THE VIRUSES OF VARIOLA, VACCINIA, COWPOX AND ECTROMELIA.—NEUTRALIZATION TESTS ON THE CHORIO-ALLANTOIS WITH UNABSORBED AND ABSORBED IMMUNE SERA.

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IN a previous paper we recorded the results of neutralization tests with the viruses of variola, vaccinia, cowpox and ectromelia and corresponding immune sera (McCarthy and Downie, 1948). Four strains of variola, two strains each of vaccinia and cowpox and one strain each of alastrim and ectromelia viruses were examined. Immune sera from immunized fowls and rabbits and from man were used in neutralization tests made by inoculating serum-virus mixtures on the chorio-allantois of chick embryos or into the skin of rabbits. In the tests on the chorio-allantois, undiluted immune sera prepared against each of the virus strains was tested against a constant amount of virus, and in the rabbit skin constant amounts of sera were tested against varying amounts of cowpox and vaccinia viruses. These cross-neutralization tests showed the close relationship of the strains studied and failed to demonstrate distinct differences among them. In an attempt to disclose such differences we have made an estimate of the neutralizing antibody in immune sera by testing five-fold dilutions of the sera against homologous and heterologous viruses. In this extension of the previous work only immune sera prepared in fowls have been studied and all neutralization tests have been made on the chorio-allantois of chick embryos. In addition, we have carried out absorption of immune fowl sera with virus suspensions and tested the absorbed sera for neutralizing power.

MATERIAL AND METHODS.

The strains of virus and the immune fowl sera were those described in the previous paper. With the exception of one vaccinia and one cowpox strain used for the absorption of sera, only strains propagated on the chorio-allantois were studied in the present experiments.

Absorption of sera.

The immune sera prepared in fowls against cowpox, vaccinia, ectromelia and one strain of variola were absorped with virus suspensions. The absorbing suspensions of cowpox and vaccinia viruses were prepared from dermal infections in the rabbit with strains which had been propagated only in this animal, as by this method relatively pure and concentrated suspensions more suitable for absorption of sera were readily obtained. The absorbing suspensions of ectromelia and variola were prepared from infected chorio-allantoic membranes.

A suspension of vaccinia elementary bodies representing the yield from two rabbits was divided into two parts. One was equally distributed in five centrifuge tubes which were spun in an angle centrifuge at high speed to deposit the virus. After the supernatant fluids had been discarded 1·5 ml. of immune fowl serum was added to four tubes, one serum to each tube, and normal fowl serum to the fifth. The virus deposit was resuspended in the serum and the tubes left overnight in the refrigerator (2–4° C.). Next day the virus was deposited by centrifugation, and the supernatant fluids removed and treated in the same way with the virus from the remaining part of the original suspension. After the second absorption the mixtures were spun at high speed for 1½ hours and the supernatant absorbed sera removed and heated at 57° C. for 30 minutes to inactivate any virus remaining in the serum. Similar volumes of sera were absorbed with cowpox elementary body suspension from infected rabbits in a similar manner. The treatment resulted in only slight dilution of the sera.

The ectromelia virus suspension used for absorption was prepared by differential centrifugation from 30 infected chorio-allantoic membranes. The same five sera were absorbed as before in two stages, but because of the smaller amount of virus available only 0·1 ml. of each serum (diluted to 1·0 ml.) was used, and the volume was finally made up to 1·5 ml. with saline, so that the final dilution of absorbed serum was 1 in 15. In the same way variola virus from 30 membranes was used to absorb 0·1 ml. of each of the five sera. Samples of each serum were similarly treated with egg-grown herpes simplex virus. Absorption with this virus did not reduce the titre of any of the sera for variola virus which was the only virus-tested.

Technique of neutralization tests with absorbed and unabsorbed sera.

The sera which had been absorbed with vaccinia and with cowpox viruses were tested together with the corresponding unabsorbed sera against one virus at a time. The test virus suspension had been prepared from infected chorio-allantoic membranes and titrated for virus content. The dilution of virus suspension used was such that the mixture with normal serum produced between 50 and 200 lesions per egg ; 0·3 ml. of virus dilution was added to 0·3 ml. of each serum, and after the mixtures had been left at room temperature for 30 to 40 minutes each was distributed on the chorio-allantois of five or six 12-day embryos. In a similar manner the unabsorbed sera diluted 1 in 15 and the sera absorbed with variola and ectromelia were tested together against one virus at a time. Inoculated eggs were opened after 3 days, the membranes excised and placed in formol saline for the counting of lesions. In recording the results the average number of lesions per egg for any one serum-virus mixture has been expressed as a percentage of the average number of lesions produced by the same virus mixed with unabsorbed or absorbed normal fowl serum.

RESULTS.

I. *Neutralization of Viruses by Dilutions of Homologous and Heterologous Immune Fowl Sera.*

In each experiment the four sera, in five-fold dilutions, were tested simultaneously against one virus. To the dilutions of sera the virus suspension was added at intervals so that thirty minutes should elapse before the inoculation of each

mixture into eggs. Five or six 12-day embryos were inoculated with each mixture. The results of the tests with four viruses are shown in Tables I to IV.

Table I shows that all four sera neutralized cowpox virus to fairly high dilution, the variola antiserum being the strongest. Vaccinia virus (Table II) was in-

TABLE I.—*Neutralization of Cowpox Virus by Immune Fowl Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Serum dilutions.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
1/10	6	3	1	2
1/50	4	5	5	1
1/250	17	5	7	4
1/1250	50	47	40	19
1/6250	58	76	64	56

Figures in Tables I to IV and VI and VII represent average number of lesions on 5 to 6 eggs expressed as a percentage of average numbers of lesions on 5 to 6 eggs inoculated with the same amount of virus and normal fowl serum.

TABLE II.—*Neutralization of Vaccinia Virus by Immune Fowl Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Serum dilutions.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
1/10	11	3	11	3
1/50	33	5	14	6
1/250	59	7	14	7
1/1250	74	11	57	15
1/6250	—	56	—	54

TABLE III.—*Neutralization of Ectromelia Virus by Immune Fowl Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Serum dilutions.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
1/10	—	—	3	—
1/50	27	7	4	7
1/250	100	19	15	15
1/1250	93	84	70	22
1/6250	85	90	114	79

TABLE IV.—*Neutralization of Variola Virus by Immune Fowl Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Serum dilutions.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
1/10	34	8	14	1
1/50	117	10	15	3
1/250	96	21	87	5
1/1250	82	66	109	13
1/6250	—	112	—	42

hibited by high dilutions of variola and vaccinial antiserum, moderately well by ectromelia and poorly by cowpox antiserum. Against ectromelia virus the variola antiserum was again the most potent (Table III), vaccinia and ectromelia antisera were rather weaker while cowpox was relatively poor in neutralizing antibody. Against variola virus (Table IV) the order of potency of the sera was variola > vaccinia > ectromelia > cowpox. To obtain a measure of the relative strengths of the antisera, graphs were drawn on log-paper of the results shown in Tables I to IV, the percentage neutralization being plotted against the serum dilutions. The serum dilutions which reduced the number of lesions to 33·3 per cent of the controls are shown in Table V as a measure of serum titres.

It appears from Table V that the cowpox antiserum is relatively weak but specific in its neutralizing activity. The vaccinial antiserum is also relatively

TABLE V.—*Neutralizing Titres of Immune Fowl Sera, Taking as End Point the Dilution of Serum giving* 33·3 *per cent of the Lesion Count obtained with Normal Serum-Virus Mixture.*

Virus.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
Cowpox	660	890	1020	2750
Vaccinia	49	3760	660	3350
Ectromelia	63	470	560	2060
Variola	9	460	102	4470

specific but is much more potent. The ectromelia antiserum neutralizes cowpox rather better than it neutralizes the homologous virus although it is less effective against variola virus. The variola antiserum appears to be the strongest but is not very specific, the titre of antibody for the other three viruses being relatively high.

In previous neutralization tests by the chorio-allantoic inoculation technique these immune fowl sera were tested undiluted but specific differences were not demonstrated (McCarthy and Downie, 1948). It is apparent, however, that tests of the sera in five-fold dilutions provide a better measure of their antibody content and elicit differences not detectable by the use of undiluted serum. Burnet, Keogh and Lush (1937) suggested that differences of neutralizing activity for vaccinia virus between weak and strong rabbit antisera might be more apparent in higher than in lower serum concentrations. However, with our relatively weak immune sera prepared in fowls differences in neutralizing activity against related viruses were more readily demonstrable in higher serum dilutions.

II. *Cross Absorption Experiments.*

The immune fowl sera absorbed with the four viruses, as described under methods, were tested for neutralizing activity in a single concentration. More accurate assessment of the effects of absorption might have been obtained if each absorbed serum had been titrated for neutralizing antibody by the dilution method. This would have entailed a much greater expenditure on materials and it seems doubtful whether the results would have justified the labour involved. The results of neutralization tests with unabsorbed and absorbed sera are shown in Tables VI and VII. The figures again represent the average number of lesion on five or six membranes expressed as a percentage of the average number on

TABLE VI.—*Neutralization Tests with Unabsorbed and Absorbed Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Antisera.	Infecting virus.														
	Cowpox.			Vaccinia.			Ectromelia.				Variola.				
	Virus used for absorption of sera.														
	None.	Cowpox.	Vaccinia.	None.	Cowpox.	Vaccinia.	None.	Cowpox.	Vaccinia.		None.	Cowpox.	Vaccinia.		
Cowpox	10	48	26	5	112	80	6	60	43		2	83	74		
Vaccinia	14	54	75	7	9	31	9	6	48		5	15	80		
Ectromelia	10	99	28	10	63	44	8	11	5		10	29	37		
Variola	20	32	31	5	7	36	8	12	8		4	5	6		

TABLE VII.—*Neutralization Tests with Unabsorbed and Absorbed Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Antisera.	Infecting virus.														
	Cowpox.			Vaccinia.			Ectromelia.				Variola.				
	Virus used for absorption of sera.														
	None.	Ectro-melia.	Variola.				None.	Ectro-melia.	Variola.		None.	Ectro-melia.	Variola.		
Cowpox 1/15	9	60	36				4	65	24		25	103	84		
Vaccinia 1/15	15	33	40		Not tested		1	19	14		6	15	91		
Ectromelia 1/15	6	82	23				1	101	6		3	124	127		
Variola 1/15	13	20	77				7	30	97		5	6	47		

membranes receiving the same amount of virus mixed with normal fowl serum. In Table VI are shown the findings in four separate experiments, all sera being tested against one virus at a time ; similarly the results of testing the sera absorb with variola and ectromelia viruses are given in Table VII. The neutralizing activity of the various sera was also tested against alastrim virus, but as the results did not differ materially from those with variola virus they have not been included.

In considering Tables VI and VII the neutralizing power of the unabsorbed sera as shown in Table V should be kept in mind. It may be noted that, in general, absorption of a serum by homologous virus reduced the neutralizing activity of the serum for both homologous and heterologous viruses and that each virus absorbed antibodies for itself from all sera. Quantitative differences in the degree of absorption in different experiments are apparent but these are difficult to analyse because of the variation in antibody content of the individual sera. We have abstracted from Tables VI and VII the figures which show the effect of absorption with the four viruses on the antibody content of each serum for its homologous virus. These are shown in Table VIII, figures in the left half being

TABLE VIII.—*Absorption by Various Viruses of Antibody to Virus used to Prepare the Sera—Data from Tables VI and VII.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Antisera.	Virus used for absorption of sera.					
	None.	Cowpox.	Vaccinia.	None.	Ectromelia.	Variola.
Cowpox . .	10	*48*	26	9	60	36
Vaccinia . .	7	9	*31*		not tested	
Ectromelia . .	8	11	5	1	*101*	6
Variola . . .	4	5	6	5	6	*47*

Figures indicate percentage " survival " of virus homologous to serum before and after absorption.

extracted from Table VI and in the right half from Table VII. The effects of absorption on the antibody content of the sera for their homologous viruses, with the exception of cowpox serum, show that significant reduction was achieved by absorption only with the homologous virus. This suggests a specificity of antibody not demonstrable by neutralization tests with undiluted unabsorbed sera. Cowpox antibody was partially removed from cowpox antiserum by absorption with all four viruses. The effects of absorption with ectromelia and variola viruses are not, however, strictly comparable with those due to absorption with cowpox and vaccinia as the serum absorbed by the first two was diluted 1 in 15 and less virus was used. Moreover, as shown in Table V the titre of the cowpox serum for cowpox virus was less than the titres of antivaccinial and antivariola serum for their homologous viruses.

From the data in Table VI and VII we attempted to analyse the results of reciprocal absorption of pairs of antisera with their respective viruses but in view of the differences in antibody content of the unabsorbed sera it is difficult to interpret the results. However, there is no evidence of complete reciprocal cross absorption between any pair of these viruses and their antisera, and it seems that no two of them are antigenically identical.

DISCUSSION.

When the neutralizing power of immune sera was estimated by testing serum dilutions against constant amounts of virus differences between the sera were detected which had not been shown in tests with undiluted sera (McCarthy and Downie, 1948). With the exception of the ectromelia antiserum each immune serum neutralized the homologous virus to higher titre than it neutralized heterologous virus. Absorption tests confirmed these findings and also demonstrated a difference between ectromelia virus and other members of the group. With the exception of the cowpox serum, which was relatively weak, antibodies to the virus homologous to each serum were considerably reduced only by absorption with that virus.

The close antigenic relationship of ectromelia virus to other members of the group, first observed by Burnet and Boake (1946), has been again confirmed by the present experiments. Downie and Macdonald (1950) using the same immune sera and soluble antigens (LS) from these viruses in inhibition of complement fixation tests found that ectromelia was more closely related to cowpox than it was to vaccinia or smallpox. The same association is shown less clearly by neutralization tests with absorbed and unabsorbed sera. Previous work with vaccinia has shown that the neutralizing power of antivaccinial sera does not run parallel with *in vitro* reactivity with LS antigen (Parker and Rivers, 1936; Salaman, 1937) and therefore the lack of complete correlation of the results of *in vitro* tests involving LS antigens and neutralization tests is not surprising. The immunological differences between cowpox and vaccinia indicated by the present results are in accord with the results of neutralization tests made in rabbits with absorbed sera (Downie, 1939). Differences between vaccinia and variola were not demonstrable by the inhibition of complement fixation test using fowl immune sera and LS antigens from chick embryos or other animal sources (Downie and Macdonald, 1950). The present experiments, however, demonstrate minor antigenic differences between the strains studied in that the titre of neutralizing antibody was higher for the homologous virus; and absorption with vaccinia virus failed to reduce significantly the antibody for variola virus in variola antiserum. The relationship of alastrim to variola virus was not examined in such detail; all the immune fowl sera were, however, tested for neutralizing activity against alastrim before and after absorption with vaccinia and with cowpox virus. Although these viruses removed antibody for alastrim from cowpox, vaccinia and ectromelia immune sera they did not appreciably affect the neutralizing power of variola antiserum for alastrim virus. These findings, as far as they go, do not suggest antigenic difference between the alastrim and the variola strains examined. On the basis of their results in cross immunity experiments in monkeys with the viruses of vaccinia, variola and alastrim, Horgan and Haseeb (1939) put forward the view that vaccinia virus is a degraded antigenic variant of variola and that variola virus possesses, in addition to a basic antigen shared by vaccinia, a specific antigen the presence of which is associated with a high degree of virulence for man. Our own observations suggest that there are minor antigenic differences between the viruses of vaccinia and variola, but whether this antigenic difference determines the infectivity and virulence for different animal hosts is a highly speculative question on which we have no information.

It may be noted that although the techniques used have served to indicate minor antigenic differences in this group these differences are no greater than those observed among different strains of some other viruses, e.g. influenza A. It is known that influenza viruses become altered in their immunological characters on propagation in the laboratory but we have had as yet no convincing evidence of such alteration in the strains of virus under study.

SUMMARY.

A comparative serological study has been made of the viruses of ectromelia, cowpox, vaccinia and variola by means of neutralization tests on the chick chorio-allantois using virus strains propagated in this tissue and immune sera prepared in fowls.

Although cross neutralization tests using undiluted sera had failed to distinguish these viruses from each other, differences were revealed by titrating each serum for neutralizing activity against each virus strain. Differences were also demonstrable by the use of absorbed sera for neutralization tests. There was no complete cross absorption between any pair of these viruses and their antisera and it seems that no two of them are antigenically identical. Our experiments failed to differentiate the viruses of variola and alastrim.

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