Isolation of Cowpox Virus Clones Deficient in Production of Type A Inclusions: Relationship to the Production of Diffusible LS Antigen

(Accepted 13 January 1981)

SUMMARY

Cowpox virus clones (A\textsuperscript{−} clones) deficient in production of type A inclusions were isolated from two cowpox strains, Amsterdam and 53. These clones did not differ from their parents in major markers such as pock morphology in chorioallantoic membranes and pathogenicity in the rabbit skin. However, the LS antigens induced by A\textsuperscript{−} clones developed precipitin lines in agar gel diffusion tests, while the antigens from their parents failed to precipitate. Immunofluorescence and agar gel diffusion tests revealed that antigens detectable by antiserum against purified type A inclusions and LS antigens were closely related to each other. These findings suggest that the A\textsuperscript{−} clones might be variants of cowpox virus which have lost the ability to assemble LS antigens into type A inclusions.

Some poxviruses induce two kinds of cytoplasmic inclusions, which are known as type A and type B inclusion bodies (Kato \textit{et al.}, 1959, 1963; Kato & Kamahora, 1962). The type B inclusion is the site of virus replication and is seen in cells infected with the members in the genus orthopoxvirus (Kato \textit{et al.}, 1959; Kato & Kamahora, 1962). This type of inclusion appears at an early stage in the replication cycle and stains basophilically with haematoxylin–eosin (HE) or Giemsa. Among orthopoxviruses, cowpox and ectromelia viruses induce eosinophilic type A inclusions which are also known as Downie (Downie, 1939) and Marchal (Marchal, 1930) bodies respectively, and appear late in the replication cycle in almost 100% of infected cells (Kato & Kamahora, 1962). The role of type A inclusions in poxvirus replication is not yet clear, but their presence is considered as one of the markers which differentiate cowpox and ectromelia viruses from other orthopoxvirus species. Even though Kamahora \textit{et al.} (1958) reported that a vaccinia virus strain also induces type A inclusions in infected cells, and we also observed type A inclusions in cells infected with many other strains of vaccinia, variola, monkeypox and rabbitpox viruses (unpublished data), the frequency was so low (<0.1%) that the significance of the type A inclusion as a biological marker for differentiation of cowpox and ectromelia viruses from other orthopoxvirus species has not been decreased.

In the course of our studies on cowpox virus, we isolated virus clones deficient in production of type A inclusions (A\textsuperscript{−} clones). This report describes the properties of such clones and the inverse relationship between production of type A inclusions and diffusibility of LS antigens in agar.

The Amsterdam strain was obtained from Dr S. Kato, Institute for Microbial Diseases, Osaka University, Osaka, Japan and Dr R. Gispen, Rijks Instituut voor de Volksgesondheid, Bilthoven, The Netherlands. The strains 50, 51, 53, 58, 60, 61 and 71-14033 were gifts from Dr R. Gispen. Brighton (LB) strain and its white pock variant were made available to us by Dr A. W. Downie, Liverpool University, Liverpool, U.K. The viruses were propagated in chorioallantoic membranes (CAM) of 12-day-old eggs and used within two passages in our laboratory. Strains of some other species of orthopoxvirus kept in our laboratory were also used.
We tried to isolate A− clones from 10 strains of cowpox virus by plaque and/or pock picking. Clones isolated were purified by five successive single pock or plaque passages and examined in HeLa S3 cells for the type A inclusion-inducing ability by HE or Giemsa staining. The direct immunofluorescence technique with hyperimmune sera against vaccinia virus was also used to demonstrate inclusions. As reported for uncloned cowpox viruses (Kato et al., 1963), A+ clones produced the inclusions in almost 100% of infected cells regardless of pock colour, while A− clones did so in less than 0-1% of infected cells, even though degenerating type B inclusions were observed in the cytoplasm of almost all cells by direct immunofluorescence. We could not isolate clones which induced the inclusions at an intermediate frequency. From the Amsterdam strain, 18 clones were isolated by pock and plaque picking. Of the nine clones isolated by the former technique, six were white and three were red. A white clone was A+ and five white and three red clones were A−. All the nine clones isolated by the latter technique were red and five were A+ and four were A−. From strain 53, nine clones were isolated by plaque picking and they were all red clones, of which four were A+ and five were A−. Of three white pock clones isolated from strain 53, two were A+ and one was A−. A− clones were not isolated from the other cowpox strains examined.

The pathogenicity of A− cowpox clones was compared with that of wild-type cowpox virus and other orthopoxviruses in the skin of Japanese albino rabbits. Wild-type strains and all red clones of cowpox virus produced prominent haemorrhagic and necrotic reactions even with small inoculation doses, regardless of their ability to induce type A inclusions, while the white clones did not induce marked haemorrhage and necrosis. Rabbits inoculated with rabbitpox or neurovaccinia viruses showed deep and widespread induration with less haemorrhage and necrosis compared to that produced by red cowpox virus strains. Thus, the prominent haemorrhagic and necrotic reaction in the rabbit skin was related to the red pock character of cowpox virus clones as reported by Baxby (1969) but not to the ability to induce type A inclusions.

It is well known that extracts of CAM infected with vaccinia virus contain the LS antigen complex which can be demonstrated by agar gel diffusion tests with anti-vaccinia or anti-cowpox serum. However, extracts of CAM infected with cowpox viruses fail to develop the LS lines with either sera (Gispen, 1955; Rondle & Dumbell, 1962; Rondle & Williamson, 1968). We examined whether A− cowpox viruses could induce diffusible LS antigens and whether they could induce antigens associated with type A inclusions. Anti-LS and anti-A sera were prepared as follows. LS antigens were prepared from dermal pulp of the rabbits infected with the RK cell-adapted Elstree strain of vaccinia virus by the method of Shedlovsky & Smadel (1942) with slight modifications (Cohen & Wilcox, 1966). Type A inclusions were isolated from HeLa S3 cells infected with the LB-white of cowpox virus and purified by the method of Shida et al. (1977) with slight modifications. The antigen preparations in Freund’s complete adjuvant were injected into rabbits two or three times at appropriate intervals. The rabbits were bled 7 days after the last injection. Both types of antiserum had no neutralizing ability when tested by plaque reduction neutralization tests on chick fibroblasts. Antigens for agar gel diffusion tests were prepared by homogenization of infected CAM in phosphate-buffered saline (PBS, pH 7-2, 1 CAM/ml) in a Sorval omnimixer for 3 min at the maximum speed. After centrifugation at 30 000 rev/min for 2 h, the supernatants were concentrated fivefold by the Amicon B-15. Holes of 5 mm diam. were punched out on 1% Noble agar in PBS with a distance of 10 mm between the centres of the neighbouring holes. As shown in Fig. 1 (a), the extracts of CAM infected with A− cowpox clones developed two precipitin lines with anti-LS serum. The lines produced by A− clone linked with that produced by vaccinia virus but the major line was not fully identical with that antigen induced by vaccinia virus. The LS lines formed by vaccinia virus were fully identical with those formed by neurovaccinia, monkeypox and rabbitpox virus antigens (not shown). The A− white pock variants gave the
same results as A− red clones. All A− clones produced diffusible LS antigens. Thus, we found that the production of LS antigens diffusible in agar and the inability to induce type A inclusions were related characters in cowpox clones. Although the antigens produced by A+ cowpox failed to develop these lines (Fig. 1 a), trypsinization of A+ cowpox antigens resulted in the appearance of the LS lines (Fig. 1 b) as reported earlier (Rondle & Dumbell, 1962; Rondle & Williamson, 1968) and the lines linked with those of vaccinia. The precipitation patterns of vaccinia and A− cowpox antigens were not changed by this treatment.

As shown in Fig. 1 (c), A− cowpox and vaccinia antigens formed two precipitin lines with anti-A serum, while antigens of A+ cowpox strains LB-white and 53 gave no precipitin line. The A+ Amsterdam antigen gave one of the two ‘A’ lines even without trypsinization. This observation suggested that the antigens associated with type A inclusions were not diffusible through the agar, with the exception of A+ Amsterdam antigen. Here again, we examined whether trypsinized A+ cowpox antigens could develop precipitin lines against anti-A serum. As shown in Fig. 1 (d), trypsinized A+ cowpox antigens gave precipitin lines which linked with those of vaccinia and A− cowpox antigens. ‘A’ line patterns with vaccinia and A− cowpox antigens were not changed.
Fig. 2. The antigenic relationship between LS antigens and antigens associated with type A inclusions. Centre: anti-A and anti-LS sera. vac Elst, vaccinia virus Elstree; neuro IHD—, neurovaccinia virus IHD(−); vac Tai, vaccinia virus Taikyu strain; cpv A−Am, cowpox A− red clone Am-R2; A t, trypsin-solubilized type A inclusions from cells infected with LB-white.

Thus, antigens detected by anti-LS and anti-A sera behaved very similarly in agar gel diffusion tests. Therefore, we examined the immunological relationship between LS and A antigens by placing both types of antiserum on the same agar plate. As shown in Fig. 2, the lines formed with anti-LS serum and various antigens linked with the lines formed with anti-A serum and the same antigens, although spurs were observed at some junctions of precipitin lines. The results indicate that a close relationship exists between LS and A antigens and this was confirmed by indirect immunofluorescence with anti-LS and anti-A sera. With both sera, diffuse cytoplasmic fluorescence was observed in cells infected with either vaccinia or A− cowpox viruses, while the fluorescence was concentrated at the periphery of type A inclusions in cells infected with A+ cowpox clones, although diffuse cytoplasmic fluorescence was also observed. Distribution of antigens in infected cells was the same when detected by either serum. Both types of sera did not stain type B inclusions. These observations suggest that the LS antigens and the antigens associated with type A inclusions are basically the same antigenically.

The red A− clones were identified as cowpox virus by pock colour and pathogenicity in the rabbit skin. The red A− clones yielded white variants as did the parent cowpox virus. Therefore, it seems reasonable to assume that white A− clones were also cowpox.

The most prominent change associated with the loss of the ability of cowpox virus to induce type A inclusions was that the LS antigens became diffusible. Even though they lost the ability to induce type A inclusions, A− clones could induce the antigens associated with type A inclusions. It was also demonstrated by agar gel diffusion and immunofluorescence tests that LS and A antigens were immunologically almost the same. These facts suggest that A− clones are variants of cowpox virus which can induce the synthesis of all or major parts of materials for type A inclusions or LS antigens but they lack the ability to induce factor(s) which leads to the assembly of these materials into type A inclusions.

Baxby & Rondle (1968) reported that cowpox virus produced diffusible LS antigens in RK 13 cells. In addition to the concentrated antigens at the periphery of type A inclusions, anti-LS serum revealed the diffuse cytoplasmic localization of LS antigens in cowpoxvirus-infected...
HeLa cells. LS antigens may be present also in diffusible form in some cell culture. Marennikova et al. (1976) reported that variola virus strain Kuait-5-67 failed to produce diffusible LS antigens. It would be interesting to examine whether or not this strain produces type A inclusions.

Dr Tagaya passed away before the final manuscript of this paper was completed. I will remember his remarkable personality and great contributions to public health and virology. I am grateful to Dr Y. Tsuchiya of our laboratory, for valuable discussion and for critical reading of this manuscript. This work was partly supported by a Grant from the Ministry of Education, Science and Culture, Japan.

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REFERENCES


(Received 11 August 1980)