

LABORATORY CHARACTERISTICS OF POXVIRUSES ISOLATED FROM CAPTIVE ELEPHANTS IN GERMANY(*)

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SUMMARY

Poxviruses isolated from captive elephants in Germany have been characterized. Although related to vaccinia and even more closely to cowpox virus, the separate identity of elephantpox virus was established by both biological and serological methods. Elephantpox virus produces A-type inclusions in infected cells, as did cowpox, but had a lower ceiling temperature, was more heat resistant and affected rabbits differently. Cross neutralization tests on absorbed sera indicated that elephantpox, cowpox and vaccinia viruses shared one surface antigen, that elephantpox and vaccinia shared an antigen absent from cowpox, and that vaccinia virus had a surface antigen absent from elephantpox and cowpox viruses.

INTRODUCTION

Poxvirus infections of circus and zoo elephants have been reported from both the Federal and Democratic Republics of Germany. The causative agent was originally thought to be vaccinia virus (Gehring, Mahnel & Mayer, 1972). However, further studies of the original and subsequent isolates indicated that they could be differentiated from vaccinia and it has been suggested that they are variants of vaccinia virus (Mahnel, 1974; H. Mahnel, personal communication).

We are interested in the characterization of animal poxviruses and Professor Mahnel kindly allowed us to examine 2 isolates of 'elephantpox

(*) Reprinted from J. gen. Virol. (1977) 37, 407-414,.

virus'. Our results indicate a close relationship to both vaccinia and cowpox viruses, but we propose the separate identity of elephantpox virus.

METHODS

Virus strains. Elephantpox strain EP-1 was used at the third passage on the chorioallantoic membrane (CAM). It was isolated in 1971 from an outbreak in Stuttgart which affected 11 of 18 elephants, 1 of which died; 2 human cases of infection also occurred (Gehring *et al.* 1972). Elephantpox strain EP-2 was also used at the third CAM passage. It was isolated in 1975 from an outbreak in Nuremberg which killed 2 elephants (H. Mahnel, personal communication). The Lister (Elstree) strain of vaccinia virus, and the Brighton strain of cowpox virus were used in control experiments.

Pock production and virus titration. Viruses were grown and titrated on the CAM of 12-day-old White Leghorn embryos.

Ceiling temperatures. The effect of increased incubation temperatures on pock production was tested using special incubators regulated by 'Accuron' heaters and thermostats and monitored by 'Grant' recorders as described earlier (Baxby, 1969, 1974). The control temperature was 35°C.

Electronmicroscopy. Pocks were excised from the CAM after 3 days incubation, fixed in glutaraldehyde and osmium tetroxide and embedded in epoxy resin. Thin sections were cut and stained with lead citrate.

Rabbit inoculation. The shaved flank of a New Zealand white rabbit was inoculated intradermally with 0.1 ml volumes of tenfold dilutions of EP-2 calculated to contain 5×10^5 to 50 pock forming units (p.f.u.). The opposite flank was inoculated with tenfold dilutions of cowpox virus calculated to contain 50, 5 and 0.5 p.f.u. The rabbit was kept in isolation and examined daily.

Antiserum to elephantpox was produced by scarifying the shaved flanks of rabbits with high titre virus which had been passaged in RK13 cells. The rabbits were re-inoculated 4 weeks later and serum obtained 10 days after that.

Tissue culture techniques. Plaque production was tested in monolayers of RK13 and VERO cells. In addition, the yields of virus and haemagglutinin (HA) produced by heavy infection of VERO cells was measured using methods previously devised for cowpox virus (Baxby, 1975a).

Heat resistance. The reduction of infectivity caused by heating virus suspensions at 56°C for 20 min was determined as described earlier (Baxby, 1975a).

Haemagglutination-inhibition (HAI). HAI antibody was detected by reacting 4 units of HA with antiserum dilutions at 35°C for 1 h before adding 1% fowl erythrocytes suspended in 1% normal rabbit serum.

Gel diffusion. The soluble antigens produced by elephantpox virus in heavily infected CAM were compared with those of vaccinia and cowpox by the Ouchterlony technique, as modified by Rondle & Dumbell (1962).

Virus neutralization. Neutralization tests were done by a standard method (Boulter, 1957), in which serum dilutions and virus were reacted for 2 h at 37°C, at which time residual infective virus was detected by CAM inoculation. All antisera were inactivated at 56°C for 20 min before use.

Serum absorption. Antisera were absorbed with virus which had been purified from infected CAM by differential centrifugation (Baxby, 1972a). A virus pellet, the yield from 50 to 60 CAM, was resuspended in the antiserum (usually 5 ml of a 1/5 dilution), and incubated overnight at 4°C. The virus was sedimented at 25000 g, and the supernatant used to resuspend another pellet of the same virus. When the antiserum had been completely absorbed, which usually took three pellets, it was passed through a 200 nm 'Millipore filter to remove any residual infective virus. An antiserum was considered to be completely absorbed when it would no longer neutralize the absorbing virus, and when further absorption did not reduce the titre of any residual antibody to another virus.

RESULTS

Biological characteristics

Pock production

The pocks produced by both strains of elephantpox were haemorrhagic and ulcerated. The size tended to vary. In most cases they were approx. 1.0 mm after 3 days and resembled those produced by monkeypox virus. In some cases they were larger (about 1.5 to 2.0 mm), and indistinguishable from those produced by cowpox virus. This variation in size was often noticed in different membranes from the same batch of embryos, and was due to the variability of chick CAM noticed earlier for other poxviruses (Baxby, 1969; Gispén & Brand-Saathof, 1972). Occasional white pocks were seen. These were presumed to be analogous to those produced by white pock mutants of cowpox (Downie & Haddock, 1952) and monkeypox (Gispén & Brand-Saathof, 1972) viruses, but due to the variability of response of the CAM cloning experiments were not attempted.

Pock production at raised temperature

Using inocula of 100 to 150 p.f.u., both strains of elephantpox produced pocks at 39.5 °C (Table 1). At 40°C no evidence of virus multiplication was

obtained by sub-culture of membranes. At 39°C the pock size was considerably reduced but pock number was only slightly reduced. At 39.5 °C the efficiency of pock production was 10% ; the pocks were small but their specificity was confirmed by sub-culture. Cowpox produced pocks at 40°C although efficiency of pock production was suppressed more at 39 °C than it was with elephantpox. The rate of decline in pock production differs for different viruses and is not always linear (Bedson & Dumbell, 1964). Vaccinia strains produce pocks above 40 °C (Bedson & Dumbell, 1961 ; Baxby & Hill, 1971) and so these results tend to distinguish elephantpox virus from both vaccinia and cowpox viruses.

Electronmicroscopy

Cells infected with elephantpox virus had the large, oval-round homogeneous, cytoplasmic inclusions first demonstrated by Downie (1939) for cowpox using light microscopy and subsequently referred to as A-type inclusions (Kato *et al.* 1959). Mature virions were seen inside these inclusions (Fig. 1), and so they can be referred to as the V+ variety of A-type inclusion (Kato *et al.* 1963). Of the members of the variola/vaccinia subgroup of poxviruses so far studied, only cowpox and ectromelia viruses produce A-type inclusions (Baxby, 1975*b*) and this result perhaps suggests a closer relationship to those viruses than to the other members.

Rabbit inoculation

The lesions produced by EP-2 developed more quickly than those of cowpox virus, and although inflamed and oedematous, lacked the purple-black necrotic centre so typical of cowpox (Downie, 1939). Although precise estimates of the minimum infective doses were not made, the rabbit was infected by 5×10^2 p.f.u. of EP-2 but not by 50 p.f.u. and also infected by 0.5 p.f.u. of cowpox virus, the lowest dose tested. Thus, rabbit inoculation enabled us to differentiate elephantpox and cowpox as previously suggested (Mahnel, 1974).

Tissue culture techniques

Although detailed studies were not made, both strains of elephantpox virus produced plaques in both RK13 and VERO cells. The plaques were possibly smaller than those of vaccinia and cowpox viruses but not so consistently or obviously as to permit identification.

Heat resistance

Under the conditions of test, which were rigorously controlled (Baxby, 1975*a*), the infectivity of suspensions of elephantpox viruses was reduced by

0.5 log when heated at 56°C for 20 min. Under identical conditions, strains of cowpox virus lost at least 1.9 logs.

Table 1. *Production of pocks by elephantpox and cowpox at raised incubation temperatures*

Virus		Incubation temperature (°C)			
		35*	39	39.5	40
Cowpox	%	100	33	8	2
Cowpox	Size	+++	+++	++	++
EP-1	%	100	83	10	0
EP-1	Size	++	+	(+)	—
EP-2	%	100	93	10	0
EP-2	Size	++	+	(+)	—

* Pock count at 35 °C (usually 100 to 150) reduced to 100 %, other values adjusted accordingly. (+) = Very small pocks, seen and counted only with difficulty.

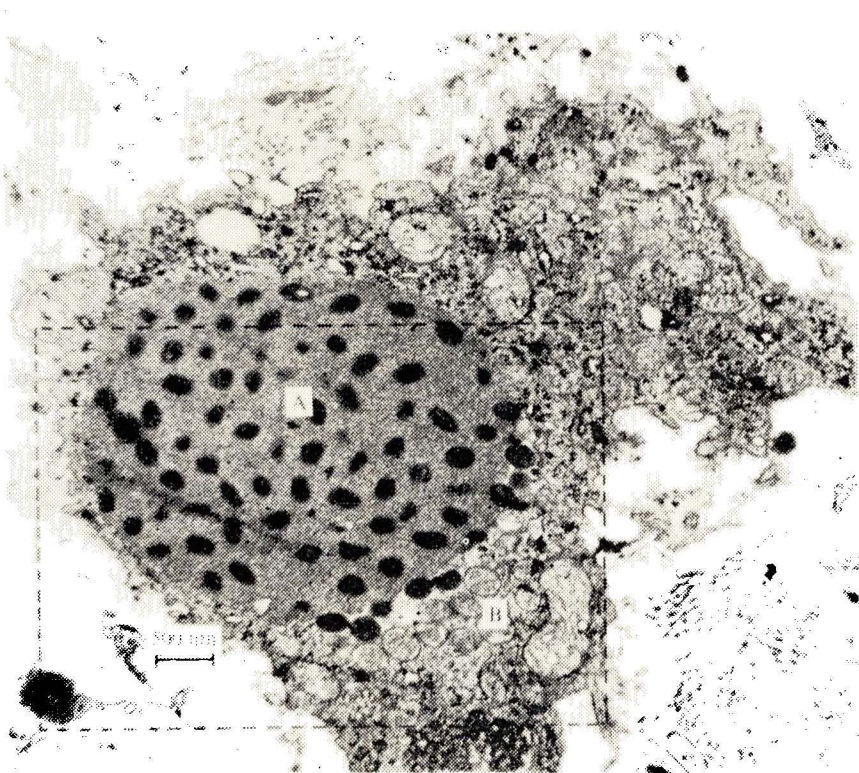


Fig. 1. Thin section of cytoplasm of CAM cell infected with elephantpox virus (FP-2). A = A-type inclusion containing numerous mature virions. B = B-type inclusion showing virions in various stages of maturation.

Antigenic characteristics

Haemagglutination-inhibition

Antisera made against cowpox, elephantpox and vaccinia viruses gave similar HAI titres against the different viruses so, although the HAI test may be of some value in differentiating certain poxviruses (Rondle & Sayeed, 1972), it was of no value here.

Gel diffusion

Extracts of elephantpox-infected CAM failed to develop the 'LS' line when tested against antisera known to possess anti-LS. In this respect elephantpox resembles cowpox which also fails to produce the LS line whereas vaccinia does (Rondle & Dumbell, 1962; Baxby, 1972*a*). Also like cowpox some, but not all, antisera to elephantpox have antibody to LS. The presence of an antigen, 'd' possibly specific for cowpox has been reported (Rondle & Dumbell, 1962). Unfortunately none of the antisera used in the present study contained sufficient amounts of anti- 'd' to give a satisfactory control result, and the presence or absence of 'd' in elephantpox could not be investigated by gel diffusion.

Cross-neutralization

The results of cross-neutralization tests with various antisera are shown in Table 2. Antisera to live vaccinia and live elephantpox viruses gave higher titers with the homologous virus. The antiserum to live cowpox gave similar results with all the viruses. Previous work Baxby (1975*a*) indicated that antisera made against heated vaccinia and cowpox viruses show a degree of specificity for the homologous virus, and when tested in this respect elephantpox resembles cowpox (Table 2). The results summarized in Table 2 suggest that despite the close relationship between the viruses, there may be minor antigenic differences between them. This was confirmed by serum absorption tests.

Serum absorption tests

The results of cross-neutralization tests on absorbed antisera are summarized in Table 3. The titres obtained with the antisera before absorption are as shown in Table 2. Absorption of each antiserum with the homologous virus removed all antibody to it and to the other viruses.

Anti-vaccinia serum absorbed with cowpox virus would still neutralize vaccinia, as reported previously (Baxby, 1972*a*). This antiserum would also

neutralize elephantpox, suggesting that elephantpox shares with vaccinia a surface antigen lacking in cowpox. However, after absorption with elephantpox virus, the anti-vaccinia serum did not neutralize elephantpox or cowpox viruses but did neutralize vaccinia. This suggests that vaccinia virus has a specific surface antigen in addition to the one shared with elephantpox virus.

After absorption with cowpox virus the anti-elephantpox serum would neutralize elephantpox and vaccinia virus and after absorption with vaccinia virus it would neutralize elephantpox to low titre. The absence of any elephantpox-specific antigen was shown by the failure of the anti-elephantpox serum to neutralize the virus after being absorbed with both vaccinia and cowpox viruses.

The anti-cowpox serum when absorbed with vaccinia virus would neutralize elephantpox to low titre but not cowpox. After absorption with cowpox it would neutralize none of the viruses.

These results indicate that elephantpox, cowpox and vaccinia viruses although antigenically related can be differentiated by serum absorption studies, and an antigenic formula for the three viruses is discussed below.

Table 2. *Cross-neutralization tests on elephantpox, cowpox and vaccinia viruses*

Antiserum to	Test virus*			
	EP-1	EP-2	Vaccinia	Cowpox
Live vaccinia	3.8	3.9	4.6	3.7
Heated vaccinia	< 1.4	< 1.4	3.3	< 1.4
Live cowpox	NT†	4.4	4.1	4.4
Heated cowpox	NT	4.2	2.7	4.2
Live EP-2	3.8	3.6	2.9	2.4

* Figures give log of reciprocal of antiserum dilution reducing poek count to 50%.

† NT = Not tested.

Table 3. *Neutralization of elephantpox, cowpox and vaccinia viruses by adsorbed antisera**

Antiserum to	Absorbing virus	Test virus		
		Vaccinia	Cowpox	EP-2
Vaccinia	Cowpox	4.3†	< 1.4	3.8
Vaccinia	EP-2	4.4	< 1.4	< 1.4
Cowpox	Vaccinia	< 1.4	< 1.4	2.7
Cowpox	EP-2	< 1.4	< 1.4	< 1.4
EP-2	Vaccinia	< 1.4	< 1.4	2.7
EP-2	Cowpox	3.0	< 1.4	2.7
EP-2	Cowpox + vaccinia	< 1.4	< 1.4	< 1.4

* Values for titres of antisera before absorption are given in Table 2. In each case absorption of an antiserum with homologous virus removed all neutralizing antibody to it and the other viruses.

† Figures give log of reciprocal of antiserum dilution giving 50% virus

Different strains of elephantpox virus

Whereas monkeypox (Rondle & Sayeed 1972) and camelpox (Baxby, 1972*b*, 1975*b*) seem to be homogeneous entities, differences have been reported among strains of smallpox (Dumbell & Huq, 1975) and cowpox (Baxby 1975*a*) viruses which may be of epidemiological value. No significant differences were found between the two strains of elephantpox virus by using methods such as ceiling temperature, heat inactivation and inclusion type described above which have proved valuable with smallpox and cowpox.

DISCUSSION

Although 'elephantpox' was originally thought to be caused by accidental infection with vaccinia virus (Gehring *et al.* 1972) more recent studies have demonstrated some differences between elephantpox and vaccinia viruses (Mahnel, 1974). We have extended this work and describe here the results of a number of simple conventional tests which have enabled us to differentiate elephantpox virus from vaccinia and cowpox viruses. However, we were impressed by the close relationship of elephantpox to cowpox virus. In particular the two viruses produce A-type inclusions and serological tests on unabsorbed antisera failed to separate them.

Elephantpox and cowpox viruses could be differentiated by examining pock production on CAM of embryos incubated at raised temperatures. Cowpoxvirus produced pocks at and below 40°C, elephantpox only at and below 39.5°C. There are slight differences in the ceiling temperature of strains of smallpox (Bedson, Dumbell & Thomas 1963; Dumbell & Huq 1975) and vaccinia viruses (Baxby, 1974). However, cowpox (D. Baxby unpublished data) monkeypox (Rondle & Sayeed 1972) and camelpox viruses (Baxby, 1972*b*) seem homogeneous in this respect. Consequently it appears that this test supports the separation of cowpox and elephantpox virus. Although the heat resistance of cowpox virus strains varies, the results obtained with elephantpox indicate

Table 4. Suggested antigenic notation for surface antigens of elephantpox, cowpox and vaccinia viruses based on results of cross-neutralization tests using absorbed antisera

Virus	Antigen*			
	x	y	z	d?
Vaccinia	+	+	+	-
Elephantpox	+	+	+	+
Cowpox	+	-	-	(+)

+ = Antigen on surface; antibody to it neutralizes. (+) = Antigen on surface; antibody to it not neutralize. - = Antigen not on surface.

that it is more heat resistant than the most heat resistant strain of cowpox virus so far examined.

Further support for the separate identity of elephantpox virus comes from the results of cross-neutralization tests using absorbed antisera. From the results with anti-vaccinia serum variously absorbed it can be deduced that elephantpox, cowpox and vaccinia virus all share at least one surface antigen that elephantpox and vaccinia share a surface antigen (or antigens) not present on the other two viruses. A possible antigenic formula for the three viruses is shown in Table 4. The 'd' antigen of Rondle & Dumbell (1962) could not be identified by gel diffusion due to lack of a sufficiently potent antiserum. However it is known that 'd' antigen is present on the surface of cowpox virus but not vaccinia, and that anti-'d' will not neutralize cowpox (Baxby, 1972*a*). The low neutralization titres against elephantpox virus obtained with cowpox and elephantpox antisera absorbed with vaccinia virus could be explained by postulating that they contain small amounts of anti-'d' too low to be detected by the insensitive gel diffusion technique, that elephantpox virus has 'd' antigen on its surface and that it is neutralized by anti-'d'.

All members of the variola/vaccinia subgroup of poxviruses are closely-related antigenically, and differences between them may only be of a minor nature. However, serological techniques are being used to show differences between existing members (Gispen & Brand-Saathof, 1974) and it is possible that antigenic analysis may aid the characterization of newly-isolated strains. Electrophoresis of virus-induced polypeptides in polyacrylamide gels is now being used to aid poxvirus characterization (Thomas *et al.* 1975) and it is possible that techniques such as this may provide information on the relationship between closely-related viruses such as elephantpox and cowpox.

The natural history of 'elephantpox' is poorly understood. The evidence available indicates that the virus may well have another reservoir, and that elephants act as indicator hosts becoming infected only occasionally. A similar situation is now thought to occur in the infections commonly referred to as cowpox, monkeypox and carnivorepox (Baxby 1977).

With the W.H.O. Smallpox Eradication Campaign on the verge of success, it is important that the natural history of animal poxviruses, particularly those capable of infecting man, is thoroughly investigated. In particular future work should be directed towards further characterization of newly-isolated viruses and towards identifying their natural reservoirs.

We would like to thank Professor H. Mahnel for supplying both strains of elephantpox virus and for supplying unpublished information.

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