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Studies on monkeypox virus *

C. J. M. RONDLE [1] & K. A. R. SAYEED [2]

The growth characteristics, including ceiling temperatures for growth, of three strains of monkeypox virus were studied on chick chorioallantoic membrane, growth and plaque formation were studied in RK13 cells, and growth was studied in rabbit dermis. The three strains could not be distinguished by these tests but could be differentiated from variola, vaccinia, and cowpox viruses. Haemagglutination-inhibition tests with homologous and heterologous antisera also showed that the monkeypox strains were indistinguishable, although they could be differentiated from vaccinia and cowpox, but not from variola, viruses. Similar results were obtained in neutralization tests. It is suggested that the monkeypox strains constitute a homogeneous poxvirus entity. The strains can be differentiated from other poxviruses by their cultural characteristics but serologically they are more closely related to variola than to vaccinia or cowpox viruses.

ıkeypox was first described by Magnus et al.). The virus was isolated from monkeys with ɔtular disease similar in distribution to variola in and it was recognized as a poxvirus by its ,pearance under the electron microscope and by ; serological relationship to vaccinia. Other iso- ıations of monkeypox virus have since been reported; these reports are summarized by Arita & Henderson (1968). Marennikova et al. (1971) compared viruses isolated by Magnus et al. (1959), Gispen et al. (1967), and McConnell et al. (1962). It was found that morphologically 4 of the 5 viruses studied formed a homologous group different in some respects from other poxviruses. The other virus, which was isolated from apparently healthy monkey kidney, could not be distinguished from variola virus.

We have studied three of the strains investigated by Marennikova et al. (1971), comparing them morphologically and serologically with each other and with recognized strains of variola, vaccinia, and cowpox viruses. The results generally confirm and extend the earlier observations.

MATERIALS AND METHODS

Monkeypox virus strains

" *Denmark* " *(MpD)*. This strain was isolated by Magnus et al. (1959) from diseased monkeys and was passaged 9 times in our laboratory in chick chorioallantoic membrane (CAM).

" *Holland* " *(MpH)*. This strain was described by Peters (1966) and Gispen et al. (1967). It was isolated from a giant anteater with a pox-like disease. The virus was passaged 3 times in CAM in our laboratory.

" *Washington* " *(MpW)*. The strain was described by McConnell et al. (1962) and the virus was passaged 4 times in CAM in our laboratory.

All these strains were obtained from Professor K. R. Dumbell, St Mary's Hospital Medical School, London, England.

Variola virus strain

The international reference strain " Harvey " (SpH) was used.

Vaccinia virus strains

Most of the studies were made with the " Lister " strain (VLS) but the " Western Reserve " (VWR) and " Levaditi " (VLV) strains were also used occasionally.

Cowpox virus strain

The international reference strain " Brighton " (CBR) was used.

Growth of virus

Virus was propagated at 35°C by conventional methods in CAM of 12-day old fertile eggs. For haemagglutination (HA) tests, confluently infected

* From the London School of Hygiene and Tropical Medicine, London, England.
[1] Senior Lecturer.
[2] Research student.

membranes were ground with sand, using 1 ml of saline containing 50 μg of chloramphenicol per ml for each CAM. The preparations were subjected to ultrasonic vibration for 1 min by means of an MSE–Mullard disintegrator operated with a small probe at approximately 17.5 kHz with an output of 50 W and then clarified by centrifugation at 31 000 rev/min for 10 min. Partially purified virus was obtained from such extracts by several cycles of differential centrifugation at low g values and then at 8 000 g for 30 min.

For ceiling temperature tests, batches of 6–7 eggs were used for each temperature. The inoculum of 0.1 ml was adjusted to contain 80–100 CAM pock-forming units of virus, although for the higher temperatures several dilutions of virus were used. Incubators were kept in a constant-temperature room at 35°C and they were opened only twice a day to check the temperature by means of thermometers immersed in beakers of water inside. No temperature variations greater than 0.2 degC were noted.

Virus was also grown in RK13 cells (Beale et al., 1963). Conditions of growth for maximum virus yield and for plaque production have been described by Baxby & Rondle (1967). Virus and haemagglutinating and soluble antigens were released from suspensions of infected cells by means of the ultrasonic treatment described above.

The growth of monkeypox virus in rabbit dermis was studied by inoculating depilated rabbits intradermally with virus passaged 4 times in RK13 cells. Inoculations, using 0.1 ml of suspension, were made in duplicate at random on each flank of the animal. The inoculum contained 10, 20, 40, or 80 RK13 plaque forming units of virus.

Antisera

Antisera to vaccinia (aVLS) and cowpox (aCp) were prepared as described by Rondle & Dumbell (1962). Antisera to monkeypox (aMpD, aMpH, aMpW) were prepared similarly except that virus passaged 4 times in RK13 cells was used instead of virus grown in rabbit dermis. Pooled serum from convalescent cases was used as an anti-variola (aSp) serum.

Haemagglutination and haemagglutination-inhibition tests

Haemagglutination and haemagglutination-inhibition (HI) tests were made by conventional methods using WHO Perspex plates instead of agglutination tubes. In all tests, susceptible fowl cells (0.5%) were suspended in 0.9% saline containing pre-immunization or " normal " rabbit serum. In HI tests 4 HA units were used.

Neutralization tests

These tests were made on CAM as described by Downie & McCarthy (1950) but using the egg punch of McCarthy & Dumbell (1961). Virus dilutions were made with 0.9% saline or with water containing 50 μg of chloramphenicol per ml. Whenever possible, the diluent contained 10% of pre-immunization serum corresponding to the serum under test; when such serum was not available, e.g., for human convalescent serum, serum from uninoculated rabbits was used.

Gel-diffusion tests

These were carried out by the method described by Rondle & Dumbell (1962), except that 1 : 5 sodium azide was used as preservative ins thiomersal. The soluble antigens studied were prepared for HA and HI tests.

RESULTS

Growth on CAM

No difference was observed between the 3 isolates of monkeypox that were tested. Pocks were not visible after incubation for 24 h. After incubation for 48 h, minute nonhaemorrhagic pocks were seen. After 72 h, pocks were approximately 0.5 mm in diameter and could be counted with the naked eye. Even after incubation for 96 h no pocks exceeded 1 mm in diameter. At this time secondary pocks appeared. The pocks were similar to those produced by variola and alastrim although they were slower to develop and some showed a pink central zone. The pink zone was quite distinct from the haemorrhage evident in cowpox pocks, and in size, time of development, and general appearance the pocks were easily distinguishable from those produced by vaccinia virus. These findings agree with those for MpD (Magnus et al, 1959) and MpW (McConnell et al. 1962), and are similar to those reported by Marennikova et al. (1971) who has described a central haemorrhage as characteristic of monkeypox pocks on CAM.

Monkeypox isolates were tested for ceiling growth temperature. The results of the most comprehensive experiment are shown in Table 1. These results indicate that the ceiling temperature for all the viruses

Table 1. Ceiling temperatures for growth of monkeypox virus

Virus strain	Relative pock count at the following temperatures (°C) [a]				
	35	38	39	39.5	40
MpD	100	77	61	0.1	0
MpH	100	52	62	0	0
MpW	100	95	81	0	0

[a] The mean count at 35°C (70–80 pocks per membrane) is taken as 100%; other counts are expressed as a percentage of this. Readings were taken after incubation for 72 h.

lay between 39°C and 39.5°C. This agrees with a reported ceiling temperature for MpD of 39°C (Bedson & Dumbell, 1961) and does not conflict with a reported ceiling temperature of not less than 38.3°C for one of the monkeypox strains isolated by Gispen et al (1967). Marennikova et al. (1971) reported a ceiling temperature for monkeypox of 39–40°C. These temperatures distinguish the strains of monkeypox tested from variola, vaccinia, and cowpox viruses.

Growth in RK13 cells

The three monkeypox isolates grew well in RK13 cells and the cytopathic changes observed were identical, being characterized by a rounding of the affected cells, which subsequently became granulated and condensed. The changes were observed 24–48 h after infection. Cells later vacuolated, degenerated, and had become detached from the glass substratum in 5–6 days. The results were similar to those obtained for vaccinia and cowpox viruses, and to those reported for a monkeypox virus in rabbit kidney epithelium by Prier & Sauer (1960) and for MpD in newborn rabbit kidney cells by Marennikova et al. (1971).

As shown by growth on CAM, virus was readily recovered after 4 passages in RK13 cells. Most virus was associated with the cells but was easily released by ultrasonic treatment.

Virus titrations in RK13 cells were as sensitive as those on CAM, provided the virus was absorbed for 2 h at 37°C and that monolayers were incubated at the temperature. Absorption for 1 h at 35°C and incubation at the same temperature gave rise to plaque counts significantly lower than pock counts on CAM.

Growth in rabbit dermis

All strains behaved in a similar way. With small inocula of virus (10 CAM pock-forming units) no lesions appeared. With larger doses of virus papules formed and became haemorrhagic, progressing to necrotic ulceration. Results obtained with MpD are given in Table 2.

These results agree with those reported by Magnus et al. (1959) for MpD and by Gispen et al. (1967) for MpH. Prier & Sauer (1960) and McConnell et al. (1962) also passaged monkeypox in rabbit dermis without difficulty.

Thus monkeypox is seen to behave like cowpox when grown in rabbit dermis. The lesions are quite distinct from those produced by vaccinia virus, and the ease with which the virus grows distinguishes it from variola virus.

Table 2. Development of papules in a rabbit inoculated with MpD.

Day	No. of pock-forming units in the inoculum		
	20	40	80
2	red papule 3 mm in diameter	red papule 4 mm in diameter	red papule 6 mm in diameter
4	red papule 4 mm in diameter	red papule 6 mm in diameter	red papule 7 mm in diameter
6	papule subsided	papule 8 mm in diameter, purple centre 3 mm in diameter	papule 10 mm in diameter, purple centre 5 mm in diameter
9	healed, no scar	subsiding, red-brown crustation	haemorrhage at centre
12		healed, no scar	lesion black, necrotic
17			healed, scarred

Table 3. Results of haemagglutination-inhibition tests

Sera	Reciprocal HI titres of serum tested against:							
	MpD	MpH	MpW	SpH	VLS	VWR	VLEV	CBR
aMpD	2 560	1 280	2 560	2 560	2 560	2 560	2 560	320
aMpH	2 560	2 560	1 280	2 560	2 560	2 560	2 560	640
aMpW	2 560	2 560	2 560	5 720	2 560	2 560	2 560	320
aVLS	640	320	320	160	5 120	5 120	5 720	640
aCp	320	320	320	80	1 280	1 280	1 280	2 560

Haemagglutination and haemagglutination-inhibition tests

Prepared as described and tested against the most sensitive fowl cells available, haemagglutinating antigen from all three strains of monkeypox virus had titres of 1 : 68–1 : 128. This compares with titres of 1 : 256–1 : 512 for vaccinia virus, 1 : 128–1 : 256 for cowpox virus, and 1 : 16–1 : 32 for variola virus. It could not be decided from the data if monkeypox virus regularly produced more haemagglutinating antigen than variola virus and less than vaccinia or cowpox viruses.

The HI titres of various sera were tested against standard preparations of haemagglutinating antigen. The results of one experiment are shown in Table 3. In this test, antisera to any of the three strains of monkeypox would not distinguish between homologous and heterologous strains, nor would they distinguish monkeypox from variola or vaccinia viruses, but they did distinguish those viruses from cowpox virus. The vaccinia serum reacted in a significantly higher titre to haemagglutinating antigen of the homologous virus than to that of the other viruses. The cowpox serum reacted significantly more strongly to cowpox and vaccinia haemagglutinating antigens than to the monkeypox and variola antigens. These results suggest that with respect to haemagglutination monkeypox and variola viruses are more closely related to each other than they are to vaccinia or cowpox viruses.

Neutralization tests

Antisera to the three strains of monkeypox virus were tested against each strain for neutralizing antibody. The results of the most comprehensive experiment are shown in Table 4. No distinction can be made between the strains and the sera chosen for study; all had an end point of approximately 10^{-4}.

In one experiment an attempt was made to absorb antisera with the other strains of virus. Two absorptions of partially purified virus grown on CAM were used. Absorption of a MpD, a MpH, or a MpW sera by any of the monkeypox viruses reduced the neutralization titre to less than 50% at serum dilutions of 10^{-1}.

Monkeypox sera were tested also against variola, vaccinia, and cowpox viruses. Human convalescent variola serum and rabbit antisera to vaccinia and cowpox were included in the study. Typical results are given in Table 5. In this table, results with aMpH, aMpW. MpH, and MpW sera are omitted since they were essentially the same as those obtained with aMpD and MpD. The four variola convalescent sera that were tested also behaved similarly and results for one only are included. Insufficient variola

Table 4. Results of neutralization tests on three strains of monkeypox virus

Serum and data		Virus strains used		
		MpD	MpH	MpW
MpD	10^{-1}	100 [a]	100	100
	10^{-2}	93	91	90
	10^{-3}	72	72	70
	10^{-4}	56	54	52
MpH	10^{-1}	100	100	100
	10^{-2}	92	98	93
	10^{-3}	70	78	67
	10^{-4}	47	50	43
MpW	10^{-1}	100	100	100
	10^{-2}	92	95	95
	10^{-3}	70	73	81
	10^{-4}	47	48	58

[a] Percentage neutralization of virus with respect to control counts.

Table 5. Results of neutralization tests on various poxviruses

Serum and dilution	Virus used			
	MpD [a]	SpH	VLS	CBR
aMpD				
10^{-1}	100 [b]	100	86	78
10^{-2}	93	94	54	47
10^{-3}	72	71	18	32
10^{-4}	56	53	4	9
V53 [c]				
10^{-1}	100	100	61	—
10^{-2}	89	92	36	—
10^{-3}	51	53	24	—
10^{-4}	19	22	7	—
aVLS				
10^{-1}	76	95	100	82
10^{-2}	56	71	98	74
10^{-3}	34	41	77	55
10^{-4}	16	14	53	16
aCP				
10^{-1}	78	85	97	100
10^{-2}	62	70	88	85
10^{-3}	46	48	66	74
10^{-4}	25	28	40	48

[a] Some values are taken from Table 4.
[b] Percentage neutralization of virus with respect to control counts.
[c] Smallpox convalescent serum.

serum was available to include cowpox virus in these tests.

It is evident that aMpD is significantly more active against monkeypox and variola than against vaccinia or cowpox viruses. Convalescent variola serum neutralized monkeypox and variola viruses more effectively than it neutralized vaccinia viruses. Vaccinia serum was more active against vaccinia and cowpox viruses than against monkeypox and variola viruses, and cowpox serum had a wide spectrum of activity. These results again suggest a closer relationship between monkeypox and variola than between monkeypox and vaccinia or cowpox.

Gel-diffusion tests

Using reagents described by Rondle & Williamson (1968), line pattern components corresponding to the "L" and "S" antigens of vaccinia were detected in monkeypox soluble antigen. These serological specificities are almost invariably absent from cowpox soluble antigen, although one or other is usually present in cowpox antisera (Rondle, unpublished data). No qualitative differences were observed between monkeypox, variola, and vaccinia, although in those experiments only 6–7 line pattern components were detected.

DISCUSSION

We have examined three strains of monkeypox virus isolated at various times in different places. Where comparisons were possible our results have generally agreed with those reported by other authors. Although a variety of techniques were used, it was not possible to distinguish between the three viruses that were examined, and it seems reasonable to conclude that they constitute a homogeneous poxvirus entity.

Growth on CAM, including ceiling temperatures and pock characteristics, differentiated these viruses from variola, vaccinia, and cowpox viruses. There are, however, some discrepancies in descriptions of the appearance of pocks on CAM. Marennikova et al. (1971) have stressed that haemorrhage is a characteristic feature of monkeypox, especially in cases of confluent lesions. We noted pink centres in some pocks but agree with Magnus et al. (1969) that many pocks were similar to those of variola and alastrim. These differences could be due to the experimental conditions, including the types of egg used to culture the virus.

Growth in RK13 cells was similar to that of vaccinia and cowpox viruses, but optimum conditions for quantitative plaque assays comparable with titrations on CAM were different from those determined for those viruses by Baxby & Rondle (1967). Unlike variola virus, monkeypox virus grew readily in rabbit dermis, producing haemorrhagic and necrotic lesions similar to those observed with cowpox.

Production of haemagglutinating antigen by monkeypox virus grown in CAM was generally greater than that of variola, and less than that of vaccinia and cowpox viruses. In HI tests appropriate sera distinguished monkeypox from vaccinia and cowpox but not from variola viruses. Neutralization tests reinforced the results of HI tests and again distinguished monkeypox from vaccinia and cowpox but not from variola viruses.

It is interesting to note that Douglas et al. (1969) studied the surface of MpH by means of an electrophoretic technique. They concluded that the surface

of MpH was dissimilar to that of vaccinia and cowpox viruses and more like that of variola and alastrim viruses.

These results suggest that the monkeypox viruses examined constitute a separate poxvirus entity. They have certain distinctive cultural properties but serologically they are related more closely to variola than to vaccinia or cowpox viruses.

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RÉSUMÉ

ETUDES SUR LE VIRUS DU MONKEYPOX

Les présentes recherches ont porté sur trois souches de virus du monkeypox d'origine géographique différente: Danemark, Pays-Bas et Etats-Unis d'Amérique. De nombreux tests ont été utilisés pour les comparer entre elles et avec des souches de référence du virus variolique, du virus vaccinal et du virus du cowpox: développement, aspect des pustules et température limite de croissance sur membrane chorio-allantoïde (MCA) de l'embryon de poulet; croissance et formation de plages sur cultures cellulaires RK 13; croissance dans le derme du lapin; production d'hémagglutinine, inhibition de l'hémagglutination et neutralisation.

Malgré la diversité de ces techniques, on n'a pas réussi à différencier les trois souches de monkeypox étudiées, et il semble raisonnable de les considérer comme constituant une entité homogène au sein du groupe des poxvirus.

Les modalités de la croissance sur MCA et l'aspect des pustules permettent de différencier le virus du monkeypox du virus variolique, du virus vaccinal et du virus du cowpox. Les pustules produites par le virus du monkeypox sont nettement visibles après 72 heures d'incubation. Elles ressemblent à celles produites par le virus variolique, mais nombre d'entre elles présentent une zone centrale rosée. Leur diamètre n'excède pas 1 mm, même après 96 heures d'incubation. La température limite de croissance est de 39°C-39,5°C.

Sur culture cellulaire RK 13, la croissance du virus du monkeypox ne diffère pas de celle du virus du cowpox et du virus vaccinal. Dans la peau du lapin, le virus du monkeypox — contrairement au virus variolique — se développe aisément, produisant des lésions hémorragiques et nécrotiques semblables à celles que provoque le virus du cowpox.

Cultivé sur MCA, le virus du monkeypox fournit en général des titres d'hémagglutinine supérieurs à ceux du virus variolique, mais inférieurs à ceux du virus vaccinal et du virus du cowpox.

En épreuves d'inhibition de l'hémagglutination, pratiquées avec des antisérums homologues et hétérologues, le virus du monkeypox n'a pu être différencié du virus variolique, mais bien du virus vaccinal et du virus du cowpox. Les épreuves de neutralisation ont également montré que le virus du monkeypox était plus proche du virus variolique que du virus vaccinal et du virus du cowpox.

Des épreuves de diffusion en gel ont révélé que le virus du monkeypox renfermait les antigènes solubles décelés dans les tissus infectés par le virus variolique et le virus vaccinal.

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