Comparison of the Soluble Antigens and Virus Particle Antigens of Vaccinia Virus

BY J. C. N. WESTWOOD,* H. T. ZWARTOUW, G. APPELEYARD
AND D. H. J. TITMUSS

Microbiological Research Establishment, Porton, Nr. Salisbury, Wiltshire

(Received 27 July 1964)

SUMMARY

Soluble antigens extracted from rabbit skin infected with vaccinia virus produced immunodiffusion patterns containing up to 17 lines. At least 5 of the components which produced lines were labile when heated at 60°. Soluble material obtained from purified vaccinia virus particles produced 8 precipitin lines and an additional virus component was detected with antiserum prepared against inactivated virus particles. Seven of the virus particle precipitin lines were identical with soluble antigen lines, but up to 10 of the soluble antigen lines did not appear to represent components of the virus particle. It is suggested that they represent specific substances (perhaps enzymes) required for virus replication but not incorporated into the virus.

INTRODUCTION

Early studies of vaccinia virus antigens by classical methods of serology were reviewed by Smadel & Hoagland (1942). The serological activity of filtered extracts of vaccinia virus infected tissue (soluble antigen) was attributed to a single substance called LS-antigen. This antigen had two specificities, one of which was labile and the other stable when heated. Vaccinia virus particles were considered to have LS-antigen as a surface component as well as another antigen X-agglutininogen and to contain an internal nucleoprotein antigen. To account for the production of virus neutralizing antibody and immunity to infection a further antigen was also postulated. Application of immunodiffusion techniques has revealed that poxvirus soluble antigen is more complex than the earlier classical methods could show. Gispen (1955) found up to 6 precipitin lines and Rondle & Dumbell (1962) detected at least 9 precipitin lines produced by extracts from vaccinia virus infected tissue. Appleyard, Westwood & Zwartouw (1962) found that a total of about 15 precipitin lines was produced by the partially separated soluble components in extracts of tissue cultures infected with rabbit poxvirus.

The simplest explanation for the existence of the soluble antigens is that they represent excess synthesis of virus particle components which have not been incorporated into complete virus. However, some early experiments of Salaman (1937) and Downie (1939) suggest that this may not be the complete explanation. These authors found that antiserum which had been absorbed with virus particles still reacted with vaccinia soluble antigen preparations. We have investigated this problem by immunodiffusion comparison of soluble antigens with antigens obtained

* Present address: Department of Bacteriology, University of Ottawa.

from vaccinia virus particles. The method for extracting nucleoprotein antigen from virus described by Smadel, Rivers & Hoagland (1942) destroys some viral antigens and nucleoprotein antigen is believed to be an artifact. Soluble components which produce up to 8 precipitin lines can, however, be obtained from vaccinia virus particles, as described in the previous paper (Zwartouw, Westwood & Harris, 1964).

METHODS

Antigens

Vaccinia virus soluble antigens. Infected dermal pulp was obtained from the skin of infected rabbits, as described by Hoagland, Smadel & Rivers (1940). The material from each rabbit was collected into 25 ml. of 0.004M-McIlvaine buffer (pH 7.8) containing 0.1% sodium azide (to inhibit growth of contaminating organisms) and dispersed by shaking with glass beads. The suspension was clarified by centrifugation at 800 g for 10 min. and the deposit re-extracted with half the original volume of buffer. The total suspension was then centrifuged at 35,000 g for 30 min. to sediment virus particles. The supernatant fluid, which contained between 5 and 7 mg. protein/ml., was the material used as soluble antigens.

Purified vaccinia virus particles. The virus sedimented from the soluble antigen solution was washed and purified by centrifugation in a sucrose density gradient, as described by Zwartouw, Westwood & Appleyard (1962). The purified virus produced no precipitin lines in immunodiffusion tests.

Extraction of antigens from vaccinia virus particles. Antigens were obtained from purified virus particle suspensions (1 mg. dry-weight/ml.) in 0.01M-phosphate buffer (pH 8.0) by incubation with 0.001% crystallized trypsin (Armour & Co. Ltd.) for 3 hr at 37°. Clear solutions containing about 20% of the virus N were obtained after centrifugation at 30,000 g for 15 min. (Zwartouw *et al.* 1964).

Antisera

Complete vaccinia antiserum. Rabbits were initially vaccinated on the skin and then hyperimmunized with two to four intravenous injections of 5×10^9 pock forming units of virus from the skins of rabbits. Sera from nine rabbits were pooled. The method of preparation made the possibility of any reaction with rabbit tissue antigens very unlikely and this was confirmed by the absence of precipitin lines when the antiserum was tested against extracts from uninfected rabbit skin.

Soluble antigen antiserum. Soluble antigen was freed from infective virus by two Seitz filtrations followed by two filtrations through collodion membranes (average pore diameter 330 m μ). Rabbits were injected subcutaneously with 4 weekly doses of 1 ml. of the filtered soluble antigen emulsified with Freund's complete adjuvant (Difco Laboratories).

Virus particle antiserum. Two alternative methods were used to inactivate purified virus for immunization. (1) Formaldehyde was added to an aqueous virus suspension to make final concentrations of 0.4% formaldehyde and 1.3 mg. dry weight of virus/ml. The mixture was kept at 20° for 1 hr. (2) An aqueous virus suspension (1.3 mg./ml. dry weight) was irradiated as a layer 1.7 mm. in depth and gently agitated by rocking at 34 cm. from a 15 W. Phillips ultraviolet lamp for 5 min. No infective virus was detected when either type of preparation was tested in chick embryos. The inactivated virus suspensions were emulsified with 1.5 vol.

of Freund's complete adjuvant and 2 ml. volumes of the mixtures (containing 1 mg. dry weight of virus) injected intramuscularly into rabbits. A second injection was given subcutaneously 6 weeks later.

Immunodiffusion

Double diffusion tests in gels were carried out on microscope slides as described by Crowle (1958). The diffusion medium was 1 % Ion-agar (Oxoid) in 0.9 % NaCl and antigen reservoirs were spaced in the Perspex templates either 4 or 6 mm. from a central antiserum reservoir. After 3 days at room temperature, the gel layers were washed and stained and the precipitin lines recorded photographically.

RESULTS

Soluble antigens

Immunodiffusion patterns produced by the complete vaccinia antiserum with samples of the soluble extract from infected tissue were very complex. It was difficult to resolve all the precipitin lines and the full number was not always distinguishable. The maximum number counted was 17. Samples of soluble antigen differed in that the components which produced the 4 lines nearest the antigen reservoirs were not present in all samples. These particular components, which were subsequently found to be important in the comparison with virus particle antigens, were detected in four out of the six soluble antigen preparations tested. One of the deficient samples had been Seitz filtered, a process which was generally applied by earlier workers to remove virus particles from soluble antigen, and this appeared to be a cause of the deficiency. Filtration of soluble antigen through a collodion membrane (average pore diameter 350 m μ) produced a similar loss. Although the unfiltered material was not completely free from virus particles, the small number present could not account for the extra precipitin lines. No precipitin lines were produced by purified intact virus particles at 1000-times the concentration present in soluble antigen.

In order to relate the precipitin line pattern to the earlier concept of a heat-labile and a heat-stable group on what was regarded as the single precipitating soluble antigen, the stability of the components revealed by immunodiffusion was similarly tested. A comparison was made of untreated soluble antigen with the same material after heating at 60° for 90 min. The stability of components producing some of the fainter lines could not be assessed, but the presence of five labile and six stable components was demonstrated.

Comparison of soluble antigens with virus particle antigens

The material obtained in solution by tryptic digestion of purified virus particles produced up to 8 precipitin lines with the complete vaccinia antiserum. When these lines were compared with the lines produced by filtered soluble antigen samples, no linkages could be detected between the two sets of lines. Thus, there appeared to be no immunological identity between any of the components in the two sets of antigens. However, when the comparison was made with unfiltered soluble antigen samples, linkages were detected between the extra soluble antigen lines (the 4 nearest

the antigen reservoir) and the centre 4 lines of the group produced by the virus particle antigens.

Since evidence of immunological identity with virus components was shown only by the extra soluble antigen components, the possibility was considered that their presence in soluble antigen might be due to breakdown of virus particles during preparation of the materials. Incubation of purified virus suspensions at 37° produced a slow release over 72 hr of the same antigens that were rapidly released from virus particles by tryptic digestion (Zwartouw *et al.* 1964). Preparations of soluble antigen and virus were normally obtained from batches of ten or more rabbits. On this scale, extraction of the material from infected skin and subsequent clarification extended over 1–2 days before most of the virus was separated from the soluble antigens by centrifugation. Although the material was kept at 4° for most of this time, some release of antigens from the virus into the soluble fraction could have occurred. We tried to eliminate this possibility by rapidly processing material obtained from single rabbits. In this way, virus was separated from soluble antigens within 4 hr of harvesting the material from infected rabbit skin. Two samples of rapidly processed soluble antigen both contained the components which produced lines linking with virus particle antigen lines as in the previous patterns. Further tests were carried out to detect any release of antigens from the small amount of virus remaining in unfiltered soluble antigen after centrifugation. Samples of the rapidly processed soluble antigen were incubated for 72 hr at 37°. This treatment caused no apparent increase in any of the components producing precipitin lines but some loss of one component was noted.

Components detected with soluble antigen antiserum

It was impossible to be certain that no linkages occurred between some of the fainter lines produced by soluble antigens and virus particle antigens in the complex patterns obtained with the complete vaccinia antiserum. Further evidence was sought by testing an antiserum prepared against soluble antigen. To avoid any antibodies produced directly against virus particles, the antiserum was prepared by injecting filtered soluble antigen. This preliminary filtration probably accounted for the inability of the antiserum to produce all the soluble antigen lines. In particular, it did not reveal the extra components of unfiltered soluble antigen or the stronger virus antigen lines which formed linkages in the previous tests. However, the immunodiffusion pattern obtained with the soluble antigen antiserum confirmed that some soluble antigen components were not present in the extract from virus particles. It also revealed that two weak lines produced by the virus particle antigens appeared to link with soluble antigen lines.

Components detected with virus particle antiserum

An attempt to detect any further virus particle antigens, which were not represented in the soluble extract prepared from virus particles, was made by testing antiserum against inactivated whole virus particles. Two alternative methods of inactivation, formaldehyde treatment and ultraviolet irradiation, were used to reduce the possibility of antigen destruction by the inactivation treatment. Although no infective virus could be detected in the treated preparations, addi-

tional evidence that virus replication had not occurred in the immunized rabbits was obtained by testing the antisera for anti-haemagglutinin. A vaccinia haemagglutination test system was inhibited by a 1/160 dilution of the complete vaccinia antiserum, but not by 1/10 dilutions of the virus particle antisera. On the other hand, the sera had high 50 % neutralization titres (200,000–500,000) when tested with infective vaccinia virus by the method of Boulter (1957). Immunodiffusion patterns produced by antiserum prepared against virus particles inactivated by either method were similar. Only one weak precipitin line was produced by the soluble antigens and the same component was not detectable as a linking precipitin line produced by the virus particle extract. Thus the line indicated a component present in both soluble antigens and virus particles which had not been obtained in solution by tryptic digestion of the virus.

DISCUSSION

Immunodiffusion analysis has shown that soluble antigens produced in poxvirus infections contain a large number of different components. Seventeen precipitin lines were produced by extracts from rabbit skin infected with vaccinia virus and recently Appleyard & Westwood (1964) detected over 20 lines produced by extracts of tissue cultures infected with rabbitpox virus. Heating the vaccinia virus soluble antigens at 60° demonstrated five labile and six stable components. Some of the components were probably not included in the earlier investigations of filtered infected tissue extracts since losses occur during filtration. However, it seems likely that the original concept of a single LS-antigen was an expression of the collective behaviour of a number of antigens.

Table 1. *Summary of immunodiffusion tests*

Antigen	Antiserum	Precipitin lines
Soluble antigens		
Total detected	Complete	17
Linkages with extracted components of virus	Complete	4
Linkages with extracted components of virus	Soluble antigen	2
Present in virus but not in extract of virus	Virus particle	1
Total shared with virus particle		7
Virus particle antigens		
Present in extract of virus	Complete	8
Detected as antibody	Virus particle	1
Total detected		9

We have attempted to determine whether the soluble antigens represent excess synthesis of virus particle components by testing for their presence in virus particles. In direct comparisons with the lines produced by soluble material obtained from the virus particles, the appearance of immunodiffusion patterns suggests that the major components of virus particle extracts were identical with minor components of soluble antigen. Evidence for identity of two minor components in both sets of antigens was obtained with soluble antigen antiserum. However, the strongest lines produced by the soluble antigens clearly did not correspond to any component in

the solution of virus particle antigens. The antigen solution obtained from virus particles contained about 20 % of the total viral N. Although no further precipitin components were detected when larger amounts of the virus were obtained in solution (Zwartouw *et al.* 1964), it was possible that some antigens were destroyed by the treatments used. If such antigens were also present in the soluble antigen mixture, they should have been detectable as precipitin lines formed by soluble antigen with antiserum prepared against inactivated virus particles. When this system was tested, only one weak precipitin line was produced by soluble antigen. A summary of the results is shown in Table 1.

Two vaccinia virus antigens could be detected by specific properties which showed that one was present in both virus particle and soluble antigen but the other only in soluble antigen. The first is the antigen reacting with virus neutralizing antibody which was demonstrated in soluble antigen by Appleyard (1961). The second is the haemagglutinin which is present in infected tissue extracts. Purified virus does not haemagglutinate (Zwartouw *et al.* 1962) and antiserum prepared against inactivated virus particles does not inhibit the specific haemagglutination. It is possible that these two antigens were not represented in the precipitin line patterns. Appleyard, Zwartouw & Westwood (1964) were not able to correlate the serum blocking activity of soluble antigen with any specific precipitin line and the relatively large size (65 m μ) of the haemagglutinin (Chu, 1948) may have prevented this antigen from diffusing in the gel system. Most of the virus particle antigens were also detected in the soluble antigen mixture. These soluble antigen components are readily explained as excess virus particle components which have not been utilized to form complete virus. However, there appear to be up to ten further components in soluble antigen which could not be detected in virus particles. A possible explanation for these is that they are specific enzymes for synthesis of new virus material and their formation in cells is initiated by the infecting virus. Increased amounts of several enzymes concerned with DNA synthesis in vaccinia virus infected cells have been reported and were reviewed by Cohen (1963). These increased amounts might be due to enzymes with virus rather than host cell specificity. Furthermore, Appleyard & Westwood (1964) have recently shown that some soluble antigens are produced in tissue cultures infected with rabbitpox before the viral DNA is replicated.

REFERENCES

- APPLEYARD, G. (1961). An immunising antigen from rabbit-pox and vaccinia viruses. *Nature, Lond.* **190**, 465.
- APPLEYARD, G. & WESTWOOD, J. C. N. (1964). The growth of rabbitpox virus in tissue culture. *J. gen. Microbiol.* **37**, 3.
- APPLEYARD, G., WESTWOOD, J. C. N. & ZWARTOUW, H. T. (1962). The toxic effect of rabbitpox virus in tissue culture. *Virology*, **18**, 159.
- APPLEYARD, G., ZWARTOUW, H. T. & WESTWOOD, J. C. N. (1964). A protective antigen from the pox-viruses: I. Reaction with neutralising antibody. *Brit. J. exp. Path.* **45**, 150.
- BOULTER, E. A. (1957). The titration of vaccinal neutralising antibody on chorio-allantoic membranes. *J. Hyg., Camb.* **55**, 502.
- CHU, C. M. (1948). Studies on vaccinia haemagglutinin: I. Some physicochemical properties. *J. Hyg., Camb.* **46**, 42.
- COHEN, S. S. (1963). The biochemistry of viruses. *Annu. Rev. Biochem.* **32**, 83.

- CROWLE, A. J. (1958). A simplified micro double-diffusion agar precipitin technique. *J. Lab. clin. Med.* **52**, 784.
- DOWNIE, A. W. (1939). The immunological relationship of the virus of spontaneous cow-pox to vaccinia virus. *Brit. J. exp. Path.* **20**, 158.
- GISPEN, R. (1955). Analysis of pox virus antigens by means of double diffusion. *J. Immunol.* **74**, 134.
- HOAGLAND, C. L., SMADEL, J. E. & RIVERS, T. M. (1940). Constituents of elementary bodies of vaccinia: I. Certain basic analyses and observations on lipid components of the virus. *J. exp. Med.* **71**, 737.
- RONDLE, C. J. M. & DUMBELL, K. R. (1962). Antigens of cow-pox virus. *J. Hyg., Camb.* **60**, 41.
- SALAMAN, M. H. (1937). The combining properties of vaccinia virus with the antibodies demonstrable in anti-vaccinial serum. *Brit. J. exp. Path.* **18**, 245.
- SMADEL, J. E. & HOAGLAND, C. L. (1942). Elementary bodies of vaccinia. *Bact. Rev.* **6**, 79.
- SMADEL, J. E., RIVERS, T. M. & HOAGLAND, C. L. (1942). Nucleoprotein antigen of vaccine virus: I. A new antigen obtained from elementary bodies of vaccinia. *Arch. Path.* **34**, 275.
- ZWARTOUW, H. T., WESTWOOD, J. C. N. & APPELYARD, G. (1962). Purification of pox viruses by density gradient centrifugation. *J. gen. Microbiol.* **29**, 523.
- ZWARTOUW, H. T., WESTWOOD, J. C. N. & HARRIS, W. J. (1964). Antigens from vaccinia virus particles. *J. gen. Microbiol.* **38**, 1.