THE COMPLEMENT-FIXATION REACTION IN EXPERIMENTAL VACCINIA

SPECIAL REFERENCE TO ANTIBODY-RESPONSE FOLLOWING THE PAUL TEST

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INTRODUCTION

For diagnosis of smallpox, the complement-fixation test, hemagglutination inhibition test, chick embryo-inoculation and the Paul test are the essential techniques nowadays (Parker, 1948; Smadel, 1948)[1)2)]. Recently (1951), Tokuda[3)] discussed many reports that had been published since Jobling[4)] on the complement-fixation reaction for diagnosis of smallpox and vaccinia, and he himself presented details of the benzene extracted testicular antigen applied to this test.

On the other hand, studies on the antibody-production following the injection of antigen directly into the cornea, such as the Paul test, have been very limited.

In view of these reports, the present experiments simply deal with the use of the chorioallantoic membranes infected with vaccinia virus as an antigen in the study of the complement-fixation test of vaccinia, using sera of rabbits immunized with the testicular strain of vaccinia. Further investigations were performed with these antigens to demonstrate the rise, persistence and decline of complement-fixing antibodies in the sera of rabbits that had been inoculated with testicular strains of vaccinia into their scarified corneas.

MATERIALS AND METTHODS

Hemolysin. Sera of rabbits immunized with goat's red cell were used. This hemolysin was inactivated in the water bath at 56°C for 30 minutes, and stored in the refrigerator added with glycerine in dilution of 50%.

Three units of hemolysin in 0.25 ml were used for the test.

Red Cell Suspension. A 3% of goat's red cell was prepared in saline after being washed 3 times and centrifuged at 2,000 rpm for 10 minutes at the last time. The sensitized red cell suspension was made mixing equal parts of a 3% suspension of goat's red cell and 3 units of the hemolysin in 0.25 ml and letting stand it at room temperature for 30 minutes before use.

Complement. The complement used in this work was a pooled fresh serum from at least 3 guinea pigs. After keeping it in the refrigerator overnight, the

complement was titrated with and without the antigens, and then 2 units of complement in the presence of the antigens were used in the test proper.

Antigen. Two kinds of antigens were prepared. (1) Coarse chorioallantoic membrane antigen. This antigen was made as follows: chorioallantoic membranes of chick embryos infected with vaccinia were harvested, weighed and ground in a mortar with sea sand.

A 10% suspension in sterile saline was made of these membranes. This suspension was spun at 2,000 rpm for 10 minutes. The supernatant fluid was employed as an antigen mostly to test preliminarily the antigen titer in shorter fixation. The chick embryo technique was that of Goodpasture and Buddingh (1948)[5].

A 10% suspension of infected chorioallantoic membrane in nutrient broth (pH 7.4) was inoculated onto the chorioallantois (0.2 ml) or into the chorioallantoic cavity (0.1 ml). (2) Supercentrifuged antigen. The coarse antigen was re-centrifuged in an angle head centrifuge at about 10,000 rpm for 30 minutes. The supernate was used as an antigen in longer fixation to test the convalescent sera of infected rabbits.

As controls 2 kinds of antigen from non-infected tissues were prepared in the same manner.

These antigens were stored in the refrigerator after merzonine was added in dilution of 1:10,000.

Serum. The Hyperimmune serum as positive control was made in the following way: rabbits were inoculated intratesticularly with 2 ml of a 10% suspension of testicular vaccinia virus in saline and on the 8th day after inoculation the same amount of the virus was injected intravenously. On the 7th day after this second inoculation, the rabbits were bled from the *carotis* to death. This hyperimmune serum was kept in the refrigerator after merzonine was added in dilution of 1 : 10,000.

Technique of the Complement-Fixation Reaction. A little modified technique described by Casals and Palacios (1941)[6], Tokuda (1951)[3], and Smadel (1948)[2] was employed. This was as follows: 0.25 ml of antigen, 0.25 ml of serum, and 0.5 ml of complement diluted to contain 2 units were incubated in the incubator at 37°C for 2.5 hours in the shorter fixation, in the refrigerator at 5°C for 16–18 hours in the longer fixation, and then the hemolytic system was added.

The total volume in each tube was 1.5 ml. The tubes were then incubated in a water bath at 37°C for 0.5 hour and the reaction was read. Complete hemolysis was expressed as 0, absence of hemolysis as 4, ±, 1, 2, and 3 indicated intermediate degrees of hemolysis. As controls, titration of complement and the hyperimmune serum were involved in the test. The titer of sera and antigens was taken as the last dilution giving 2 or better fixation.

Rabbits' sera were inactivated in a water bath at 60°C for 20 minutes except

in special cases.

In order to obtain the proper units of antigen and hyperimmune serum to be used in the test, the box-titration was carried out (see: Experimental).

Virus. The vaccinia virus used in this work was kindly delivered from Dr. Shimizu (Microbiological Institute, Faculty of Medicine, Kyoto University). This was received as the 8th rabbit testicular passage.

Virus Inoculation. The technique of virus inoculation into the scarified cornea was according to the method described by Parker (1948)[1], the intratesticular inoculation was that of Noguchi (1915)[7].

Virus Titration. This was carried out by intradermal titration on rabbits' backs according to Parker (1939)[8].

Eggs. Eleven- to thirteen-day-old embryonated eggs of white leghorn were used. These eggs were incubated in this laboratory from the beginning.

Experimental

Chorioallantoic Membrane Antigen

The testicular strain of vaccinia virus from Kyoto University which had been preserved in glycerine-saline for more than a half year, had titer of $10^{-3.07}$ (ID50). This was inoculated into a testicle of a rabbit. This new virus had ID50 of $10^{-7.0}$. A 10% suspension in nutrient broth (pH 7.4) from this newly passaged virus was inoculated into 4 embryonated eggs. After 3 days of incubation at 37°C, chorioallantoic membranes were harvested and examined for lesions. No lesions were seen on the first passaged membranes. These membranes were mixed and ground in a mortar, to make a 10% suspension in saline. A portion of this was preserved as a coarse antigen and the rest was employed as an inoculum to the next passage. Thus, 6 serial passages using embryonated eggs were made at 3 days' interval. On the 2nd passage, lesions made their first appearance on the inoculated sites of the membranes of a few eggs. After the 3rd passage, some eggs had one confluent and others many scattered lesions all over the surface of the membranes. More than 3 membranes chosen from each passage were mixed and coarse antigens were made of them. Antigen titration in shorter fixation with 1:16 diluted hyperimmune rabbits' serum was performed. The results are given in Table I. As shown in Table I, the antigen titer began to increase at the 3rd passage and attained a high level after the 5th passage.

Table I

Antigen titration by shorter fixation using rabbits' hyperimmune serum in dilution of 1:16

No. of passage	I	II	III	IV	V	VI
Antigen titer expressed as reciprocals	0	0	8*	<4	32	32

*A little anticomplementary.

After the 5th passage, the infected eggs regularly died on the 3rd to 4th day after the inoculation onto the chorioallantois, and on the 3rd to 7th day in the case of inoculation into the chorioallantoic cavity. After the 6th passage, no tendency to the elevation of an antigen titer was obtained (now 13th passage). From the infected eggs observed daily, those which were dead within two days were discarded, and eggs killed thereafter were examined for their lesions on the membranes. Then coarse antigens were made from membranes showing lesions. Such an antigen was titrated preliminarily in shorter fixation, for there were occasionally seen non-specific lesions. Thus, the membranes that gave an antigen-titer of more than 1 : 16 in shorter fixation were employed as an antigen in the test proper.

Table II (A) and Table III (A) gave examples of box-titration employing these antigens. Table II (A) showed a box-titration of a coarse antigen and Table III (A) that of super-centrifuged antigen made from the same infected membranes. Table II (B) and Table (III) (B) expressed the titration of complement used in these box-titrations respectively. As shown in Table II (A) and Table III (A), an antigen titer of the coarse antigen was a little higher than that of the super-centrifuged antigen, the coarse antigen, however, had a slight anti-complementary effect as given in Table II (B). On the other hand, the super-centrifuged antigen was not anticomplementary (Table III (B)).

Thus, the coarse antigen occasionally had an anti-complementary effect, therefore, in most cases, the super-centrifuged antigen was prepared.

TABLE II (A)

Box-titration of a coarse antigen made of vaccinia-infected chorioallantois of chick embryo using rabbits' hyperimmune serum inactivated at 60°C for 20 minutes by longer fixation at 5°C for 18 hours

Dilution of antigen	Original dilution of immune serum					
	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256
1 : 2	4	4	4	4	4	1
1 : 4	4	4	4	4	4	1
1 : 8	4	4	4	4	2	±
1 : 16	4	4	3	2	1	0
1 : 32	4	3	2	±	0	0
1 : 64	2	2	±	0	0	0

TABLE II (B)

Titration of complement incubated in the ice box along with the test proper

	Amounts (ml) of complement (1 : 18) in 1.5 ml						
	0.4	0.3	0.25	0.2	0.15	0.1	saline
In the presence of antigen	0	0	±	2	3	4	4
Saline	0	0	0	±	2	4	4

0=complete hemolysis, 4=no hemolysis, ±, 1, 2, 3=intermediate degrees

TABLE III (A)

Box-titration of a super-centrifuged antigen made of vaccinia-infected chorioallantois of chick embryo using rabbits' hyperimmune serum inactivated at 60°C for 20 minutes by longer fixation at 5°C for 18 hours

Dilution of antigen	Original dilution of immune serum					
	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256
1 : 2	4	4	4	4	3	1
1 : 4	4	4	4	4	2	±
1 : 8	4	4	4	2	±	0
1 : 16	4	4	2	0	0	0
1 : 32	2	2	0	0	0	0
1 : 64	0	0	0	0	0	0

TABLE III (B)

Titration of complement incubated in the ice box along with the test proper

	Amounts (ml) of complement (1 : 33) in 1.5 ml						
	0.4	0.3	0.25	0.2	0.15	0.1	saline
In the presence of antigen	0	0	0	2	4	4	4
Saline	0	0	0	2	4	4	4

Foot notes as in Table II

Furthermore, Table III (A) shows that to titrate an antigen-titer, a more than 1 : 16 dilution of serum was to be used, and more than a 1 : 4 dilution of antigen to titrate a serum-titer. Both antigens gave no non-specific reactions, thus the controls of this kind were omitted from the tables.

ANTIBODY RESPONSE IN RABBITS' SERA FOLLOWING THE PAUL TEST

By the use of these membranal antigens, complement-fixing antibodies in the sera of rabbits inoculated with testicular vaccinia virus into their scarified corneas were tested at intervals. Figure 1 was prepared using these data.

Complement-fixing antibodies in the sera of the infected rabbits began to appear between the 8th and 12th day, showed the highest levels on the 15th to 16th day, and persisted at significant titers until the 25th to 30th day with slightly declining curves.

The inoculum employed for the inoculation of R1 and R4 was 1,000 ID50 on the rabbit's back titration and that for R5, R7 and R10 was 10,000 ID 50. The inoculum was injected to the left eye of the rabbit according to the above mentioned technique. The right eye was dropped with the nutrient broth used in virus dilution in the same manner.

R7, as control, was inoculated intratesticularly with 1.0 ml amount of virus. When the virus was inoculated on the scarified cornea, the corneal reaction began to appear within 48 hours. After several days, it developed a severe kerato-con-

junctivitis and healed between 2 to 3 weeks after corneal injection.

Of 4 rabbits infected according to the Paul test, R1 and R5 showed severe symptoms, the former died on the 18th day after inoculation with the low level (1 : 4) of antibody-response on the 15th day, the latter, however, attained the highest level (1 : 256) of response with a sharp curve which seemed to be parallel with the corneal reactions. R4 and R10 had a relatively mild illness and their curves were somewhat similar to that of R7 (control).

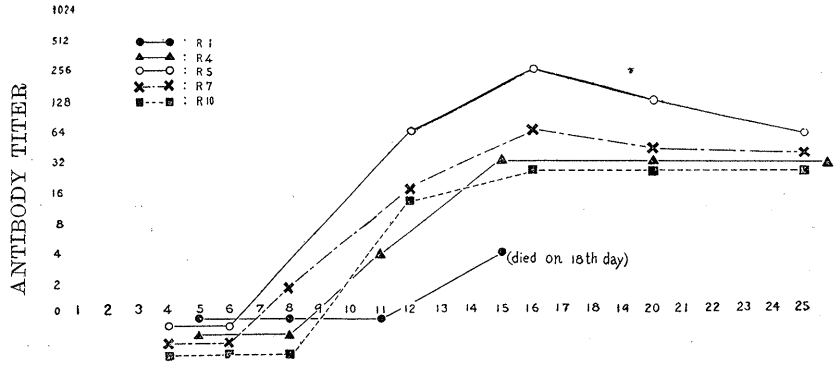

Fig. 1

Discussion

Recently, Thompson and Olson, (1951)[9] reported that when the ovalbumin was injected into the centre of the rabbits' cornea, antibodies began to appear in the serum at 192 hours following the injection. The two papers of Bolin, Anderson and Leymaster (1950)[10] and Bolin and Leymaster (1952)[11] dealt with the data that the injection of mumps virus into the guinea pig eye produced corneal opacity and that subsequent to the injection of this virus into the eye of guinea pigs, complement-fixing antibodies in the convalescent sera appeared between the 4th and 8th day, persisted at significant levels until the 20th to 24th day, and declined.

All these three papers reported the positive results of antibody production after the antigen was injected into the cornea directly.

Of many reports on the complement-fixation reaction in vaccinia (see Tokuda[3]), Parker (1934)[12] reported that there were great individual variations in regard to the time of appearance and the subsequent development of the complement-fixing antibodies, employing 8 rabbits inoculated with vaccinia intracutaneously or intratesticularly. By him, the first appearance of antibody was on the 8th to 15th day, the maximum titer in half of the cases was attained after 3 weeks, while the rest showed a gradual increase until the sixth week. According to Tokuda (1951)[3] complement-fixing antibodies appeared as early as on the 8th

day and reached the high titer on the 12th and 14th day after intratesticular or intracutaneous inoculations. In the author's experiments unexpected results were not obtained compared with these two reports. Only R5, in spite of its severe reaction of cornea, had a late appearing antibody of low level and died. As to such a case, Collier *et al.* (1950)[13] noted that patients of smallpox who died in the early eruptive stage showed a poor antibody-response as estimated by hem-agglutinin titration. And Tokuda (1951)[14], studying on the patients' sera of Japanese B encephalitis, stated that the positive rate of complement fixation tests at the patients who died of this disease was under 30% and that the titer of positive cases was of a low level.

Summary

When the embryonated eggs were infected with the testicular vaccinia virus, suitable antigens could be obtained from the chorioallantois after 5 passages.

A 10% suspension of the infected chorioallantois spun at 10,000 rpm for 30 minutes was no more anticomplementary as an antigen for use in antibody titration with sera of rabbits infected with the testicular vaccinia.

Using these antigens, the antibody-response in sera of rabbits that had been inoculated onto their scarified corneas with the testicular vaccinia according to the Paul test was estimated. Complement-fixng antibodies made their appearance within the 12th day, reached the highest levels on the 15th to 16th day, and persisted at significant titers until the 25th day following inoculation.

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