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Antigens of cowpox virus

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INTRODUCTION

The term 'filterable precipitable substance' was used by Craigie (1932) to describe that material present in virus-free extracts of vaccinia-infected tissues which would react with anti-vaccinia sera. Since this time the term has degenerated to 'soluble antigen' and has been applied to virus-free, serologically reactive extracts obtained from virus-infected tissues.

Much is known about the immunological behaviour of pox-virus soluble antigens in complement fixation and neutralization studies, a summary of this information being given by Downie & Dumbell (1956).

The behaviour of vaccinia and cowpox soluble antigens in gel-diffusion tests was investigated by Gispen (1955) using a modified Oudin technique. It was found that vaccinia soluble antigen contained five or six different precipitable substances and that at least four of these substances were present in cowpox soluble antigen. One major component (Fraction I) of vaccinia soluble antigen could be demonstrated only poorly in cowpox soluble antigen and it was suggested that this difference could be used to distinguish vaccinia from cowpox.

This paper describes an investigation of cowpox soluble antigen by the gel-diffusion technique of Ouchterlony (1948). Cowpox was chosen for study because of the interest of this Department in the variant strains of cowpox virus first reported by Downie & Haddock (1952). It will be shown *inter alia* that an antigenic difference can be demonstrated between strains of cowpox virus and their 'white' variants.

MATERIALS AND METHODS

Virus strains

Most work has been done with the 'Brighton' strain of cowpox (Downie, 1939) which has been designated as the international reference strain of this virus (Fenner & Burnet, 1957). Six other strains of cowpox, isolated from cow or human material in Great Britain, have also been studied. The vaccinia strains used were obtained by dermal rabbit passage of commercial vaccine lymph from the Lister Institute and Evans Medical Ltd.

Soluble antigens

Viruses were propagated on the chick chorioallantois or on rabbit skin. Twelve-day, fertile eggs were infected and incubated 3 days at 35° C. After harvesting, pooled batches of infected membranes were suspended either in (*a*) 0·004M phosphate-citrate buffer pH 7·4, or in (*b*) 0·15M phosphate-phosphate buffer

pH 7·4, diluted with an equal volume of isotonic saline. For each membrane 1 ml. of buffer was used. The suspensions were shaken 5 min. at 20° C., left 16 hr. at 4° C., and then centrifuged 10 min. at 2000 r.p.m. to remove membrane debris. On occasion (see below) membranes were extracted up to six times successively.

Rabbit skins were harvested 3 days after infection as described by Parker & Rivers (1935), but using 18–22 ml. phosphate-citrate or phosphate-phosphate buffer for each skin. The pulp obtained was extracted in the same way as the egg membranes.

Buffer extracts from egg membranes and rabbit skin were centrifuged 30 min. at 6500 *g* in a Spinco Model L centrifuge to remove most of the virus. Portions of the centrifuged extracts were stored at 4° C. and at −20° C., but the bulk of the material was dialysed against water until free from salt and dried from the frozen state.

In some experiments other methods of extraction were used; these are described in the text.

Antisera

Rabbits convalescent from cowpox infection were hyperimmunized with three to five intravenous injections of partially purified cowpox virus suspensions. These suspensions were always prepared from rabbit-passed virus to avoid interspecies antibody production. Rabbits were bled 8–10 days after the final injection.

Antisera to the white variants of cowpox and to vaccinia were prepared in a similar way.

Diffusion analysis

The technique used was modified from Ouchterlony (1948). Petri dishes, 10 cm. diam., were filled to a depth of approximately 3 mm. with 20 ml. of diffusion medium (final concentrations: agar, 1 %; NaCl, 0·9 %; phosphate-phosphate buffer pH 7·4, 0·02 M; Merthiolate, 0·001 %). Reagent reservoirs 9 mm. diam. were cut with a hollow punch in isometric patterns, there being 14 mm. between reservoir centres. Developed precipitation patterns were recorded photographically after careful visual examination. No line pattern component seen by eye was absent from the final photographic print.

RESULTS

Examination of buffer extracts

The line patterns given by hyperimmune anti-cowpox rabbit sera against 10 different extracts of infected rabbit skin and 30 different extracts of infected egg membranes have been examined. There were at least seven components in all the line patterns observed. All seven components were present in every preparation from both rabbit and chick embryo although each component was not always represented by a discrete line and on several occasions some components could be seen only when concentrated tissue extracts were used. In some experiments the line patterns obtained could not be explained on the basis of seven components. One such experiment, illustrated in Pl. 1, fig. 1, required nine components to explain the line patterns seen. These nine components were labelled *a*, *b*, *c*, *d*, *e*,

g, *h*, *j* and *k*. In this experiment two different soluble antigens and two different antisera were used at predetermined concentrations. The reagents were chosen and placed to give the best possible resolution of different pattern components and the photograph does not therefore represent a typical reaction. Typical reactions are shown incidentally in other Figures.

In addition six other strains of cowpox have been tested against antisera to the 'Brighton' strain. Each virus was propagated on chick chorioallantois and extracts made as previously described. The line patterns given by each of these strains was identical with that given by the reference strain.

Fluid extracts varied in their content of indiffusible material. The rabbit extracts contained 5–25 mg. indiffusible material per ml., the chick embryo extracts 5–15 mg. indiffusible material per ml. No significant differences were observed between the two buffers used. Successive extraction of infected tissues removed progressively smaller amounts of indiffusible material, and for both rabbit skin and chick chorioallantois the first extract contained about half of the total quantity of material removed in six successive extractions.

The maximum number of discrete lines in precipitation patterns was obtained when extracts of either rabbit skin or chick chorioallantois were used at a concentration of 8–12 mg. indiffusible material per ml. At lower concentrations some line pattern components could not always be seen while at higher concentrations zoning of the lines obscured the clarity of the pattern. It was established that fluid extracts of infected tissues containing 8–12 mg. indiffusible material per ml. gave line patterns identical with samples of these materials which were dialysed until free from salt, dried from the frozen state, and reconstituted to their original volume in 0·02M phosphate-phosphate buffer pH 7·4. Dried materials had the advantage that they gave line patterns unchanged after storage for 3 years at 4° C. This was not true of the fluid extracts.

Sera were not expected to be uniform in behaviour and of the 19 sera examined only seven gave seven or more lines in diffusion tests, the remainder gave simpler line patterns.

It is believed that the reacting substances in the extracts are specifically associated with cowpox infection because preparations from infected rabbit skin and infected chick chorioallantois gave identical results. Normal rabbit serum did not react with the egg preparations used nor did the anti-cowpox sera react with extracts of uninfected rabbit or egg tissue. The reactions observed with rabbit preparations could not be due to unavoidable bacterial contaminants because the egg preparations were free from viable bacteria. The identical behaviour of the two types of preparation also excludes reactions due to any hypothetical breakdown products of rabbit tissue which might be antigenic in that animal.

Line patterns given by white cowpox soluble antigens

Using a white variant of the Brighton strain of cowpox virus, one extract of infected rabbit skin and seven extracts of infected chick chorioallantois were made. These extracts were examined against the antisera used previously. The line patterns given by the white preparations showed all but one of the components found in

the parent strain line patterns; line *d* was absent. Even when the white preparations were examined at a concentration of 35 mg./ml. (approximately × 5) line *d* was not seen.

Four hyperimmune rabbit antisera, prepared against Brighton white cowpox, were examined against extracts of tissues infected with either Brighton white cowpox or the parent strain. With both types of extract the sera gave similar line patterns, line *d* being absent. Two of the sera were chosen for further study; serum 'globulins' were precipitated by addition of ammonium sulphate to 50% saturation at 4° C. and after dialysis, reconstituted to one-fifth of their original volume. The fivefold concentrated antisera failed to give line *d* when tested against extracts of tissues infected with the parent strain of virus.

These results, which are illustrated in Pl. 2, fig. 2, suggest that the ability to produce a line *d* reaction is lost by Brighton cowpox virus when it undergoes variation to the white form.

Preliminary experiments on extracts of egg membranes infected with white variants from each of six other strains of cowpox suggest that this may be a general phenomenon.

Line patterns given by vaccinia soluble antigens

The line patterns given by extracts of cowpox-infected tissue against anti-cowpox sera were compared with the line patterns given by these extracts against anti-vaccinia sera and with the line patterns given by extracts of vaccinia-infected tissue against both anti-cowpox and anti-vaccinia sera. In all, 16 extracts of cowpox-infected tissue (six rabbit and 10 egg) and 14 extracts of vaccinia-infected tissue (six rabbit, six egg, and two sheep) have been examined against six rabbit anti-vaccinia sera and the four rabbit anti-cowpox sera adjudged from previous experiments to contain the widest antibody spectra. An extended account of the homologous reactions of vaccinia will be given in a subsequent paper; for the present study three observations from the work described are of importance.

In one experiment or another, but not consistently, the line pattern components *a*, *b*, *c*, *e*, *g*, *h*, *j* and *k* of homologous cowpox systems have been detected in extracts of vaccinia-infected tissue examined against both anti-cowpox and anti-vaccinia sera.

The line pattern component *d* has never been demonstrated unequivocally in any reaction involving either extracts of vaccinia-infected tissue or vaccinia antisera in their native state. The examination of concentrated extracts of vaccinia-infected tissue has also failed to show pattern component *d*, but as seen in Pl. 2, fig. 3 the reaction of extracts of cowpox-infected tissue with concentrated anti-vaccinia sera does lead to the appearance of this component.

Extracts of vaccinia-infected tissue when tested against either vaccinia or cowpox antisera have invariably shown a line pattern component which cannot be detected in extracts of cowpox-infected tissue even when these extracts are concentrated fivefold. The phenomenon is also seen in Pl. 2, fig. 3 where the component is labelled *f*.

Examination of cowpox-infected tissue for f

Since cowpox antisera will give a pattern component *f* when tested against extracts of vaccinia-infected tissue it must be assumed that in the course of cowpox infection a material is produced which elicits the formation of anti-*f* antibody. The failure of extracts of cowpox-infected tissues to give pattern component *f* when tested against either cowpox or vaccinia antisera might therefore be due to the time of harvesting of infected tissues, to the method of extraction employed, or to a difference in physical state or serological reactivity of those materials produced in cowpox and vaccinia infections which give rise to apparently identical antibodies. Distinction between these possibilities was attempted by experiment and for convenience attention was confined to cowpox-infected chick chorioallantois.

Buffer extracts of egg membranes harvested at 6, 12, 24 and 36 hr. after infection failed to show line *f*, and recourse was had to different methods of extraction.

Eggs were infected and harvested as previously described. Separate batches of infected membranes were then subjected to different methods of extraction. One batch of membranes was suspended in 0·15 M phosphate-phosphate buffer pH 7·4 (1 ml./membrane) and shaken with an equal volume of Arcton 63 (Kaplan & Valentine, 1959); a second batch was suspended in buffer as before and then subjected to vibration with a M.S.E.–Mullard Ultrasonic Disintegrator for differing lengths of time; a third batch of membranes was ground in a mortar precooled to $-20°$ C. and then subjected to buffer extraction. In this last experiment the tissue debris was re-extracted after standing 16 hr. at 4° C. Extracts obtained by these procedures were clarified by centrifugation for 15 min. at 3000 r.p.m. and were then examined against the appropriate antisera. In all extracts line pattern components *a*, *b*, *c*, *d*, *e*, *g* and *h* were observed; in two of the five extracts obtained by ultrasonic vibration the additional line pattern components *j* and *k* were clearly seen and in the examination of second extracts of ground membranes as many as twelve components were present in the line patterns obtained. No one of the extracts however gave a pattern component *f* although the extracts obtained by ultrasonic vibration and by grinding could be used to 'block' the appearance of this line pattern component in homologous vaccinia systems. Such a 'blocking' reaction is shown in Pl. 2, fig. 4, where partially purified *f* substance from vaccinia-infected tissue is shown tested against both a diluted vaccinia antiserum and the undiluted serum admixed with an ultrasonic extract of cowpox-infected tissue. The blocking effect was critically dependent upon the reagents used and the proportions in which they were mixed; it was never demonstrated when appropriate antisera were admixed with extracts of uninfected membranes, or with extracts of infected membranes after centrifugation at 10,000 *g* for 30 min., or with purified cowpox virus suspensions.

In a second series of experiments separate batches of infected membranes were extracted as described under 'Methods' but the phosphate-phosphate buffer was replaced by veronal buffers at pH 7·5, 8·0, 8·5, 9·0, 9·5 and 10·0. Each of the six extracts obtained was then examined against an appropriate antiserum on a series of Ouchterlony plates buffered at each of the pH values used for extraction. In

one experiment at pH 9·0 line *f* was clearly seen (Pl. 2, fig. 5), but despite repeated trial the phenomenon could not be reproduced.

In a third series of experiments separate batches of infected membranes were ground and suspended at 1 membrane/ml. in enzyme solutions buffered at pH 7·4. The suspensions were heated 30 min. at 37° C. and then clarified in the usual way. The use of crystalline trypsin, and crude ficin, mycozyme, and papain (all obtained from L. Light & Co. Ltd, Colnbrook, Bucks) was investigated and each enzyme was studied at a range of concentrations. Results varied from experiment to experiment but fairly consistent observations were made when trypsin was used at a final concentration of 0·05–0·15 %, ficin and papain at a final concentration of 1 % in 3 % aqueous cysteine hydrochloride, and mycozyme at a final concentration of 3 %.

Cowpox extracts obtained using trypsin and mycozyme almost invariably gave line *f* when tested against cowpox or vaccinia antisera. In such extracts also the intensity of line *a* was enhanced and that of lines *b* and *c* diminished or abolished. A typical result with trypsin is shown in Pl. 2, fig. 6. Subsequent experiments showed that although the diminution in intensity of lines *b* and *c* occurred when simple buffer extracts of infected tissues were treated with trypsin, line *a* was not enhanced and line *f* did not appear. Moreover, treatment of purified cowpox virus suspensions with trypsin failed to give evidence for line *f*.

Using ficin and papain the extracts obtained did not give line *f* when tested against the appropriate antisera. In all such extracts, however, and also in correspondingly treated buffer extracts the ability to give pattern components *c* and *h* was diminished and the ability to give pattern components *a* and *b* was abolished.

These results suggest that under defined conditions of examination, line pattern component *f* can be detected in homologous cowpox systems. Moreover, the material present in those extracts of cowpox-infected tissue which give rise to line *f* does not differ in serological activity from the line *f* material found in vaccinia-infected tissue.

Further examination of white cowpox and vaccinia

Following the results given above it seemed essential to examine white cowpox antisera and white cowpox-infected tissue for pattern component *f* and to re-examine extracts of white cowpox- and vaccinia-infected tissue for pattern component *d*.

It was readily demonstrated that three of the four white cowpox antisera used previously gave line *f* when tested against extracts of vaccinia-infected tissue. Moreover, two different trypsin extracts of white cowpox-infected tissue (egg) gave line *f* when tested against suitable antisera, although extracts of infected chorioallantois obtained by buffer extraction, ultrasonic treatment, or extraction of ground membranes did not. In addition, examination against cowpox antisera of extracts of white cowpox or vaccinia-infected tissue (egg) prepared by buffer extraction, Arcton treatment, ultrasonic vibration, trituration of ground membranes, successive extraction of ground membranes stored at 4° C., or tryptic digestion failed to give evidence for pattern component *d*. For extracts of white

cowpox-infected tissue these findings were not unexpected; for extracts of vaccinia-infected tissue however, where anti-*d* has been found in concentrated antisera, it is evident that further work is required.

DISCUSSION

It is apparent from the work of Gispen (1955) and again from the present study that the soluble antigens of cowpox and vaccinia are mixtures of some complexity. In this paper a study of homologous cowpox systems by the Ouchterlony technique has shown that a seven-component line pattern is regularly obtained. If it is assumed that each line pattern component represents one discrete antibody-antigen or hapten pair then it may be inferred that antigens or haptens *a*, *b*, *c*, *d*, *e*, *g* and *h* are regularly present in simple buffer extracts of cowpox-infected tissues (i.e. soluble antigens). Moreover, from a study of the reactions between cowpox antisera and vaccinia soluble antigen an antigen *f* must be postulated to be produced in cowpox infection. Examination of cowpox-infected tissue using a variety of extraction procedures has given good evidence for the presence of antigen or hapten *f* in these materials; in addition line pattern components *j* and *k* have been detected irregularly in cowpox soluble antigen and two of the more vigorous methods of extraction have resulted on occasion in materials giving line patterns having twelve or more components.

Evidence is available that pattern components *a* to *h* inclusive are specifically associated with cowpox and vaccinia infections; such evidence is not available as yet for the less readily detected line pattern components but there seems little reason to doubt their specific induction by virus infection. It would seem, therefore, that cowpox infection can lead to the production of at least twelve different, precipitating antibodies and that by suitable conditions of test at least twelve different precipitable substances can be demonstrated in cowpox-infected tissues, each of which can diffuse independently in 1 % agar. At the present time it is not known what relationships these substances might bear to one another or to the cowpox virus particle. Attempts have been made to obtain line patterns with well washed, purified cowpox virus suspensions, but these attempts have been fruitless. Even after enzyme treatment, purified virus suspensions have failed to give line patterns when tested against suitable antisera. It is difficult to believe that no component of cowpox soluble antigen is intimately associated with the virus particle, more probably it would seem that the conditions of virus breakdown are critical and not as yet achieved.

Evidently much additional work is required before the significance of the various components of cowpox soluble antigen can be appreciated. One obvious approach is the isolation of these materials in a purified state and a careful study of their serological and other properties; this work is now in progress.

Although the present study has failed to indicate precisely the number of substances of distinct serological specificity produced in cowpox infection, the comparison of cowpox with its white variant and with vaccinia has not been without interest and three findings might be discussed.

First, component *d* of cowpox line patterns does not appear in reactions involving either white cowpox antisera or extracts of white cowpox-infected tissue. It is not possible to prove the absence of an antigen or hapten conclusively, but it is tempting to suggest that in variation to the white form a genetic change occurs in cowpox virus and the ability to produce antigen *d* is lost. For vaccinia, small amounts of anti-*d* have been detected in some antisera, but line pattern component *d* has never been given by extracts of infected tissue. In this case, provided the conditions of test have been adequate, more subtle genetic differences would seem to be involved.

With respect to line pattern component *f*, anti-*f* antibody is readily detected in antisera to cowpox virus, to its white variant, and to vaccinia virus. A precipitable pattern component *f* however is detected readily only in extracts of vaccinia-infected tissue, its demonstration in cowpox infections being achieved with difficulty. The behaviour of *f* corresponds to the behaviour of 'Fraction I' of Gispen (1955) and from unpublished observations would seem to be the LS antigen of vaccinia (cf. Craigie & Wishart, 1934; Shedlovsky & Smadel, 1942). Evidence for this statement will be published subsequently. Gispen (1955) suggested that 'Fraction I' of cowpox differed from 'Fraction I' of vaccinia in diffusibility or precipitability. The present results tend to favour the former possibility and strongly suggest that in cowpox-infected tissue an *f* substance is bound in a non-diffusible state which can be rendered diffusible by trypsin treatment.

Finally, Downie (1939) showed that although the soluble antigens of cowpox and vaccinia fixed complement with both the homologous and heterologous antisera, it was possible to distinguish between the two viruses using antisera absorbed with washed suspensions of the heterologous virus. The difference between the viruses was shown even more clearly in agglutination tests and the results support the view that the phenomena observed were due to differences in the surface components of the washed elementary bodies. Some of the results could be partly explained on the assumption that pattern component *d* was a major surface substance of cowpox elementary bodies and pattern component *f* was a major surface substance of vaccinia elementary bodies (cf. Craigie & Wishart, 1936). It would be unwise to comment further until gel-diffusion studies on absorbed antisera have been completed.

SUMMARY

Cowpox soluble antigen is shown by gel-diffusion studies to contain at least nine precipitable substances whose presence is induced by virus infection. One of these substances, pattern component *d*, is not produced in white cowpox infections, and its production in vaccinia infection can be inferred only from the presence of small amounts of anti-*d* in anti-vaccinia sera.

Comparison of cowpox with vaccinia has led to the recognition of a further line pattern component *f* which is readily demonstrated as a soluble precipitable substance in simple buffer extracts of vaccinia-infected tissue, but is demonstrated in extracts of cowpox-infected tissue only with difficulty; anti-*f* antibody is readily detected in antisera prepared against cowpox, white cowpox, and vaccinia.

REFERENCES

CRAIGIE, J. (1932). *Brit. J. exp. Path.* **13**, 259.
CRAIGIE, J. & WISHART, F. O. (1934). *Brit. J. exp. Path.* **15**, 390.
CRAIGIE, J. & WISHART, F. O. (1936). *J. exp. Med.* **64**, 803.
DOWNIE, A. W. (1939). *Brit. J. exp. Path.* **20**, 158.
DOWNIE, A. W. & DUMBELL, K. R. (1956). *Ann. Rev. Microbiol.* **10**, 237.
DOWNIE, A. W. & HADDOCK, D. W. (1952). *Lancet* i, 1049.
FENNER, F. & BURNET, F. M. (1957). *Virology*, **4**, 305.
GISPEN, R. (1955). *J. Immunol.* **74**, 134.
KAPLAN, C. & VALENTINE, R. C. (1959). *J. gen. Microbiol.* **20**, 612.
OUCHTERLONY, Ö. (1948). *Ark. kemi. min. geol.* **26**B, 1.
PARKER, R. F. & RIVERS, T. M. (1935). *J. exp. Med.* **62**, 65.
SHEDLOVSKY, T. & SMADEL, J. E. (1942). *J. exp. Med.* **75**, 165.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Line patterns given by cowpox soluble antigens against hyperimmune rabbit antisera. Sera, S1 and S2; soluble antigens, CA1 and CA2. The several line pattern components are labelled *a*, *b*, *c*, *d*, *e*, *g*, *h*, *j* and *k*. Further details are given in the text.

PLATE 2

Fig. 2. Comparison of cowpox Brighton with its white variant. Cowpox soluble antigen, CA; white cowpox soluble antigen, CAW. Cowpox antiserum, CS; white cowpox antiserum, CSW. Fivefold concentrated reagents are labelled '$\times 5$'. It is apparent that line *d* is not given by CAW or by CSW.

Fig. 3. Comparison of cowpox and vaccinia soluble antigens and antisera. Cowpox soluble antigen, CA; cowpox antiserum, CS. Vaccinia soluble antigen, VA; fivefold concentrated vaccinia antiserum, VS $\times$ 5. Line *d* is not given by VA, but is given by VS $\times$ 5 against CA. Line *f* is given by CS against VA, but not by CA.

Fig. 4. Blocking of anti-*f* by an extract of cowpox-infected tissue. Vaccinia antiserum, VS; the same antiserum admixed with cowpox extract, VS/CA; an *f* preparation so labelled.

Fig. 5. Line *f* given by an alkaline extract of cowpox-infected tissue. Vaccinia soluble antigen, VA; vaccinia antiserum, VS; alkaline extract of cowpox-infected tissue, CApH9. Line *f* is clearly seen.

Fig. 6. Line *f* given by a trypsin extract of cowpox-infected tissue. Vaccinia antiserum, VS; vaccinia soluble antigen, VA. Cowpox soluble antigen, CA; trypsin extract of cowpox-infected tissue, CAT. Line *f* is not given by CA but is given by CAT.