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Studies on the Non-specific Inhibition by Some Body Fluids against Vaccinia Haemagglutination

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Introduced into the organism, the haemagglutinins of the vaccinia virus release the production of specific antihaemagglutinins, i. e. haemagglutination-inhibiting (HAI) antibodies (1). They appear in the serum 7 to 14 days after the inoculation of the virus, reach their highest concentration between the 2nd and 4th week, and show a gradual diminution thereafter (2—12). These antibodies are extremely persistent so that, in some cases, their titre in the serum is still considerably high even after the lapse of 60 years (13). Normal human and animal sera do not inhibit haemagglutination in dilutions above 1 : 10 (14, 3) even if only one haemagglutination (HA) unit of the virus is used for the inhibition test (3).

Also other substances than specific antibodies produce inhibition against vaccinia HA, e. g. crude calf lymph (15, 16), normal lung-tissue extract (17) or mouse-tumor fluid (18). Non-specific inhibitors have been found in the serum collected from the umbilical cord of vaccinated mothers (19, 20). *Szathmáry* and *Holik* (21, 22, 23) succeeded in demonstrating non-specific inhibitors in the cerebrospinal fluid which was thermostable at a temperature of 56° C and contained a pathologically high amount of protein; they demonstrated them also in the fluids collected from the pleural and peritoneal cavities, from the joints, the hydrocele of testis and, in certain cases, in the fluid obtained from ovarian cysts. These authors found that about 50 per cent of the examined ovarian cyst-fluids and all of the other examined body fluids inhibited vaccinia HA in dilutions over a range from 1 : 4 to 1 : 8000. Inhibition seemed to be independent of whether 1) the body fluid proceeded from patients who had not yet been vaccinated and whether their blood contained passive maternal HAI antibodies or not; 2) it made no difference if the body fluid (pleural exudate in one of

the cases) was obtained three weeks after vaccination when the blood of the patients contained a great amount of specific active HAI antibodies; 3) inhibitory activity was likewise uninfluenced by the fact that—as proved by their typical vaccination scar—some of the patients from whom body fluids were collected had undergone both primary vaccination and revaccination and that their blood contained just demonstrable or higher titres of HAI antibodies. There seems to be no close parallelism between the grade of inhibition and the protein content of the body fluids (0,7 to 6,6 per cent); as a rule, body fluids with a higher level of protein produce inhibition in higher titres. Of albumin and globulin fractions separated by means of salting-out with ammonium sulphate, only the latter possesses inhibitory activity. Specific HAI antibodies contained in hyperimmune rabbit serum are likewise in the globulin fraction (3).

Non-specific inhibitors in human ascites were demonstrated by *Cassel* and *Fater* (24).

The experiments described in this paper were concerned with the study of non-specific inhibitors contained in the above-mentioned body fluids, viz. the cerebrospinal fluid with pathologically high protein level, in the pleural, peritoneal and synovial fluids as also in the hydrocele of testis and ovarian cysts.

Material and Methods

We obtained our test material from various hospitals, most of it from the Municipal Hospital of Balassagyarmat. The centrifuged material was first inactivated for 30 minutes at 56° C and then stored at −12 to 15° C. We performed the HAI-test with *Takátsy*'s (25) micromethod by means of a 0,025 ml. spiral loop and instillator. This method has already been described in detail in an earlier communication (22). The phosphate-buffered saline suspension of confluent pocks developed on the chorio-allantoic membrane of chick embryos infected with the usual bovine vaccine served as haemagglutinin.

We used 2 haemagglutinating units for the HAI test, i. e. the double of that virus concentration which completely agglutinated fowl erythrocytes sensitive to vaccinia.

Having prepared serial twofold dilutions of the test material and mixed it with the virus, we incubated this mixture for an hour at 37° C and added then 1% fowl-erythrocyte suspension. Readings were taken after half an hour at room temperature. The highest dilution giving complete inhibition was regarded as the HAI-titre. The reciprocals of the initial dilutions of the body fluids are indicated as titres in our tables.

Hyperimmune rabbit serum, obtained after repeated intraperitoneal and subcutaneous immunization, served as reference.

Crystalline trypsin, prepared by the Chemical Department of our Institute, was used for the treatment of serum and body fluids. Trypsin solutions of 1 and 2 per cent—made with phosphate buffer of pH 7,6—were mixed with the test fluid at the ratio 1 : 1. Body fluids diluted at the same ratio with phosphate buffer served as controls. The mixtures were incubated at 37° C

Table 1. Effect of temperature and time of incubation on the inhibitory titre of body fluids

Test fluid	6° C				22° C				37° C			
	0′	30′	60′	120′	0′	30′	60′	120′	0′	30′	60′	120′
Hyperimmune rabbit serum No. 998/999	32	128	128	256	32	128	256	256	32	128	256	512
Pleural exudate No. 113	64	128	128	512	64	256	512	1024	32	512	1024	2048
Ascites No. 219	16	64	64	128	8	64	128	256	16	128	512	512
Hydrocele of testis No. 224	4	16	32	32	8	16	64	128	4	64	128	256
Cerebrospinal fluid No. 244	<4	4	8	16	<4	8	16	32	<4	16	32	32

for different lengths of time, and then heated at 56° C for 30 minutes with a view to inactivating the trypsin.

An amount of 0.5 g. of commercial kaolin was added to each ml. of the test fluid. After shaking it during 15 minutes at room temperature, we centrifuged the suspension for 10 minutes at 2000 r. p. m., and performed the reaction with the clear supernate fluid.

Results

Effect of the duration and temperature of incubation on inhibition. *Chu*'s (3) experimental results seem to prove that if—before the admixture of the erythrocytes—the mixture of haemagglutinin and specific serum, kept at room temperature, is incubated for a longer time, the inhibitory titre will become higher. *Wigand* (26), on the other hand, observed but an insignificant rise of titre after lengthening the time of incubation at room temperature. We performed our tests at various temperatures and let the mixtures (haemagglutinin-specific serum or haemagglutinin-body fluid) stand for from zero to 2 hours, sometimes even 4 hours, before adding the erythrocyte suspension. The results of an individual test

in respect of each experiment are assembled in Table 1. It shows that reaction between specific inhibitors and virus sets in immediately at the examined temperatures, and that—with a fluctuation amounting to a single degree of dilution—the time of incubation is directly proportional to the height of the serum's inhibitory titre. With incubations of equal length, one generally obtains higher titres at higher, and lower titres at lower temperatures. After an incubation of 4 hours, the titre either remains unchanged or rises by one degree of dilution; in none of our experiments did we observe a diminution of the titre. Non-specific inhibitors contained in the body fluids show a

Table 2. Effect of haemagglutinin concentration on inhibitory titre

Concentration of virus	HAI titre			
	Hyperimmune rabbit serum No. 998/999	Pleural exudate No. 113	Ascites No. 219	Hydrocele of testis No. 288
1	512	2048	1024	512
2	256	>512 <1024	512	256
4	>64 <128	512	256	128
8	64	256	128	>32 <64

similar behaviour, with the difference that an incubation of 10 to 30 minutes is needed for the reaction to become demonstrable in the case of body fluids with low HAI titre.

Effect of haemagglutinin concentration on inhibition. According to literature (1, 14, 3, 26), the titre of specific serum is inversely proportional to the dose of haemagglutinin. The situation is similar in regard of body fluids: apart from a fluctuation amounting to half a degree of dilution, a doubling of haemagglutinin concentration reduces the HAI titre by 50 per cent (Table 2). Also the non-specific inhibitors of the body fluids combine with haemagglutinins according to the law of multiple proportions.

Sensitization of erythrocytes by inhibitors. With a view to ascertaining whether the erythrocytes or the haemagglutinins are affected by non-specific inhibitors, we designed the following experiment: the body fluids and hyperimmune rabbit serum were kept at 6° C and at room temperature with fowl erythrocytes, in each case for an hour; after washing them three times with saline, a 1% erythrocyte suspension was made and added to the virus dilutions. Haemagglutination did not differ in any case from that reached with non-sensitized red corpuscles. Like specific HAI antibodies, non-specific inhibitors are not adsorbed to erythrocytes.

Inhibition against the thermostable haemagglutinin of the virus. The vaccinia virus has two different haemagglutinins: a thermolabile, which is destroyed by being kept at 56° C for 30 to 45 minutes, and a more thermostable component which can be exposed to higher temperatures with no or but a slight loss of potency; the latter is completely destroyed only by being autoclaved or kept at 100° C during 90 minutes (3, 27, 28). Therefore, we instituted comparative tests: we examined the inhibitory effect of body fluids and immune rabit serum on thermostable haemagglutinins that had been exposed to temperatures of 56° C and 80° C for 30 minutes. The HA titre of the virus suspension used in the experiments was 1:256 without heating, while the titre was 1:128 and 1:32 after heat treatment. The inhibitory effect of non-specific substances upon thermostable haemagglutinins, tested against 2 HA units, was not significantly different from the inhibition observed against untreated haemagglutinins which comprise both thermolabile and thermostable components. Tests with immune rabbit serum yielded similar results.

Elution of haemagglutinins bound by inhibitors. It was demonstrated by *Chu* (3) that it is possible to utilize the different heat sensitivity of haemagglutinins and antihaemagglutinins for their separation. The thermostable component of haemagglutinins is not or is hardly destroyed by exposure to a temperature of 100° C, whereas HAI antibodies undergo complete destruction long before such temperature is reached. If a mixture of virus and immune serum which contains all haemagglutinins in the adsorbed state is boiled for a short time, the antihaemagglutinins suffer complete destruction, and fowl erythrocytes added to the system will be agglutinated by the eluted haemagglutinins. We utilized this reaction for the differentiation of specific and non-specific inhibition but failed to observe any significant difference between the amount of haemagglutinins eluted from non-specific substances and that of haemagglutinins eluted from specific inhibitors.

Thermoresistance of specific and non-specific inhibitors. In addition to studying the stability of non-specific inhibitors, we examined—for purposes of comparison—also that of undoubtedly specific HAI antibodies of various origins. The results of our experiments are assembled in Table 3.

Specific inhibitors contained in hyperimmune rabbit sera remain stable up to a temperature of 70° C, an observation which agrees with literary data (29, 3). The titre is reduced about 2 degrees of dilution at 75° C. The antibodies of *human sera* collected 3 to 4 weeks after both primary vaccination and revaccination are somewhat less heat resisting than those contained in hyperimmune rabbit serum. A temperature of 70° C reduces the titre of the sera by 1 to 2 degrees of dilution. One of our cases gave us the possibility of examining the stability of a serum obtained from a

patient who had been vaccinated 40 years before. Repeated tests proved this serum to produce—presumably non-specific—inhibition in a titre exceeding the normal value, even in a dilution of 1:128. Inhibitors contained in this serum were seriously damaged by exposure to a temperature of 70° C: the titre went down four degrees of dilution. The inhibitory titre

Table 3. Thermoresistance of specific and non-specific vaccinia haemagglutination inhibitors

Material tested	No. of cases	Mean titre	Diminution of mean HAI titres after a heating of 30 minutes at				
			56° C	60° C	65° C	70° C	75° C
Hyperimmune rabbit sera ..	8	8.6	0.1	0.2	0.2	0.8	2.2
Human sera after primary vaccination.....	6	8.2	0.2	0.2	1.0	2.0	3.0
Human sera after revaccination	5	6.2	0	0	0.6	1.0	2.6
Human serum about 40 years after vaccination	1	7	0	0	2	4	>4
Human placental sera	4	8.5	0	1.7	3.0	3.2	4.2
Pleural fluid, 21 days after primary vaccination. Titre of serum: 2^{-9}	1	10	1	1	2	2	3
Pleural fluids obtained many years after vaccination. Titres of sera: $2^{-3}-2^{-4}$	6	9.7	0.5	1.2	1.9	2.5	3.9
Pleural fluid. Non vaccinated baby. No antibody in serum	1	12	1	2	3	4	4
Ascitic fluids. Titres of sera: $2^{-3}-2^{-5}$	4	7	0	1	1.7	2	2.2
Cerebrospinal fluids. Titres of sera: $2^{-3}-2^{-5}$	3	5.7	0.4	1	1.7	2	3.7

The titres are expressed as neg. log. of dilutions. Heating was performed in a dilution of 1:8, excepted the cerebrospinal fluids, which were diluted in 1:2.

of placental sera collected from vaccinated mothers is much more sensitive to heat than that of immune sera, provided the inhibitory titre of the placental serum exceeds that of the maternal one.

The thermoresistance of undoubtedly non-specific inhibitors contained in body fluids is weaker than that of specific antibodies. Apart from occasional fluctuations within the limits of one degree of dilution, they are thermostable up to 56° C, but their titre goes down 1 to 2 degrees at 60° C,

while the reduction of the titre amounts to 2 to 3, in one case even to 4 degrees of dilution if the temperature rises to 70° C.

At 80° C the specific HA inhibiting antibodies contained in hyperimmune rabbit and in human sera are destroyed in ⅓ of the sera, the non-specific inhibitors in the body fluids about in half of the cases. A tempera-

Table 4. Effect of treatment with trypsin and kaolin on the HAI titre of immune rabbit serum and various body fluids

Test fluid	Trypsin treatment				Kaolin treatment	
	Diluent 1:1	Kept at 37° C for				
		0′	60′	120′	Before	After
Immune rabbit serum No. 306	Phosphate buffer	512	512	512	1024	1024
	1% trypsin solution	512	512	512		
	2% trypsin solution	512	512	512		
Pleural fluid No. 279	Phosphate buffer	512	512	256	1024	1024
	1% trypsin solution	512	256	512		
	2% trypsin solution	512	512	256		
Pleural fluid No. 310	Phosphate buffer	64	32	64	128	< 2
	1% trypsin solution	64	16	< 2		
	2% trypsin solution	4	4	2		
Ascites No. 311	Phosphate buffer	32	16	32	64	< 2
	1% trypsin solution	2	2	2		
	2% trypsin solution	4	4	2		
Cerebrospinal fluid No. 317	Phosphate buffer	16	16	16	32	< 2
	1% trypsin solution	2	< 2	2		
	2% trypsin solution	4	4	4		
Fluid of ovarian cyst No. 295	Phosphate buffer	32	64	32	64	< 2
	1% trypsin solution	32	16	16		
	2% trypsin solution	4	4	2		
Phosphate buffer (Control)	1% trypsin solution	< 2	< 2	< 2		
	2% trypsin solution	4	4	2		

ture of 85° C completely destroys both specific and non-specific inhibitors.

Data collected in Table 3 are showing no correlation between the thermoresistance of the non-specific inhibitors found in the body fluids and the content of HA inhibiting antibody of patients serum. This finding agrees with the results of our earlier experiments. We found that the titer of non-specific inhibitors in body fluids is independent of the titer of HA inhibiting antibodies in serum (21, 22, 23).

It is known that non-specific inhibitors against influenza haemagglutination, contained in normal and immune sera, can be removed by means of various chemical and physical treatments. Digestion by trypsin, for instance, destroys these substances completely or almost completely, while the specific antibodies are not, or hardly, affected (30—33). Kaolin, too, is well suited for the adsorption of non-specific inhibitors (30, 32). Led by these considerations, we studied the effect of trypsin and kaolin on non-specific inhibitors and specific antibodies affecting vaccinia haemagglutination. It is evident from Table 4 that neither a treatment with trypsin nor that with kaolin produce any effect on the HAI titre of hyperimmune rabbit sera. The level of specific antibodies seemed to be unchanged even after a digestion of 6 hours. Non-specific inhibitors, on the other hand, can be eliminated in most cases by means of both trypsin and kaolin, although these treatments proved sometimes ineffective in the case of fluids drawn from the pleural cavity of ovarian cysts. When treatment by trypsin or kaolin was ineffective, in some cases higher titres in others lower titres of HA inhibiting antibodies were found in the corresponding sera. The addition of 2 per cent trypsin produced immediate maximum effect without being kept at 37° C. In this concentration, trypsin itself sufficed to inhibit the haemagglutination of vaccinia virus in the two lowest dilutions.

Discussion

Apart from a lower resistance to heat, the examined properties of non-specific inhibitors contained in the various body fluids are—if untreated—quite similar to those of specific HAI antibodies. All haemagglutination inhibiting body fluids contain protein but there is no close parallelism between the grade of inhibition and the total protein content. As a rule, higher titres are associated with body fluids of higher protein content, save in the case of the fluid proceeding from ovarian cysts. Since our last publication (23), we have repeatedly had occasion to analyse fluids drawn from ovarian cysts and found striking discrepancies between inhibitory titre and the amount of total protein. While one sample of such fluids, containing 0,7 per cent of protein, produced inhibition at a titre of 1:32, another sample—with a protein level of 2.7 per cent—altogether failed to inhibit vaccinia haemagglutination. The body fluids arise in the course of pathological processes either owing to inflammation or congestion. Their property of non-specifically inhibiting vaccinia haemagglutination has presumably to be ascribed to proteins formed during the pathological process or to those protein-like substances which are precipitated with the globulins. The amount of these pathologically produced substances is variable, and the titre in which they are able to act as inhibitors depends on their amount. Most of these substances can be eliminated from the body fluids by means of a treatment with trypsin or kaolin, and it is only

exceptionally that they fail to be digested by trypsin or adsorbed to kaolin.

It sometimes happens that the serum has an unusually high HAI titre even a long time after vaccination (13). Presumably not only specific HAI antibodies but also non-specific inhibitors are involved in this phenomenon. This theory seems to be confirmed by a case (we had, unfortunately, but a single case of this kind) in which the serum of such a patient displayed a considerably lower thermoresistance than that obtained shortly after vaccination. It is, therefore, expedient to test the heat resistance of sera which are collected long after vaccination and show a conspicuously high HAI titre.

Summary

The properties of non-specific vaccinia-haemagglutination inhibitors are compared with those of specific haemagglutination-inhibiting antibodies. Non-specific inhibitors are contained in various human body fluids, viz. in cerebrospinal fluids with pathologically high protein contents, further in pleural, peritoneal and synovial fluids as also in the hydrocele of testis and sometimes in fluids drawn from ovarian cysts. As regards prompt onset of the reaction with haemagglutinins at different temperatures, there seems to be no difference between non-specific inhibitiors and specific HAI antibodies. Also non-specific inhibitors combine proportionately with haemagglutinins and are not adsorbed to the erythrocytes. They are less heat-resistant than, but require the same temperature for complete destruction as, specific inhibitors. Digestion by trypsin and adsorption to kaolin do not affect specific HAI antibodies, while non-specific inhibitors can in most cases be removed from the body fluids by these treatments.

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