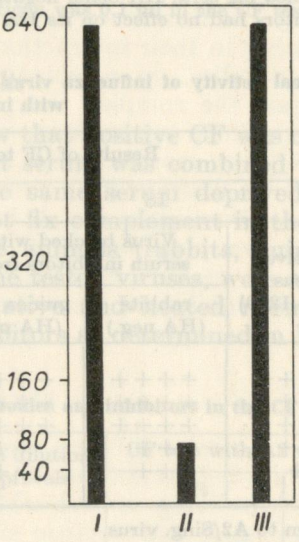

Fig. 2.

1 — Absorption of antibody from immune serum by IS A2/Singapore influenza virus
I — Before absorption
II — After absorption with untreated virus (HA titre of 1280)
III — After absorption with virus partially blocked by inhibitors from normal rabbit serum (HA titre of 160)
IV — After absorption with virus completely blocked by inhibitors from normal rabbit serum (HA negative)
2 — Absorption of inhibitors from normal rabbit serum by IS A2/Singapore influenza virus
I — Before absorption
II — After absorption with untreated virus (HA titre of 320)
III — After absorption with virus completely blocked by antibody (HA negative).

Thus, the two (CF and HI) tests yielded similar results indicating that blocking of viral haemagglutinins with serum inhibitors does not influence the ability of haemagglutinins to interact with specific antibody.

Other results were obtained when haemagglutinins of A2/Sing. virus were blocked by antibody and inhibitors subsequently added to such viral suspension. The virus deprived of HA activity after combining with antibody was incapable of absorbing inhibitors from normal rabbit serum (Fig. 2).

However, the reaction of virus particles with inhibitors did not occur only when a sufficient amount of antibody was used for the blocking of viral haemagglutinins. This conclusion was supported by the results of experiments presented in Table 3 which showed that addition of different dilutions (from 1 : 50 to 1 : 1600) of immune serum to viral suspension was accompanied by fixation of subsequently added inhibitors only if the dilution of the serum was high, i.e. from 1 : 200 to 1 : 1600. When a sufficient amount

Table 3. Interaction between influenza virus and inhibitors after blocking with specific antibody

Influenza virus (ml)	Immune serum dilution (0.1 ml)	Inhibitors — guinea pig serum 100° C (ml)	Inhibitor titre*						
			200	400	800	1600	3200	6400	12800
No virus	No serum	0.25	—	—	—	—	—	—	+++
0.5	50	0	++	+++	+++	+++	+++	+++	+++
0.5	50	0.25	—	—	—	—	—	—	+++
0.5	100	0.25	—	—	—	—	—	+	+++
0.5	200	0.25	—	—	—	—	++	+++	+++
0.5	400	0.25	—	—	++	+++	+++	+++	+++
0.5	800	0.25	—	—	++	+++	+++	+++	+++
0.5	1600	0.25	+++	+++	+++	+++	+++	+++	+++

* Determined in HI tests with 2 HA units of A2/Sing. virus; +, ++ and +++ refer to increasing degree of agglutination of human O erythrocytes; —: inhibition of haemagglutination.

of antibody (immune serum diluted 1 : 50 or 1 : 100) was added, their combination with virus prevented the latter from interaction with inhibitors, which remained unbound.

To study the peculiarities of the interaction of influenza virus with inhibitors and antibodies, simultaneously present in immune serum, the following experiments were carried out.

One volume of an A2/Sing. virus eluate was added to two volumes of immune serum, diluted 1 : 10 and 1 : 20 (the amount of virus was determined beforehand to reach excess of antibodies after their combining with virus). The mixture was held at 4° C for 24—48 hours, then centrifuged for 60 minutes at 10000—15000 rev/min. The sediment obtained was washed with chilled physiological saline (in 1/4 to 1/5 volume of the mixture) to remove unbound inhibitors and antibodies.

The supernatant fluid, washings of the sediment, and sediment resuspended in physiological saline (in 1/4 to 1/5 volume of the mixture) were tested for the presence of inhibitors and antibodies in HI tests with IS and IR strains of influenza virus. The fluids were examined unheated and heated at 68—70° C and 100° C. According to our previous experiments (Kosyakov and Rovnova, 1966), heating of the influenza virus-antibody complex at 68—70° C leads to inactivation of haemagglutinin and release of antibody. Heating of the complex up to 100° C resulted in inactivation of both viral haemagglutinin and antibody. However, thermostable inhibitors remained unchanged at this temperature. Exposure to different temperatures of the tested samples of the supernatant fluid and sediment obtained from the virus-immune serum mixture permitted us to determine which factors of the serum — antibodies or inhibitors — combined with virus.

The results of these experiments (Table 4) showed that the initial immune serum contained both antibodies to the two variants of influenza A2 virus

Table 4. Serological analysis of interactions of influenza virus with antibody and inhibitors

Results of HI tests with the given virus and serum dilutions (reciprocals)	Control of serum			Experiment — mixture of immune serum with virus								
				Supernatant			Washing of sediment			Sediment		
	I	II	III	I	II	III	I	II	III	I	II	III
IS A2/Sing.												
40	–	–	–	–	–	–	–	–	+++	+++	–	+++
80	–	–	–	–	–	–	+++	+++	+++	+++	–	+++
160	–	–	–	–	–	–	+++	+++	+++	+++	–	+++
320	–	–	–	–	–	–					–	+++
640	–	–	–	–	–	–					–	+++
1280	–	–	–	–	–	–					–	+++
2560	–	–	+++	+++	+++	+++					+++	+++
5120	–	–	+++	+++	+++	+++					+++	+++
10240	++	++	+++	+++	+++	+++					+++	+++
IR A2/57												
40	–	–	+++	–	–	+++	+++	+++	+++	+++	–	+++
80	–	–	+++	–	–	+++	+++	+++	+++	+++	–	+++
160	–	–	+++	–	–	+++	+++	+++	+++	+++	–	+++
320	–	–	+++	–	+++	+++					–	+++
640	–	–	+++	+++	+++	+++					+++	+++
1280	–	–	+++	+++	+++	+++					+++	+++
2560	–	–	+++	+++	+++	+++					+++	+++
5120	+++	+++	+++	+++	+++	+++					+++	+++

I — unheated samples; II — samples heated at 68—70° C; III — samples heated 100° C.
Haemagglutination scored as in Table 3.

and thermostable (up to 100° C) inhibitors to the IS strain of virus. With respect to the IS strain, the titre of thermostable inhibitor was 1 : 1280. Antibody titres to the IR and IS variants were 1 : 2560 and 1 : 5120, respectively. After heating of serum at 68—70° C, antibodies also reacted with virus the antibody titre remaining the same. Thermostable inhibitors caused no HI of the IR strain A2/57.

The washing of the sediment was free from inhibitors and contained a small amount of antibody.

The supernatant fluid contained both thermostable inhibitors (in a titre of 1 : 1280 with the IS strain) and antibody (in a titre of 1 : 320 with the IR strain) (excess of antibody as determined with the two variants of virus was intentionally introduced into the mixtures). As shown in Table 4, the titre of thermostable inhibitors remained unchanged after addition of virus to immune serum.

The sediment suspended in physiological saline was found to contain no free antibody to the two variants of A2 virus. Heating of the sediment at 68—70° C, during which viral haemagglutinins were destroyed, resulted in the release of antibodies that inhibited HA of both IR and IS strains of influenza virus. Heating of the sediment at 100° C led to a complete loss of its virus-inhibiting activity.

These experiments offered evidence that only antibodies were bound to virus whereas thermostable inhibitors did not react with virus in the presence of excess antibody. The results coincided with the findings obtained while examining the supernatant fluid in which no decrease in inhibitors was observed after addition of virus to immune serum.

Discussion

The investigations into the character of interactions of antibodies and inhibitors with influenza virus speak in favour of a higher avidity of antibodies as compared with serum inhibitors. Antibodies are the first to combine with virus when antibodies and inhibitors are simultaneously present in immune serum. Reaction with antibodies prevented virus from interaction with inhibitors contained in the same serum or added later on. Conversely, influenza virus when combined with inhibitors retained its ability to react with antibodies. This was found both in CF tests and by the method of antibody absorption with subsequent testing of the serum in HI tests.

The study of the virus-inhibitor complex confirmed previous suggestions that the combining of virus with inhibitors occurs without complement fixation (Friedewald *et al.*, 1947; Smorodintsev and Shishkina, 1951; Henle *et al.*, 1958).

The results of our experiments lead to the conclusion that a positive CF reaction always testifies to the presence of antibodies in the serum. On the contrary, HI may be induced both by antibodies and inhibitors.

The question as to whether the established peculiarities of interaction of antibodies and inhibitors with influenza virus are due to the existence of different receptors on the surface of the virion or are stipulated by some

peculiar properties of antibodies and inhibitors has to be solved by further investigations.

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