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A comparison of the antigens present on the surface of virus released artificially from chick cells infected with vaccinia virus, and cowpox virus and its white pock mutant

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SUMMARY

Antisera prepared against vaccinia and cowpox viruses were absorbed with purified suspensions of vaccinia virus, red cowpox and white cowpox viruses. They were then tested for their ability to neutralize the viruses, and to precipitate the virus soluble antigens.

The results showed that some virus specific antigens were not virus surface components and that some components were present on the surface of all three viruses. However, certain components were detected on the surface of vaccinia virus but not on the surface of cowpox virus, and vice versa. Some evidence for the existence of a vaccinia-specific surface component was also obtained.

Comparisons between results of cross-neutralization tests and immunodiffusion tests on the absorbed sera indicated that antibody to a number of antigens, including the classical LS, and the cowpox-specific *d* antigen play no part in the process of poxvirus neutralization.

INTRODUCTION

Despite extensive studies on the serological and immunological properties of the members of the variola-vaccinia subgroup of poxviruses, their antigenic make-up is still not clear. Downie (1939*a*) and Downie & McCarthy (1950) studied these viruses using absorbed antisera. Considerable cross-reaction was found by cross-neutralization tests but the absorption of anti-vaccinia serum with cowpox virus did not reduce its specificity for vaccinia. More recently, Madeley (1968) and McNeill (1968) have detected immunological differences between vaccinia and cowpox viruses but the antigens involved have not been identified. Although any differences between these viruses must be minor when compared to the overall cross-reaction between them, such differences are of interest. Their elucidation would contribute to our knowledge of these viruses, and might in part explain their different cell tropisms and intracellular growth patterns.

The LS antigen of vaccinia is present on the surface of that virus (Craigie & Wishart, 1934, 1936; Cohen & Wilcox, 1966, 1968). However, anti-vaccinia serum absorbed with LS still agglutinates vaccinia virus, indicating that there are

additional surface antigens, the so-called 'X-agglutinogens' (Craigie & Wishart, 1938; Smadel & Shedlovsky, 1942).

Rondle & Dumbell (1962) described a cowpox-soluble antigen *d* which was absent from the white pock mutant of cowpox and possibly from vaccinia. They suggested that Downie's results (1939*a*) could be explained by postulating that *d* and LS were major surface components of red cowpox and vaccinia viruses respectively. These assumptions are tested in the present work. An attempt has also been made to determine which antigenic components of those described by Baxby & Rondle (1968) are involved in virus neutralization.

As well as comparing vaccinia and cowpox viruses the white pock mutant of cowpox was also studied, since apart from Haddock's (1952) preliminary finding that some cross-neutralization occurred between the three viruses nothing further is known about the immunological relationship of white cowpox to its parent, or to vaccinia virus.

The principal methods used have been to prepare antisera to the viruses and to absorb them with purified virus suspensions. The antisera were then tested for residual antibody by cross-neutralization tests and by immuno-diffusion.

After the antisera had been absorbed with virus artificially released from infected cells (intracellular virus), Appleyard, Hapel & Boulter (1971) and Turner & Squires (1971) reported that vaccinia virus released naturally from infected cells (extracellular virus) possessed an immunogenic component not present in virus released artificially. They also suggested that the extracellular forms of virus were more important for detecting protective antibody than the intracellular forms studied in the present paper.

MATERIALS AND METHODS

Virus strains

The Lister Institute strain of vaccinia virus, the Brighton strain of red cowpox and a white pock mutant isolated from Brighton cowpox were used primarily. Some cross-neutralization tests were done with the 61 (Gispen, 1955) and Carmarthen strains of cowpox and their white pock mutants, and the Tashkent and Connaught Laboratory strains of vaccinia virus.

Antisera

The antisera selected for absorption were a rabbit antiserum to Lister vaccinia (VS) and a rabbit antiserum to Brighton red cowpox (CS). They were prepared in conjunction with Dr C. J. M. Rondle, by initial scarification of live virus into the shaved skin of the animals, followed by repeated intravenous injections of live virus. They are known to possess antibodies to a wide range of poxvirus antigens and have been used previously to study the production of such antigens by different poxviruses (Baxby & Rondle, 1968; Baxby & Hill, 1971). Other rabbit antisera were prepared in the same way and used unabsorbed in cross-neutralization tests. Rabbit antisera were prepared against highly purified heat-inactivated virus using the immunization schedule described by Madeley (1968).

In addition, an antiserum prepared in a calf against vaccinia virus inactivated by irradiation with ultraviolet light was kindly given by Dr E. A. Boulter of the Microbiological Research Establishment, Porton.

Preparation of virus freed from soluble antigens

When preparing suspensions of purified virus care was taken to free them from contaminating soluble antigens. Briefly, the method used involved extraction of infected chorioallantoic membranes (CAM) by shaking them with glass beads, depositing the gross debris at 1000 ***g*** for 10 min., and then depositing the virus at 30,000 ***g*** for 30 min. in the SW 39 rotor of the Spinco model L centrifuge. This cycle of differential centrifugation was repeated. The resuspended virus was then extracted with Arcton 113 (I.C.I. Ltd.) and the aqueous phase banded in a sucrose-density gradient at 39,000 ***g*** for 20 min. (Zwartouw, Westwood & Appleyard, 1962). The virus, which was consistently found in the 50% sucrose layer, was given two further cycles of differential centrifugation at 30,000 ***g*** and 1000 ***g***. At each stage the virus pellets were resuspended in 10^{-3} M-NaCl with the aid of an M.S.E./Mullard ultrasonic disintegrator.

Absorption of antisera

As antigens are known to leach from poxvirus particles into the suspending medium (Craigie & Wishart, 1936; Zwartouw, Westwood & Harris, 1965) the long absorption periods used by some previous workers were avoided. Instead, repeated short absorption periods were used as advocated by Salaman (1937). In this way it was hoped that absorption of antibody to non-surface structural components could be avoided.

Pellets of purified virus were resuspended in 1·2 ml. of antiserum and the suspensions incubated at 20° C. for 60 min. and then at 37° C. for 60 min. The virus was then deposited at 50,000 ***g***. This absorption procedure was repeated as necessary. Before testing, the absorbed antisera were spun at 50,000 ***g*** for 60 min. and the supernatant irradiated with ultraviolet light to remove and inactivate residual virus. In no instance was any residual virus detected.

Testing of absorbed antisera

The antisera were assumed to be completely absorbed when either no neutralizing antibody could be detected at a 1/30 dilution, or when additional absorption failed to reduce the neutralizing titre further. The antisera were tested for neutralizing antibody by Boulter's method (1957), in which mixtures of purified virus and serum dilutions were held at 37° C. for 2 hr. before inoculation on the CAM. When completely absorbed they were also tested for neutralizing activity against extracellular virus as described by Appleyard *et al.* (1971), using the antiserum prepared against irradiated vaccinia virus to suppress any contaminating intracellular virus.

Ouchterlony immuno-diffusion tests were done as described by Rondle & Dumbell (1962). Various preparations of soluble antigens were used, including some which contained only 1 or 2 specific line pattern components (lpc). The lpc

Table 1. *Characteristics of representative suspensions of vaccinia, red and white cowpox viruses*

Virus	Total count*	Viable count†	Total: viable	Total count/ μg. protein‡
Vaccinia	$3{\cdot}4 \times 10^9$	$2{\cdot}0 \times 10^8$	16:1	$1{\cdot}85 \times 10^8$
Red cowpox	$4{\cdot}0 \times 10^{10}$	$6{\cdot}0 \times 10^8$	66:1	$1{\cdot}7 \times 10^8$
White cowpox	$7{\cdot}0 \times 10^9$	$1{\cdot}0 \times 10^8$	70:1	$1{\cdot}75 \times 10^8$

* Counted against a reference suspension of polystyrene latex spheres (Dow Chemical Co.).
† Expressed as p.f.u./ml. on the CAM.
‡ A value of $1{\cdot}8 \times 10^8$ particles/μg. protein was obtained by Joklik (1962).

produced were identified using the reference antigens prepared and described earlier (Baxby, 1967; Baxby & Rondle, 1968). The lpc earlier referred to as 3 and 5 (Baxby & Rondle, 1968) have subsequently been shown to be the L and S antigens respectively (Rondle & Williamson, 1968) and are here so labelled.

Complement fixation tests were done as described by Macdonald & Downie (1950), except that the unit volume was 0·1 ml. Extracts of infected CAM were used as antigens either heated at 65° C. for 60 min. or unheated (Madeley, 1968).

RESULTS

The significance of the results obtained by testing absorbed antisera depends to a great extent on the purity and integrity of the virus suspensions used for absorption. Examination of purified suspensions by electron microscopy showed particles having the morphology of intact, mature poxvirus with little contaminating debris. Protein estimations gave values which were very close to those obtained by Joklik (1962) (Table 1) and the values obtained for the ratios of total/infective titres were similar to those obtained by previous workers (Table 1) (Dumbell, Downie & Valentine, 1957; Kaplan & Valentine, 1959; Joklik, 1962).

Cross-neutralization tests on antisera made against live virus

Cross-neutralization tests were performed on a series of antisera to determine whether the two selected for absorption could be regarded as 'typical'. The results (Table 2) showed that they could. High-titre neutralizing antibody was found against vaccinia and white cowpox, although titres against red cowpox were consistently lower, a feature noted earlier by Downie & McCarthy (1950).

Cross-neutralization tests on antisera made against inactivated virus

The results of cross-neutralization tests on antisera made against heat-inactivated virus are interesting as they provide information on the relative importance of heat stable antigens in virus neutralization (Table 3). The antiserum prepared against u.v.-irradiated vaccinia virus neutralized all three viruses. However, a number of antisera prepared against heated vaccinia neutralized only vaccinia virus and not cowpox viruses. This suggests that vaccinia virus has a heat-stable

Table 2. *Cross-neutralization tests on hyperimmune sera*

Serum	Virus		
	Vaccinia	Red cowpox	White cowpox
Vaccinia VS	56,000*	5,000	28,000
Vaccinia 2	50,000	6,650	44,000
Vaccinia 3	40,000	5,000	40,000
Red Cowpox CS	30,000	5,000	30,000
Red Cowpox 2	64,000	5,500	40,000
Red Cowpox 3	30,000	4,000	25,000
White Cowpox 1	15,000	3,500	17,000

* Figure is reciprocal of serum dilution giving 50 % virus survival after 2 hr. at 37° C.

Table 3. *Cross-neutralization tests on sera made against heat-inactivated virus*

Serum	Virus		
	Vaccinia	Red Cowpox	White Cowpox
Heated Vaccinia 1	18,000	25	50
Heated Vaccinia 2	3,000	20	35
Heated Red Cowpox 1	200	10,000	12,500
Heated Red Cowpox 2	75	1,000	1,250
U/V Vaccinia*	1,125,000	150,000	125,000

* Serum, supplied by Dr E. A. Boulter, prepared by inoculation of ultraviolet irradiated vaccinia virus + adjuvant into a calf.

immunogen* that is either non-immunogenic or lacking in cowpox. However, Madeley (1968) produced antisera against heated vaccinia virus which would neutralize cowpox virus. Antisera prepared against heated cowpox viruses neutralized cowpox viruses to high titre, but neutralized vaccinia virus to only very low titre. That this may be due to the different importance or amounts in these viruses of a common antigen, rather than being due to the presence of a cowpox-specific antigen, will be discussed later.

Cross-neutralization tests on absorbed antisera

Before describing the results of tests on antisera which had been completely absorbed, it is of interest to describe the unusual behaviour of sera after partial absorption with vaccinia virus (Table 4), particularly as the results obtained aid interpretation of the results obtained with antisera to heated virus. Table 4 shows that after partial absorption of VS with purified vaccinia virus, the serum had a much lower titre against cowpox viruses than against the absorbing virus. As discussed later, this suggests that shared antigens may be more important for neutralization of cowpox virus than vaccinia virus.

Tables 5 and 6 show cross-neutralization tests on CS and VS respectively after complete absorption; a concentration of 1/30 was the highest that could be used

* Immunogen = an antigen which stimulates the production of virus-neutralizing antibody.

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Table 4. *Cross-neutralization tests on vaccinia antiserum VS after partial absorption with vaccinia virus*

	Serum VS absorbed with	
Test virus	Nil	Vaccinia
Vaccinia	56,000	30,000
Red Cowpox	5,000	100
White Cowpox	28,000	250

Table 5. *Cross-neutralization tests on cowpox antiserum CS after complete absorption with purified virus*

	Absorbing virus			
Test virus	Nil	Vaccinia	Red cowpox	White cowpox
Vaccinia	30,000	< 30	< 30	< 30
Red cowpox	5,000	< 30	< 30	< 30
White cowpox	30,000	< 30	< 30	< 30

Table 6. *Cross-neutralization tests on vaccinia antiserum VS after complete absorption with purified virus*

	Absorbing virus			
Test virus	Nil	Vaccinia	Red cowpox	White cowpox
Vaccinia	56,000	< 30	25,000	21,000
Red cowpox	5,000	< 30	< 30	< 30
White cowpox	28,000	< 30	< 30	< 30

with the small volumes of antisera available. The results with vaccinia and red cowpox confirm and extend those obtained by Downie & McCarthy (1950), who did only qualitative tests for residual antibody, and provide data for white cowpox virus which has been described since then.

Absorption of the anti-cowpox serum with each of the three viruses removed all detectable neutralizing antibody to them. This result suggests that the neutralizing antibodies induced by infection with red cowpox correspond to antigens present on the surface of all three viruses.

Absorption of the anti-vaccinia serum VS with vaccinia virus removed all detectable neutralizing antibody for all three viruses, but absorption of VS with either red or white cowpox left high titres of vaccinia-neutralizing antibody. This result suggests that vaccinia virus elicits a neutralizing antibody to a vaccinia virus surface immunogen which is absent from the surface of both red and white cowpox viruses. Comparison of this result with the results obtained with antisera to heat-inactivated and irradiated vaccinia virus suggests that this vaccinia specific immunogen is heat-stable.

These results on absorbed antisera provided no evidence of any qualitative differences between the surface immunogens of red and white cowpox viruses.

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Table 7. *Cross-neutralization tests on antiserum VS after complete absorption with intracellular virus, using extracellular test virus**

Test virus (extracellular)	Antiserum absorbed with intracellular			
	Nil	Vaccinia	Red cowpox	White cowpox
Vaccinia	40,000	28,000	32,000	30,500
Red cowpox	4,300	3,200	2,700	2,950
White cowpox	27,000	19,000	22,000	18,000

* Neutralization tests were done in the presence of 1/500 antiserum to irradiated vaccinia virus, which suppressed any contaminating intracellular virus.

Cross-neutralization tests with extracellular virus on antisera absorbed with intracellular virus

After the appearance of the papers by Appleyard *et al.* (1971) and Turner & Squires (1971) on the properties of extracellular vaccinia virus, all the absorbed antisera were tested for neutralizing antibody to suspensions of extracellular vaccinia, red and white cowpox viruses. In all instances high-titre neutralizing antibody to extracellular virus was found. The results obtained with VS are shown in Table 7; similar results were obtained with the anti-cowpox serum. This not only confirms the above workers' finding that vaccinia antisera absorbed with intracellular vaccinia virus still contained neutralizing antibody for extracellular virus, but also indicates that this also occurs with cowpox viruses.

Experiments with other strains of vaccinia and cowpox virus

There are many strains of vaccinia virus which can be distinguished by suitable laboratory tests (Fenner, 1958). There is also accumulating evidence that strains of cowpox virus may be differentiated. Kato, Takahashi, Kameyama & Kamahora (1959) showed that the 'A' inclusion of cowpox, originally described for the Brighton strain by Downie (1939*b*), in some strains contains no virus particles (V^-, e.g. Brighton) and in other strains has virus particles embedded in it (V^+). We have recently described a third alternative (V^i) where the periphery of the inclusion is covered with virus particles, but no particles are present within the inclusion (Plate 1A) (D. Baxby & D. R. Moorcroft, unpublished). The possibility that immunological differences might exist among different strains of either vaccinia or cowpox was tested by doing neutralization tests on absorbed antisera with the Tashkent and Connaught Laboratories strains of vaccinia, and the Carmarthen (V^+) and 61 (V^i) strains of cowpox virus. The results obtained showed no evidence of immunological strain differences.

Complement fixation tests on absorbed antisera

Complement fixation tests were done on all absorbed antisera using heated and unheated antigens. In no case, however, were complement-fixing antibodies completely absorbed. This result becomes understandable when one considers the complexity of poxvirus soluble antigens and the fact that it is uncertain how many of these antigens fix complement. If the antibody with the highest complement-

Table 8. *Surface antigens of intracellular vaccinia and cowpox viruses detected by immuno-diffusion tests on absorbed sera*

	Antigens										
Virus	1	2	3 (L)	4	5 (S)	6	7	8	9	10	*d*
Vaccinia	−	−	+	+	+	−	+	−	−	+	−
Red cowpox	−	−	−	+	−	−	−	+	+	+	+
White cowpox	−	−	−	+	−	−	−	+	+	+	−

\+ = Antigen present on virus surface, i.e. absent from absorbed antiserum.
− = Antigen absent from virus surface.

fixing titre is specific for a non-surface antigen, exhaustive absorption with purified virus should not reduce the titre of the antiserum. If the antibody with the highest complement-fixing titre is specific for a surface antigen, absorption will reduce the overall complement-fixing titre to that determined by the highest concentration of antibody to non-surface antigens. The results obtained support the view that only antibody to surface antigens had been absorbed.

Immuno-diffusion tests on absorbed antisera

Ouchterlony immuno-diffusion analysis of the absorbed antisera was carried out in order to identify the line pattern components to which residual antibody was present. Although unequivocal results were only obtained for 11 of the components, these results were clear-cut and are summarized in Table 8. Representative experiments are also illustrated (Plate 1 B–G).

Absorption with vaccinia virus removed antibody to LS, indicating that these antigens are present on the surface of that virus (Plate 1 B). However, absorption with either red or white cowpox did not remove antibody to these components, indicating the absence of LS from the surface of these viruses (Plate 1 C). Absorption of CS with red cowpox virus removed antibody to lpc *d*, indicating the presence of this antigen on the surface of red cowpox (Plate 1 D). However, absorption with either vaccinia or white cowpox did not remove antibody to lpc *d* (Plate 1 E), indicating that this component is absent from the surface of vaccinia and white cowpox viruses. Thus Rondle & Dumbell's (1962) hypothesis regarding the location of these components is confirmed; their significance in virus neutralization will be discussed later.

Further tests indicated that lpc 1, 2 and 6 were not virus surface components whereas lpc 4 and 10 were (Plate 1 D–G). An interesting situation was found with respect to lpc 8, 9. These components were detected on the surface of red and white cowpox viruses, but not on the surface of vaccinia virus (Plate 1 F, G). This result is the reverse of that obtained with LS.

DISCUSSION

These results relate to the antigens present on the surface of vaccinia and cowpox viruses extracted from infected cells. The immunological response to poxvirus infection and the role of antibody in recovery from infection have not been

considered. The immunological response to poxvirus infection has been studied extensively since Downie (1939*a*) showed that cowpox and vaccinia viruses were not identical, and the recent work of Boulter (Boulter, 1969; Appleyard *et al.* 1971) and Turner & Squires (1971) suggests that antibody to extracellular virus may be more important than antibody to intracellular virus in recovery from infection. Nevertheless intracellular forms of poxvirus are neutralized by antisera, and it is from the study of intracellular virus that most of our knowledge of the serology and chemistry of poxviruses has been obtained. Owing to the difficulties involved in obtaining high concentrations of extracellular virus free from intracellular virus (Appleyard *et al.* 1971), intracellular virus will probably continue to be used for some purposes.

The immuno-diffusion analysis of the antisera after absorption with purified virus indicates clearly that although vaccinia and red and white cowpox viruses share some surface components the surfaces of the three viruses are not identical. Thus the LS antigens and lpc 7, with which they are often associated (Baxby, 1967), are present on the surface of vaccinia virus but not of the two cowpox viruses. In contrast, lpc 8 and 9 are present on the surface of red and white cowpox virus but not vaccinia. Antibody to lpc LS, 7, 8 and 9 are present in antisera prepared against all the viruses and lpc 8 and 9 are present as soluble antigens of all the viruses (Baxby & Rondle, 1968). However, the location of LS and 7 in cowpox has not been established, although Rondle & Dumbell (1962) extracted what is now known to be LS (D. Baxby & E. C. Smith, unpublished) from cowpox-infected tissues with trypsin. Lpc 1, 2 and 6 are not surface antigens of these viruses whilst lpc 4 and 10 are. Thus lpc 4 and 10 can be counted among the 'X-agglutinogens'.

When this distribution of surface antigens is compared with the results of neutralization tests some conclusions can be drawn about the role of different antibodies in virus neutralization. Obviously antibody to antigens such as lpc 1, 2 and 6 which are not surface components of intracellular virus can play no part in the neutralization of that virus. However, one or more of these antigens may represent the additional immunogenic component of extracellular virus described recently (Appleyard *et al.* 1971; Turner & Squires, 1971). Extracellular forms of vaccinia and cowpox viruses were neutralized by absorbed antisera, indicating that the extracellular forms of all three viruses share an additional common antigen, or antigens.

In general, the results obtained indicated that antibody to lpc L, S, *d*, 8 and 9 plays no role in the neutralization of intracellular vaccinia or cowpox viruses. Antiserum containing antibody to LS, which has been absorbed with cowpox virus, still contains anti-LS but will not neutralize vaccinia. White cowpox antisera do not have anti-*d* but will neutralize red cowpox. Also a serum containing anti-*d* which has been absorbed with white cowpox still contains anti-*d* yet will not neutralize red cowpox. An antiserum containing antibody to lpc 8, 9 which has been absorbed with vaccinia still contains anti-8, 9 but will not neutralize cowpox.

Attempts to obtain vaccinia-neutralizing antibody by immunization with LS preparations have produced varied results. Parker & Rivers (1936) and Woodroofe & Fenner (1962) were unsuccessful while Cohen & Wilcox (1966) although initially

unsuccessful later succeeded (Wilcox & Cohen, 1968). Although the LS complex can be shown by immuno-diffusion analysis to exist in its classical form, i.e. as two antigenic sites on one molecule (Rondle & Williamson, 1968), on purification it is invariably contaminated with other virus antigens (Marquardt, Holme & Lycke, 1965; Cohen & Wilcox, 1966), and it is possibly antibody to these extra antigens which neutralizes the virus.

Lpc 4 and 10 were surface components common to all three viruses and so could be neutralization sites. However, Appleyard & Westwood (1964) and Westwood, Zwartouw, Appleyard & Titmuss (1965) have detected up to 20 poxvirus antigens compared to the 11 for which results are presented here, and it is possible that there are additional common surface immunogens.

Experiments on the neutralization of poxviruses by antisera prepared against heat-inactivated virus provide information that common heat-stable antigens may vary in importance in the neutralization of different viruses, and also provide evidence for a heat-stable vaccinia specific antigen.

Antisera prepared against heated cowpox virus neutralized cowpox virus to high titre, but vaccinia virus to very low titre. However, as immune cowpox serum absorbed with vaccinia virus will not neutralize cowpox virus, the heat-stable antigen involved is not cowpox specific. This suggests that this common heat-stable antigen is relatively unimportant for the neutralization of vaccinia virus. An anti-vaccinia serum partially absorbed with vaccinia virus had very low-titre neutralizing antibody for cowpox virus, but high-titre neutralizing antibody for vaccinia virus. This also suggests that vaccinia has a major surface antigen which is more important for the neutralization of cowpox virus than vaccinia virus.

Antisera prepared against heat-inactivated vaccinia virus neutralized vaccinia virus but not cowpox virus. This suggests that the common heat-stable component discussed above is haptenic or non-immunogenic in vaccinia virus. Neter (1969) has described how a common specificity may be immunogenic in one organism and haptenic in a closely related organism. It also suggests that vaccinia virus is neutralized through a heat-stable antigen which is either absent or non-immunogenic in cowpox virus. After absorption with cowpox virus a vaccinia antiserum still neutralizes vaccinia, as originally shown by Downie (1939*a*) and Downie & McCarthy (1950), a result which suggests that the heat-stable immunogen may be absent from the surface of cowpox virus. However, Madeley (1968) produced antisera against heated vaccinia which did neutralize cowpox virus; the reason for this discrepancy is not known. The virus in Madeley's vaccine was not so rigorously purified and contained large amounts of, for example, poxvirus haemagglutinin (HA). This extra material may have protected one of the common heat-labile immunogens, and antibody to such a component may have neutralized cowpox virus. Certainly the heat resistance of some poxvirus antigens depends on their degree of purity. The purification of HA produces material which becomes progressively less heat resistant and less antigenic (Madeley, 1968; E. C. Smith & D. Baxby, unpublished). It is possible that a similar situation exists with immunogenic components.

The results obtained did not suggest that there are qualitative immunological

differences between red and white cowpox viruses, although the absence in white cowpox of the non-immunogenic component *d* has been noted. The lower titres found against red cowpox may indicate differences in the amounts or importance of shared antigens.

Examination of other strains of vaccinia and cowpox provided no evidence of serological strain differences. This result is in agreement with the work of Downie (1939*a*), Horgan & Haseeb (1945), Gispen (1955) and Rondle & Dumbell (1962). However, such is the variety of biological properties exhibited by different strains of vaccinia virus (Fenner, 1958) that the existence of serologically distinct strains cannot be ruled out.

The results presented here emphasize, as have those of other workers, that the neutralization of even intracellular pox viruses is not a simple process. The presence of certain antigens on the surface of these viruses has been determined and although it has not proved possible to identify the immunogenic components of these viruses some surface antigens, including the LS antigen, have been shown to be non-immunogenic. Despite the large degree of cross-reaction between vaccinia and red and white cowpox they are not immunologically identical. They appear to be neutralized through common heat-labile antigens, and there is evidence that heat-stable common antigens may be more important for the neutralization of cowpox than vaccinia, and that vaccinia may have a specific heat-stable immunogen. The additional immunogens acquired by extracellular virus still require identification.

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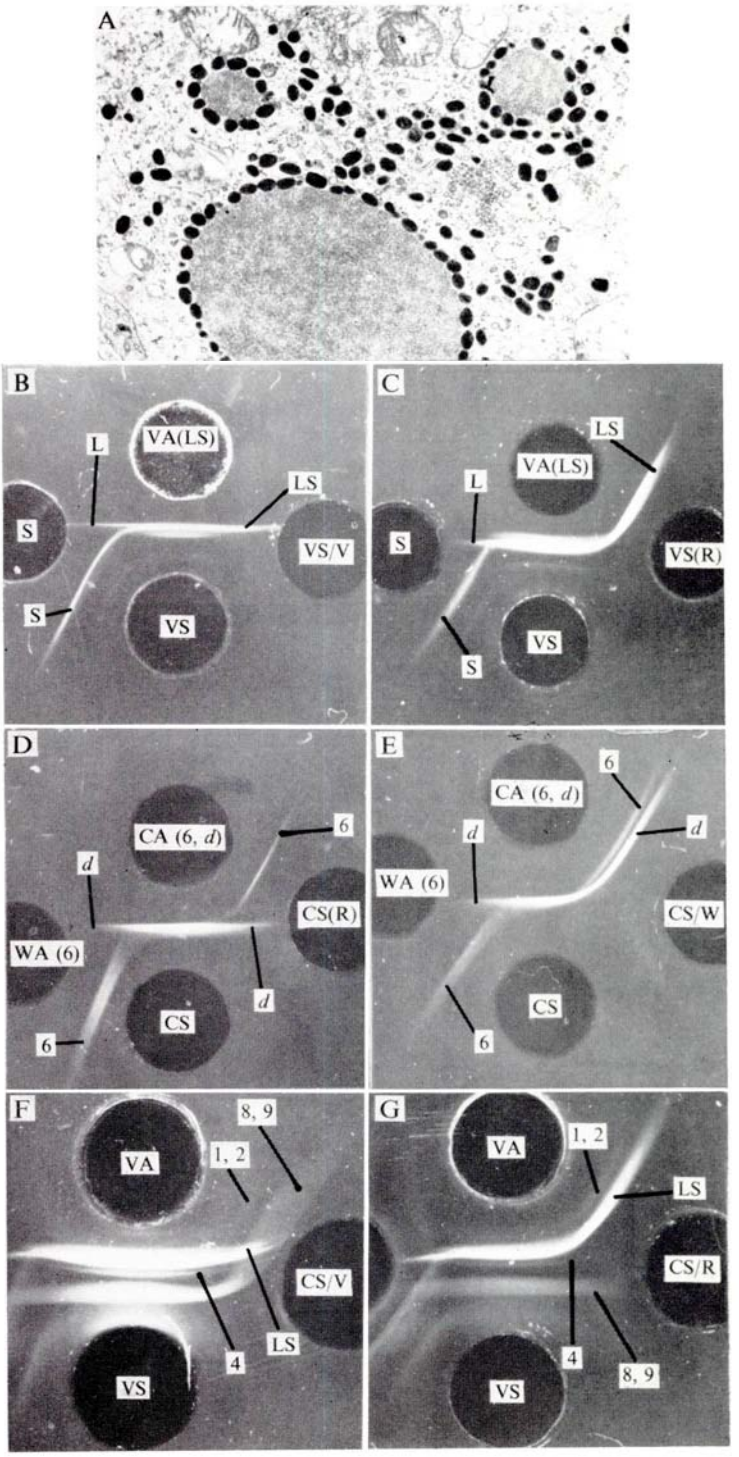

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EXPLANATION OF PLATE

Fig. A. Electron micrograph of section of 'A' inclusion produced by cowpox strain 61, showing the virus particles embedded round the periphery of the inclusion. × 11,000.

Fig. B. Absorption of antivaccinia serum (VS) with vaccinia virus (VS/V) removes antibody to LS. VA(LS) = vaccinia soluble antigen containing essentially only LS. S = heated LS containing essentially only S.

Fig. C. Absorption of VS with red cowpox (VS/R) does not remove antibody to LS.

Fig. D. Absorption of anticowpox serum (CS) with red cowpox virus (CS/R) removes antibody to *d* but not to 6. CA (6, *d*) = Red cowpox soluble antigen specific for 6 and *d*. WA (6) = White cowpox antigen specific for 6.

Fig. E. Absorption of CS with white cowpox virus (CS/W) does not remove antibody to 6 or *d*.

Fig. F. Absorption of CS with vaccinia virus (CS/V) removes antibody to LS, 4, but not to 1, 2, 8, 9. VA = complex vaccinia soluble antigen. VS = vaccinia antiserum.

Fig. G. Absorption of CS with red cowpox (CS/R) removes antibody to 4, 8, 9, but not 1, 2, LS.