THE SEROLOGICAL RELATIONSHIP BETWEEN VACCINIA AND ECTROMELIA VIRUSES

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INTRODUCTION

The discovery of Burnet & Boake (1946) that ectromelia virus gave rise to a haemagglutinin closely resembling, but not identical with, that of vaccinia first suggested the relationship between these two viruses. These workers found that sera produced by immunization of calves, rabbits, mice and men against one or other virus showed specific inhibition of haemagglutination by both types of virus, the titres being usually higher against the homologous type. Rabbits surviving an intravenous ectromelia infection were immune to intradermal vaccinia, whilst mice given living vaccinia intravenously developed antibody against both and were resistant to at least 10^4 infective doses of ectromelia. Fenner (1947) reported that the Gillard strain of vaccinia given intranasally to mice gave a high degree of immunity against the Hampstead strain of ectromelia but not the more virulent Moscow strain; the immunity was correlated with the antihaemagglutinin titre and probably with multiplication of the vaccinia virus. He was so impressed by the relationship between vaccinia and ectromelia that he has called the latter 'mouse-pox'. Andrewes, Elford & Niven (1948) recently reported that vaccinia virus inactivated by ultra-violet irradiation immunized mice against ectromelia.

The object of the work reported in the present paper was to compare the antigens of vaccinia grown on rabbit skin and chick embryo with ectromelia from mouse liver and chick embryo. Elementary body suspensions prepared from these different sources were used to immunize rabbits and the serological reactions investigated.

No difference between the antigens of the same virus grown on different hosts was detected and there was a close relationship between ectromelia and vaccinia in respect to haemagglutination and complement fixation. Potent neutralizing sera were not obtained, even the vaccinia antiserum having only slight protective properties against the homologous antigen.

METHODS

Strains

The Salaman strain of vaccinia was passaged on rabbits' backs and the Hampstead strain of ectromelia was maintained by mouse passages. The vaccinia readily produced lesions on the chorio-allantoic membrane and for the preparation of the egg antigen, second egg passage material was used. Suspensions of mouse liver did not readily produce lesions on the chorio-allantois; only one out of four eggs gave a positive result and the membrane of this egg was used to start a series of passages. Third egg passage material was used for egg antigen; it gave distinct lesions on the chorio-allantoic membrane.

Titration of virus

Egg titrations were carried out by pock counts on the chorio-allantoic membranes of 10-day eggs, the inoculated eggs being incubated for a further 2 days in the case of vaccinia and 3 days in that of ectromelia, both at 35° C. For ectromelia titrations in mice 0·25 ml. amounts of broth dilutions were given intraperitoneally and survivors killed at 15 days. The presence of a typical enlarged fatty liver, as seen macroscopically on autopsy, was the criterion for ectromelia infection. In most cases this was confirmed by haemagglutination titrations on the freshly ground livers. Suspensions (10 %) of infected livers gave titres ranging from 16 to 256; tests on infected chorio-allantoic membranes gave similar titres. Fenner's (1947) method of titration of antihaemagglutinin of mouse sera to determine infection of mice killed at the end of the experiment was very useful when there was no macroscopic evidence of infection.

Preparations of antigens

All extractions were made in McIlvain's citric acid buffer, pH 7·2, diluted 1/50 with distilled water.

Vaccinia (rabbit-skin preparation). The method of Parker & Rivers (1935) was followed, using two washings at 5000 r.p.m. The elementary body (E.B.) suspension, fraction C, had a titre by a pock count, of about 10^5–10^6 per ml.

Vaccinia (egg preparation). A 10 % ground suspension of infected chorio-allantoic membranes was centrifuged at 2000 r.p.m. for 10 min., and the deposit washed in an equal volume of diluted citrate buffer. The supernates were pooled and recentrifuged. The final supernate was known as fraction A and all later fractions were made to correspond to this 5 % suspension. Fraction A was centrifuged at 5000 r.p.m. for 1 hr. and the supernate, fraction B, was kept for

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complement fixing antigen and haemagglutinin. The virus deposit was washed twice at 5000 r.p.m. for 1 hr. and the final suspension, made up to volume, was known as fraction C. By a pock count titration it had a titre of 10^4–10^5 per ml.

Ectromelia (mouse-liver preparation). This was similar to the vaccinia (egg) preparation but was complicated by the liver tissue and fat. The first deposit, after centrifuging fraction A, was made up to volume and clarified by treatment for 10 min. with 5 % acid-washed kieselguhr, which was removed by centrifugation at 2000 r.p.m. for 5 min.; a similar treatment was used in the preparation of rickettsial antigen by Fulton & Begg (1946). After two washings at 5000 r.p.m. the final fraction was known as fraction D. Kieselguhr treatment before the first 5000 r.p.m. stage was useless. In separating fraction B, the top layer of fat was avoided.

The preparation was examined at various stages under the dark ground microscope. Fraction D looked very similar to fraction C of vaccinia, with elementary bodies of fairly uniform size. The infectivity of fraction D was compared with that of the original fraction A by mouse titrations. Fraction A killed 3/3 mice at 10^{-4} and 1/3 at 10^{-6}; fraction D killed 3/3 mice at 10^{-2} and 1/3 at 10^{-4}. This drop was not considered excessive in view of the removal of much tissue debris in the kieselguhr preparation; a more potent preparation could easily be prepared by recentrifuging at 5000 r.p.m. and adding a smaller volume of buffer.

Ectromelia (egg preparation). This was prepared in the same way as the vaccinia (egg) preparation. Fraction C had a titre by pock count of 10^4–10^5 per ml.

Normal tissue antigens. Normal mouse liver and normal chorio-allantoic membranes were extracted in parallel with the infected tissues and used as controls. Faintly opalescent C fractions were obtained in both cases, which contrasted with the very turbid preparations from infected tissues.

Preservation of fractions

It is well known that vaccinia elementary body suspensions keep well in diluted citrated buffer at 4° C., and this method of preservation was at first used for the other preparations, with 10 % ether as a preservative. However, undiluted ectromelia fraction D from mouse liver was found to be non-infective for mice when kept at 4° C. for 2 weeks, whereas the corresponding egg preparation remained active. Fraction A of ectromelia mouse liver similarly became inactive within 2 weeks at + 4° C., but retained its infectivity for mice for at least 5 months when kept at − 20° C. Elementary body suspensions could not be kept frozen as visible aggregation had occurred when the preparation thawed. Moreover, on thawing frozen A and C fractions a deposit formed, which came down at low-speed centrifugation with almost total loss of the final virus activity. It was, therefore, necessary to prepare fresh ectromelia liver elementary bodies for each experiment. Fraction A of egg preparations of ectromelia kept its infectivity at − 20° C. for at least 3 months, and fractions A and C were infective after 3 months at + 4° C. although their titres had fallen.

Preparation of antisera

After initial bleedings for control sera, all rabbits received five injections at weekly intervals. The first vaccinia injections were given by skin scarification and all the ectromelia rabbits (two for egg preparations and one each for fractions C and D of mice preparations) received 0·5 ml. intravenously, the first at 1/10 and the others at full strength. Animals were bled 1 week after the last injection. Sera were stored at − 20° C.

Serological tests

All sera were inactivated at 56° C. for 20 min.

Neutralization tests. Equal volumes of antigen dilutions and serial twofold dilutions of antisera were held at room temperature for 1½ hr. and titrated either intraperitoneally in mice for ectromelia, or by intradermal titration of 0·2 ml. in rabbits' backs for vaccinia.

Elementary body agglutination titrations. 0·2 ml. elementary body suspensions, in diluted McIlvain's buffer, were added to 0·2 ml. sera dilutions in saline; tests were read after 24 hr. incubation at 56° C.

Haemagglutinin and antihaemagglutinin titrations were carried out by the method of Salk (1944), using 0·5 % fowl cells.

If liver fractions were kept for a few days at + 4° or − 20° C. they were unsatisfactory for haemagglutination tests because they haemolysed fowl cells. Normal liver preparations on keeping behaved similarly. Titrations were therefore always made on fresh material. For antihaemagglutinin titrations, sera were serially diluted in 0·5 % fowl cells and four haemagglutinating units of ectromelia egg fraction B added. All antisera prepared from egg membrane preparations possessed agglutinins for normal fowl cells to titres of 1/320 and were therefore diluted 1/5 and absorbed with an equal volume of packed fowl cells prior to use. Pre-immunization sera failed to agglutinate fowl cells at 1/20.

Complement-fixation tests. 0·25 ml. sera dilutions, 0·25 ml. antigen and 0·25 ml. complement containing either 2·0 or 2·5 units of complement, were mixed. After overnight fixation at 4° C., 0·5 ml. of sensitized sheep cells was added (0·25 ml. of 5 % sheep cells plus 0·25 ml. haemolysin equivalent to 2½ units).

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Tests were read after 1 hr. incubation at 37° C. As a result of preliminary tests, two antigen strengths, 1/10 and 1/15, were employed, but some B fractions were anticomplementary at 1/10. Antigen, sera and normal tissue controls, according to the source of the antigen on test, were included in every experiment. Four complete separate tests were made testing each antigen against each antiserum, one with 2·0 units of complement and three with 2·5 units of complement.

RESULTS

In all tests a minus sign means a negative result at the stated dilution.

Antihaemagglutinin titrations. Fractions A and B were used as haemagglutinins in the test. Table 1 shows that haemagglutination by both viruses was inhibited by antivaccinia sera, but that antiectromelia sera were without effect on either haemagglutinin.

Table 1. *Haemagglutination tests*

	Serum dilution neutralizing 2 units haemagglutinin of		
Antisera to elementary bodies from	Vaccinia egg/A	Vaccinia rabbit/B	Ectromelia egg/B
Vaccinia (rabbit)	2560	2560	320
Vaccinia (egg)	1280	1280	320
Ectromelia (mouse/C)	−40	−40	−40
Ectromelia (mouse/D)	−40	−40	−40
Ectromelia (egg)	−40	−40	−40
Ectromelia (egg)	−40	−40	−40
Normal sera control	−40	−40	−40

Titres shown are reciprocals of dilutions of antiserum showing complete inhibition of haemagglutination in a Salk test incubated for 1½ hr. at room temperature.

Elementary body agglutination titrations. Results are given in Table 2 for antigen opacities of approximately 500×10^6 *Bact. coli* per ml. Heavier suspensions were agglutinated by normal sera. Vaccinia E.B.'s were agglutinated by antivaccinia sera but not by antiectromelia sera. Ectromelia E.B.'S were not agglutinated significantly by any sera.

Complement fixation tests. The results of one complete test are given in Table 3. The other three tests gave essentially the same results. It was found that 2·5 units of complement gave more clear cut results than 2·0 units.

Neutralization tests. Results of neutralization tests against ectromelia by the mouse-protection test and against vaccinia by skin titrations are recorded in Table 4. All the control ectromelia mice had typical symptoms of ectromelia. Of the mice which had been given immune sera, all died, but none showed macroscopic evidence of ectromelia infection and all their liver haemagglutinations were negative at 1/10. Their kidneys were, however, very enlarged, as Marchal (1930) found to be the case in ectromelia. Moreover, the sera of all animals dying after 12 days

Table 2. *Elementary body agglutination tests*

	Agglutination titre against elementary bodies from			
Antisera to elementary bodies from	Vaccinia (rabbit)	Vaccinia (egg)	Ectromelia (mouse)	Ectromelia (egg)
Vaccinia (rabbit)	160	160	20	20
Vaccinia (egg)	160	160	±20	±20
Ectromelia (mouse/C)	20	20	20	20
Ectromelia (mouse/D)	−20	−20	−20	−20
Ectromelia (egg)	20	20	−20	−20
Ectromelia (egg)	±20	−20	20	20
Normal sera control	−20	−20	−20	−20

Titres shown are reciprocals of highest dilutions showing any agglutination after incubation for 24 hr. at 56° C.

Table 3. *Complement-fixation tests*

	Antisera to elementary bodies from			
Antigen	Vaccinia (rabbit) L4	Vaccinia (egg) L12	Ectromelia (mouse) L14	Ectromelia (egg) L15
Saline	−20	40	20	20
Vaccinia (rabbit/C)	320	320	320	320
Vaccinia (rabbit/B)	160	160	40	20
Vaccinia (egg/C)	320	640	320	—
Vaccinia (egg/B)	160	160	80	—
Ectromelia (egg/C)	160	80	160	—
Ectromelia (egg/B)	80	160	80	—
Normal (egg/C)	−20	40	20	40
Normal (egg/B)	−20	40	20	40
Ectromelia (liver/D)	160	320	320	320
Ectromelia (liver/B)	80	160	160	40
Normal (liver/D)	20	40	320	20
Normal (liver/B)	20	40	80	40

Antigen controls with and without 1/20 normal sera: fractions C and D (=elementary bodies)—satisfactory at 1/10 dilution of antigen; fraction B (=6000 r.p.m. supernate)—satisfactory at 1/15 dilution for mouse liver and ectromelia; egg (B fractions) were anticomplementary at 1/10. Titres shown are reciprocals of highest serum dilution showing complete fixation of 2·5 units of complement.

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showed antihaemagglutinin titres of between 40 and 320, so that ectromelia infection had occurred. Before death, the mice were very thin and some had a crouching gait and tremors, symptoms which did not occur in the control mice of the neutralization experiments nor in other infected mice.

An attempt to demonstrate a skin lesion in rabbits inoculated with ectromelia intradermally was negative in three out of four rabbits, but the fourth rabbit, which received undiluted fraction A of an egg preparation, developed a lesion resembling that of vaccinia and on this rabbit undiluted fractions C and D injected into the skin produced local indurations. Ectromelia skin titrations on rabbits were therefore not attempted.

there has been active virus multiplication and tissue damage (Fenner, 1947; Chu, 1948).

Complement fixation with antivaccinia sera revealed a similar relationship between the two viruses, higher titres being recorded for the vaccinia virus. Results with the same virus grown on different hosts were not significantly different. This was most apparent in the case of antivaccinial serum prepared from the rabbit skin elementary body preparation, which could contain no antibody to egg or mouse tissues. All four antiectromelia sera were obviously of low antibody content as opposed to the vaccinia antisera. This was probably due to the fact that the vaccinia multiplied in the rabbit, whereas the ectromelia did not. However, complement fixation

Table 4. *Neutralization of vaccinia and ectromelia viruses*

	Vaccinia antigen (severity of skin reaction at stated serum dilution)				Ectromelia antigen (time of death in days)	
	Antigen dilution					
Antisera	10^{-1}	10^{-2}	10^{-3}	10^{-4}	1000 M.L.D. virus serum 1/20	100 M.L.D. virus serum 1/2
Control	++++ 1/2	+++ 1/2	++ 1/2	+ 1/2	5, 7, 7	6, 8
Vaccinia (rabbit)	++++ 1/2	− 1/10	− 1/10	− 1/20	8, 9, 9	13, 13
	—	+++ 1/20	++ 1/20	—	—	—
Ectromelia (mouse)	++++ 1/2	+++ 1/2	+ 1/2	− 1/2	7, 8, 9	—
Ectromelia (egg)	++++ 1/2	+++ 1/2	++ 1/2	− 1/2	5, 7, 9	11, 12
Ectromelia immune sera from mice	—	—	—	—	12, 12	—

DISCUSSION

The results of the serological tests confirm the close relationship between vaccinia and ectromelia viruses. Burnet & Boake (1946) noted the relationship between the haemagglutinins of these, viruses and their results with rabbit antivaccinia sera have been confirmed. These workers also found antihaemagglutinin in rabbits infected intravenously with ectromelia in the form of a crude egg supernate of the Hampstead strain. Two of the rabbits died and positive haemagglutination tests were given by liver and spleen extracts. In the work reported here, rabbits inoculated intravenously with elementary body suspensions showed no evidence of infection. No antihaemagglutinin was detected when the normal fowl cell agglutinins had been adsorbed from the sera prepared from egg material. Furthermore, only one of four rabbits gave a skin reaction with undiluted crude ectromelia egg supernate, whereas Burnet & Boake found definite reactions in such tests. Thus, in contrast to the results of these workers, I have found no evidence of multiplication of the ectromelia virus in rabbits, which would explain the failure of antihaemagglutinin formation in rabbits, as opposed to mice, if one assumes that vaccinia and ectromelia haemagglutinins are only produced when

results probably indicated some specific fixation by ectromelia and vaccinia elementary bodies with antiectromelia sera although there was considerable fixation due to normal mouse-liver antigen. Fraction B of all preparations failed to fix complement significantly with any ectromelia antisera, as might be expected since rabbits were inoculated with washed elementary bodies only.

Ectromelia E.B.'s were not agglutinated by antiectromelia or antivaccinia sera to significant titres, and vaccinia E.B.'s were only agglutinated by the antivaccinia sera. E.B. agglutination therefore gave no evidence of an antigenic relationship between vaccinia and ectromelia viruses. It is possible that the antigens responsible for E.B. agglutination are different from those concerned with complement fixation and haemagglutinin inhibition, but it may well be that the ectromelia E.B. suspensions used were inagglutinable for reasons unconnected with their antigenic structure. As judged by microscopic appearance and infectivity tests, the ectromelia E.B. suspensions were comparable to those of vaccinia, so that the question of their specific agglutinability remains unsettled. The unsatisfactory behaviour of the antiectromelia sera prevented further investigation of this point.

In the case of neutralizing antibody, the antivaccinial sera diluted 1/10 suppressed a skin reaction

to vaccinia antigen dilutions of 10^{-2}, 10^{-3} and 10^{-4}; the 10^{-4} dilution gave only a slight reaction with a normal serum control. The ectromelia antiserum was ineffective at 1/2. The mouse-protection tests against approximately 100 M.L.D. of ectromelia showed a greater survival period for the mice given vaccinia or ectromelia antisera, but there was no absolute protection and in view of the few mice used, the prolongation of life may not be significant. Even mouse immune sera did not protect against a challenge dose of 1000 M.L.D. The absence of macroscopic liver infection and the enlarged kidneys in all those mice which had received virus plus immune serum was very striking, especially as the livers were still infective to normal mice. Andrewes *et al.* (1948) noted a similar picture in mice which had received vaccinia virus plus ectromelia virus. Their results suggest that, in my experiments, there was some protection afforded by the antisera. My results support their explanation of the protective effect of vaccinia virus as due to orthodox immunity rather than to interference.

The instability of ectromelia elementary body suspensions prepared from mouse livers, compared with those from eggs or with those of vaccinia is interesting. It does not appear to be due to aggregation at 4° C. as, although a few flaky clumps sometimes developed in fraction D, the suspension remained macroscopically as stable as vaccinia in the diluted citrate buffer. Data concerning the stability of vaccinial liver suspensions are not available and are now being sought experimentally.

SUMMARY

1. Elementary body suspensions of vaccinia and ectromelia were obtained from rabbit skin and chick embryo or mouse liver and chick embryo respectively and used to immunize rabbits.

2. Judged by the complement fixation and haemagglutination tests, the viruses from different hosts were very similar antigenically. Ectromelia and vaccinia appeared closely related, the heterologous virus titres being usually lower than the homologous.

3. Elementary body suspension agglutination tests did not reveal a similar relationship between the two viruses, but were not satisfactory on account of the low antibody content of the antiectromelia sera.

4. Highly active ectromelia antisera were not obtained and there was no evidence of multiplication of this virus in rabbits.

5. Only slight neutralizing activity was demonstrated in the antisera.

6. Elementary body suspensions of ectromelia virus prepared from mouse livers, as well as the crude liver extracts, were soon inactivated. Egg membrane preparations were relatively stable.

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