Bull. Org. mond. Santé }
Bull. Wld Hlth Org. } 1972, **46**, 585-592

"White" poxvirus strains from monkeys *

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Pox-like disease in monkeys has been reported as being either the source or the outcome of smallpox in man. A survey of such outbreaks has been published by Arita & Henderson (1968).

Von Magnus et al. (1959) described the isolation of a simian poxvirus from an outbreak of a pox-like disease in a colony of *Cynomolgus* monkeys in Copenhagen. The virus produced pocks in chick chorioallantoic membrane similar to those of variola. The antigenic patterns of both viruses appeared to be identical in immunodiffusion tests against antivaccinia serum and cross-immunity was demonstrated in active immunization tests (von Magnus et al., 1959; Gispen, Verlinde & Zwart, 1967).

Monkeypox virus can be distinguished from variola virus by the haemorrhagic-necrotic reaction following intradermal inoculation of the virus into rabbit skin. The virus can also be transmitted through rabbits by the intradermal route; variola virus cannot.

Testing the host range of several poxviruses in different cell cultures, we found that monkeypox and variola viruses could be differentiated in RK 13 cell cultures. Monkeypox virus grew well and produced plaques in RK 13 cells for an indefinite number of subinoculations. Inoculation of variola virus led to hyperplastic foci in the first passages only. Transmission of the virus through more than a few passages failed.

Marennikova, Gurvič & Šelukina have shown recently (unpublished results) that a monkeypox virus strain (64-7275), isolated in our laboratory from monkey kidney in the absence of pox-like disease, differed from other monkeypox virus strains. Reactions caused by 64-7275 on chick chorioallantoic membranes were not haemorrhagic, whereas other strains derived from animals with lesions of monkeypox produced haemorrhagic reactions under similar conditions. Strain 64-7275 could also be differentiated from other monkeypox virus strains in a continuous cell line from pig embryo kidney. Its properties seemed to be intermediate between those of monkeypox and variola. The authors used the name "Latent" or L strain suggesting that the intermediate character might be characteristic for virus strains derived from healthy animals.

Starting from these data we investigated strain differentiation with regard to clinical and subclinical infections. This report concerns only poxviruses belonging to the variola–vaccinia subgroup that produce small pocks in chick chorioallantoic membranes (CAM).

MATERIALS AND METHODS

Viruses

A sample of monkeypox virus was obtained from Dr Preben von Magnus in 1959. This virus was maintained as a reference and is referred to as the Copenhagen strain. Eleven strains were isolated during an outbreak of monkeypox in Rotterdam from diseased apes, monkeys, and a giant anteater (Gispen, Verlinde & Zwart, 1967). A sample of a monkeypox virus strain from the National Center for Primate Biology, Davis, Calif., USA, was sent by Dr S. S. Kalter, of San Antonio, Texas. Variola major virus, strain Tilburg, was isolated from a smallpox patient during the Tilburg outbreak in 1951 and variola minor virus strain Den Haag was isolated from an alastrim patient during the epidemic in The Hague in 1953–54. Virus strains 64-7255, 64-7275 and 64-9411 were isolated from routine *Cynomolgus* kidney cell cultures, at a time when there was no pox-like disease present in the monkey colony of the institute (Gispen & Kapsenberg, 1967).

Tissue culture

Details of RK 13 cell culture have been described (Gispen & Brand-Saathof, 1967).

Virus titration

Virus was titrated by pock count in chick chorioallantoic membranes (CAM) according to a technique described by Westwood, Phipps & Boulter (1957). The diluent used was 10% skimmed milk. The inoculated eggs were incubated at 35°C for 3 days.

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Animal test

Healthy adult rabbits were depilated and inoculated one day later by intradermal injection of 0.1 ml of a 10% suspension of CAM with confluent lesions. The skin reaction was read each day for at least 1 week.

For virus transmission the animals were sacrificed 3 days after inoculation. The skin of the inoculated area was excised and emulsified to a 10% suspension in phosphate buffered saline, pH 7.2. Failure of virus growth was accepted if after one or more passages the virus could not be reisolated in CAM.

Immunofluorescence

Antigen preparations were made by inoculating RK 13 cell cultures on coverslips with about 80 pock-forming units of monkeypox virus. After 3 days of incubation the cell cultures were fixed with absolute methanol, precooled at −70°C, for 30 min at −70°C. Conjugated rabbit antivaccinia serum was used at 8 times the titre concentration for direct immunofluorescence to examine the localization of cells containing virus antigen. Further details of these methods have been published elsewhere (Gispen & Brand-Saathof, 1967).

Chick embryo lethality test

The CAMs of groups of at least 5 chick eggs were inoculated with various dilutions of the virus. The mean reciprocal survival time in days was plotted against the log virus dose. The D4 value, the log virus dose corresponding to a mean reciprocal survival time of 0.25, was estimated graphically.

RESULTS

Pock character

Poxvirus strains were placed in three categories according to whether they originated from (*a*) monkeypox lesions, (*b*) healthy monkey kidney cell cultures, or (*c*) human smallpox lesions. The strains were inoculated on to CAM and rabbit skin. The reactions are summarized in Table 1.

All strains from monkeypox lesions produced red-coloured reactions in CAM, which were most striking in membranes with confluent lesions. After inoculation at a higher dilution the reaction looked pink as a result of small dilated blood vessels in the centre of each pock. The size of the " red " pocks 3 days after inoculation was about 0.5 mm.

At the higher dilutions a few white pocks were seen among the many red ones (Fig. 1). The white mutant pocks were more prominent and had a larger diameter of about 1 mm. All 13 strains isolated from animals with pox-like lesions produced both types of pocks.

Table 1. Reactions in CAM and rabbit skin

Virus strain	Pocks on CAM		Rabbit skin	
	Red	White	Haemorrhagic–necrotic reaction in 1st passage	Virus transmission in 3rd passage
Isolated from monkeys with pox-like diseases				
Copenhagen, *Cynomolgus*	+	few	+	+
Rotterdam	+	few	+	+
California	+	few	+	+
Isolated from normal *Cynomolgus* monkey kidney cell cultures				
64–7255	−	+	+	−
64–7275	−	+	variable	−
64–9411	+	few	+	+
Isolated from human patients with smallpox or alastrim				
Variola major, Tilburg	−	+	−	−
Variola minor, Den Haag	−	+	−	−

The pock characteristics of virus strains from healthy *Cynomolgus* monkey kidney cells were heterogeneous. One strain (64-9411) was haemorrhagic and could not be distinguished from the Copenhagen strains of monkeypox virus. Two other strains (64-7255) and (64-7275) produced white confluent lesions and separate white pocks with a diameter of about 1 mm that protruded more from the CAM. These wild white strains resembled variola virus in the size and character of the pocks (Fig. 2). The ceiling temperature for growth was 38.3°C.

Reaction and growth in rabbit skin

The strains from monkeypox lesions caused a haemorrhagic–necrotic lesion after intradermal injection in rabbit skin. These strains could also be transmitted through at least 3 skin passages in rabbits (Table 1).

Strain 64-9411 derived from healthy monkey kidney could also be grown through 3 passages in rabbit skin and gave similar haemorrhagic–necrotic reactions. The other two strains from healthy monkey kidney could not be transmitted in rabbits. One of these (64-7255) caused a typical necrotic lesion in

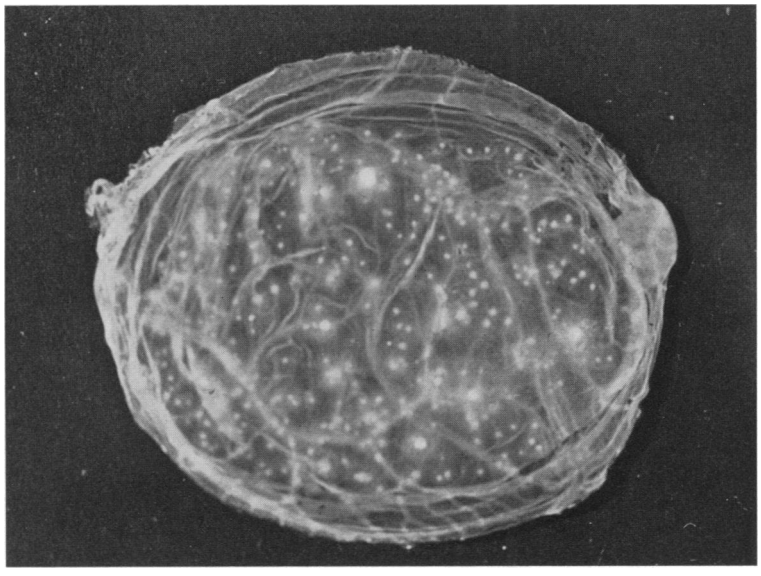

Fig. 1. Pocks of the monkeypox virus Copenhagen strain, 3 days after inoculation with a 10^{-4} dilution. A few larger white pocks (white mutants) are present (× 2).

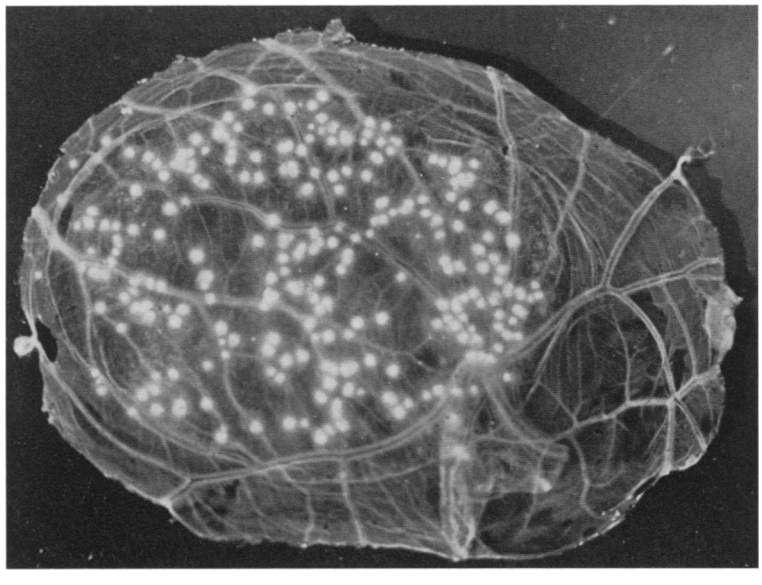

Fig. 2. Pocks of the " white " virus strain 64-7275, 3 days after inoculation with a 10^{-6} dilution (× 2).

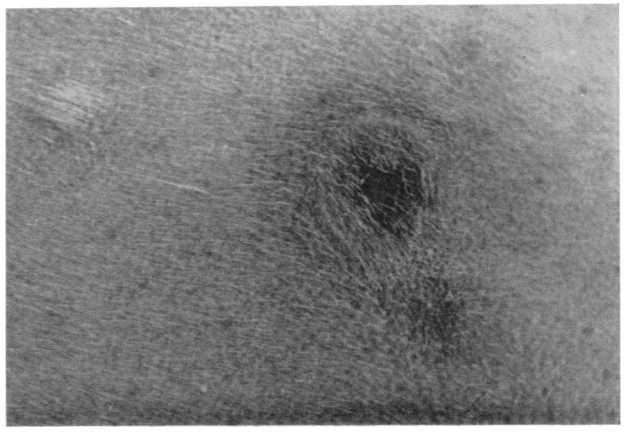

Fig. 3. Black-necrotic reaction in rabbit skin 6 days after inoculation with the " white " virus strain 64-7275: first passage.

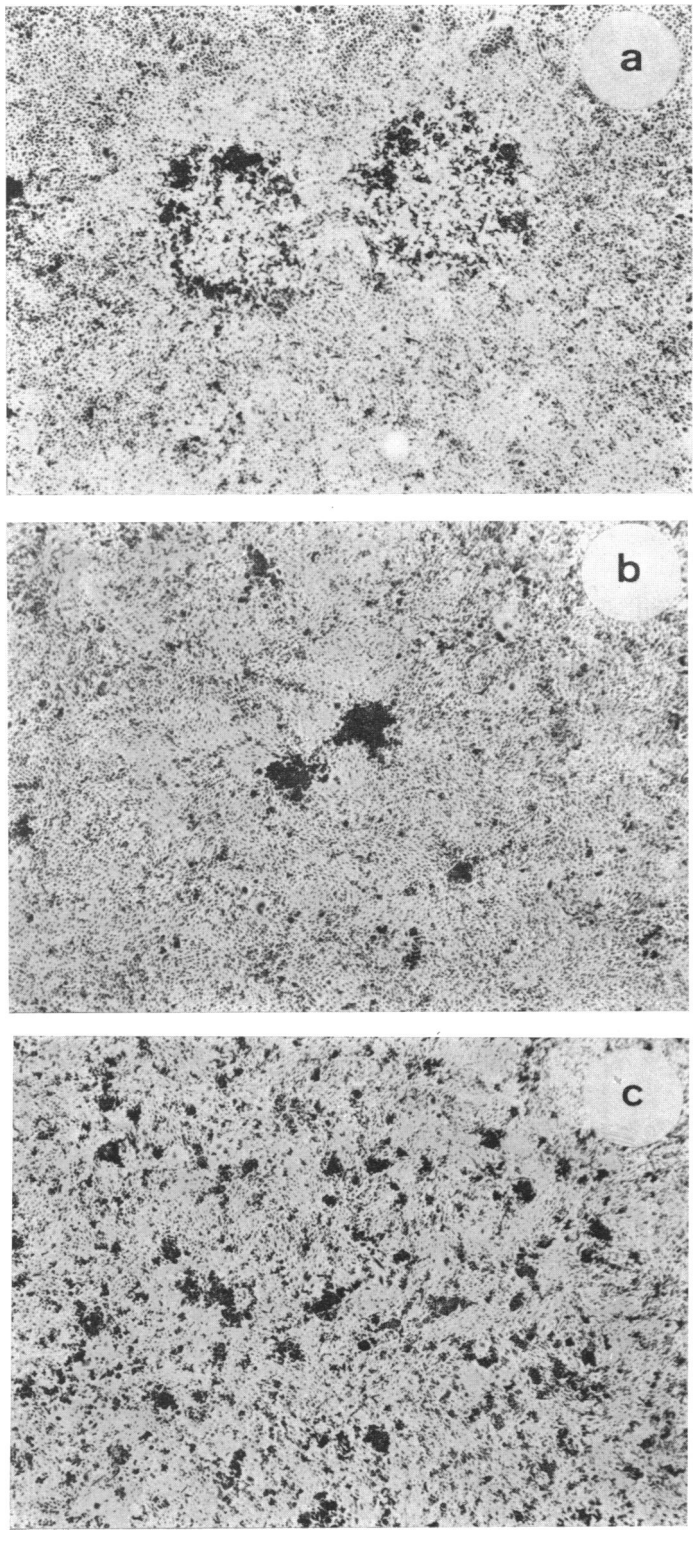

Fig. 4. Cytopathic effect in RK 13 cells induced by (*a*) monkeypox virus Copenhagen (plaques), (*b*) white virus strain 64-7275 (hyperplastic foci), and (*c*) variola major virus (hyperplastic foci) : first passages (× 33).

the first passage only. The reaction of the other (64-7275) varied from a slight red induration to a lesion with a black–brown coloured necrotic centre (Tables 1 and 2; Fig. 3).

Table 2. Rabbit skin reaction of wild white strain, 64-7275

Virus	Suspension concentration (%)	Number of rabbits	Black–brown necrosis	
			Present	Not present
64–7275	20	4	1	3
64–7275	10	7	1	6
Variola major	20	4	0	4
Variola major	10	6	0	6

Variola major and minor viruses induced a red induration in the first passage and could not be transmitted by further subinoculation in rabbits.

Cytopathic effect and growth in RK 13 cells

The viruses were inoculated in tube cultures of RK 13 cells without agar overlay. The cytopathic effect was read after 3 and 7 days of incubation (Table 3). The virus strains from monkeypox lesions caused sharply defined, small plaques within 3 days (Fig. 4). The number of plaque-forming units (PFU) determined with the Copenhagen strain were fairly reproducible in five tests. The titre of a stock virus suspension of the Copenhagen strain varied from 3.6×10^8 to 4.6×10^8 PFU/ml. Variola viruses failed to produce plaques, but hyperplastic foci were seen in the first passage in RK 13 cells. The strains from healthy monkey kidney cells caused either hyperplastic foci (64-7255 and 64-7275) or plaques (64-9411). All plaque-forming strains could be grown in RK 13 cells, whereas the viruses that produced merely hyperplastic foci were lost within 3–7 passages through RK 13 cell cultures.

Table 3. Growth in RK 13 cells

Virus strain	Cytopathic effect in 1st passage	Virus transmission: titration of 3rd–7th passage (pock-forming units/ml)
Isolated from monkeys with pox-like diseases		
Copenhagen	plaques	1.6×10^7
Rotterdam	plaques	$> 1.0 \times 10^5$
California	plaques	1.0×10^7
Isolated from normal *Cynomolgus* monkey kidney cell cultures		
64–7255 wild white strain	hyperplastic foci	negative
64–7275 wild white strain	hyperplastic foci	negative
64–9411	plaques	1.4×10^7
Isolated from human patients with smallpox or alastrim		
Variola major, Tilburg	hyperplastic foci	negative
Variola minor, Den Haag	hyperplastic foci	negative

A white mutant from the Copenhagen strain

As only a few white pocks were produced among a large majority of red ones by each of the red monkeypox strains, we tried to isolate a white mutant and to purify the strain by repeated selective subinoculations. The reaction of the Copenhagen strain in CAM appeared to vary with environmental temperature. At 33°C the reaction was definitely red, at 38°C it was predominantly white. We tried to isolate a white mutant from CAM that had been inoculated with the Copenhagen strain and incubated at 35°C. Virus from white pocks was subinoculated with a sharp needle through a series of passages in egg membranes. After 11 passages a pure white mutant was obtained which appeared to be genetically stable. The mutant produced only white pocks at either 33° or 38°C even in confluent lesions (Table 4).

The relevant properties of the mutant and parental strain were compared (Table 4). The reactions of the white mutant and the parental strain did not differ in rabbit skin, or in RK 13 cells. The white mutant grew well in both host systems in contrast to the two wild white viruses from healthy monkeys. The pocks of the white mutant remained white after 3 passages in rabbits or RK 13 cells.

Similar efforts to isolate a pure red strain were unsuccessful.

Histology

The cytopathic effects of the Copenhagen strain, the white mutant isolate 64-7275, and variola major virus were examined histologically in stained coverslip cultures of RK 13 cells. The parental Copenhagen strain induced focal necrobiosis with

Table 4. Comparison of the white mutant strain isolated from the monkeypox virus strain Copenhagen with the parental strain

Virus strain	Pock character		Rabbit skin		RK 13 cells	
	33°C	38°C	Haemorrhagic–necrotic reaction in 1st passage	Virus transmission: titration of 3rd passage (pock-forming units/ml)	Cytopathic effect	Virus transmission: titration of 3rd passage (pock-forming units/ml)
parental	red	white	+	4.5×10^5	plaques	1.4×10^6
white mutant	white	white	+	6.5×10^3	plaques	8.6×10^5

degeneration and central cytolysis. Multinucleate giant cells and cytoplasmic inclusions were present in the marginal parts of these plaques. Variola virus infection caused much less pronounced reactions: focal hyperplasia without central cytolysis, fewer inclusions, only a few giant cells, and scanty necrobiosis. The CPE of strain 64-7275 in stained coverslip cultures resembled that of variola virus. The white mutant, however, produced focal necrobiosis with central cytolysis similar to that produced by the parental Copenhagen strain.

Distribution of infected cells in RK 13 cell cultures

Coverslip cultures of RK 13 cells were examined by immunofluorescence for infected cells three days after inoculation with virus suspensions made from infected CAM. The cells infected with monkeypox virus, Copenhagen strain, were concentrated in round foci. Only a few infected cells were seen in the areas between the focal reactions. Almost all cells in the foci appeared to contain poxvirus antigen. Cells with variola virus antigen, however, were dispersed over the cell culture. There was no localization into foci comparable with that of the Copenhagen strain. Cells with 64-7275 virus antigen were partly localized in foci, but most of the cells in these foci did not contain poxvirus antigen. The scattering of infected cells outside the foci was intermediate between that found with variola and the Copenhagen strain of monkeypox virus.

Chick embryo lethality tests

Chick embryo lethality tests showed that the white poxviruses from healthy monkeys, 64-7255 and 64-7275, had about the same pathogenicity as variola major virus, their D4 values being 5.5, 5.7, and 5.1, respectively. In contrast, the white mutant derived from the Copenhagen strain of monkeypox virus was much more virulent, its D4 value being 2.4 (Fig. 5).

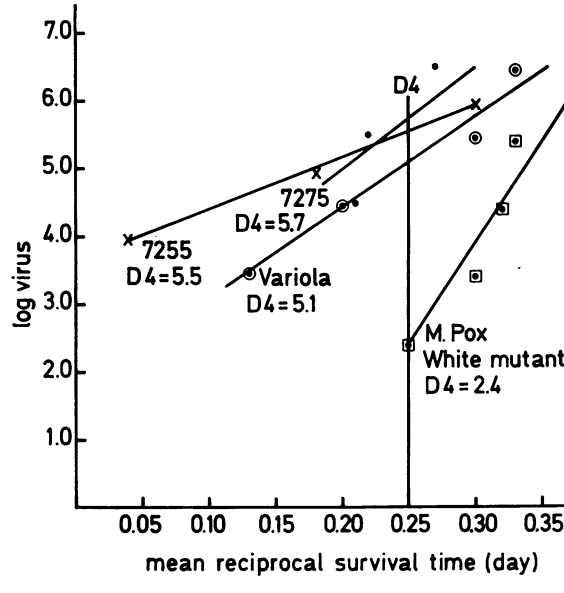

Fig. 5. Results of chick embryo lethality tests.

DISCUSSION

The heterogeneity of the poxvirus isolates from monkeys was confirmed. In our study all 13 virus strains isolated from manifest monkeypox induced red reactions in CAM at 35°C, especially when the lesions were confluent. Each of these virus strains, however, was heterogeneous, as shown by the presence of a few white pocks, which were larger than most of the red ones. The reddish colour was partly caused by small dilated blood

Table 5. Reactions of four white poxviruses of the variola subgroup with small-type pocks

Virus strain	Rabbit skin		RK 13 cells	
	Haemorrhagic–necrotic reaction in 1st passage	Virus growth	Plaques in 1st passage	Virus growth
Copenhagen, white mutant	+	+	+	+
64–7255, wild white strain	+	−	−	−
64–7275, wild white strain	±	−	−	−
Variola major, Tilburg	−	−	−	−

vessels in the centre of the pock. Separate pocks induced by higher virus dilutions showed a slight pink colour. Extravascular blood was also detected by histological examination. The influence of temperature on the pock character as reported by Baxby (1969) was obvious. The Copenhagen strain induced red pocks at 33°C and predominantly white pocks at 38°C.

By a series of selective subinoculations starting from the Copenhagen strain a pure white mutant strain was obtained, which was stable and which induced exclusively white pocks even in confluent lesions at 33°C. Similar isolates of haemorrhagic virus from the same source never became stable. The parental Copenhagen virus seems therefore to be composed of virus that continually gives rise to a few white-pock forming virus particles by mutation.

The occurrence of wild white poxvirus in healthy monkeys cannot be explained by the instability noted above. Wild white virus strains differed essentially from the white mutant strain. Three associated characters were found to be suitable to differentiate the strains: plaque formation, growth in RK 13 cells, and growth in rabbit skin. The white mutant was positive, while two wild white virus strains were negative if tested for plaque formation and associated properties. The wild white strains are unable to grow and induce focal hyperplasia in RK 13 cells only in the first passages. Similarly these wild white strains cause a haemorrhagic–necrotic reaction only in the first passage in rabbit skin. As the wild white strain does not grow, the reaction in rabbit skin is more variable. This should be taken into account when the wild white virus is being differentiated from variola virus by means of the rabbit skin reaction. The wild white poxvirus strains from monkeys form a group which seems to be more closely related to variola than to Copenhagen monkeypox virus. They resemble variola major in both chick embryo lethality tests and ceiling temperature tests.

Virus strains isolated from healthy monkeys are characterized by either white or red pocks. Wild white poxvirus strains have so far not been isolated from animals with clinical monkeypox.

ACKNOWLEDGEMENTS

The authors are indebted to Dr H. H. Vink for careful histological examination and Mr J. Wester for technical help.

RÉSUMÉ

SOUCHES « BLANCHES » DE POXVIRUS ISOLÉES CHEZ DES SINGES

On a tenté d'établir un rapport entre les différences de propriétés constatées entre souches de virus du monkeypox et l'origine épidémiologique des isolats. Cette étude comparative a porté sur une série de souches appartenant au sous-groupe variole-vaccine (13 souches isolées de lésions typiques de monkeypox et 3 souches isolées à partir de reins de singes sains) en recourant à l'inoculation de la membrane chorio-allantoïde (MCA) de l'em-

bryon de poulet, à l'inoculation intradermique au lapin et à l'inoculation de cultures cellulaires RK 13.

Toutes les souches isolées à partir de cas typiques de monkeypox ont donné sur MCA des pustules de couleur rouge surtout marquée en cas de confluence des lésions. On notait la présence, parmi les nombreuses pustules rouges, de quelques pustules blanches. Une des souches isolées de reins de singes a également produit des pustules rouges, mais les deux autres ont formé des pustules blanches (souches « blanches » sauvages).

Par passages répétés de la souche Copenhague sur MCA, on a réussi à isoler un mutant stable donnant uniquement des pustules blanches, mais des tentatives similaires pour obtenir un mutant pur donnant uniquement des pustules rouges ont échoué.

Toutes les souches « rouges », indépendamment de leur origine, provoquaient, lors de l'inoculation intradermique au lapin, une induration nécrotique et hémorragique, transmissible en série. Les deux souches « blanches » provoquaient des lésions indurées ou nécrotiques, non transmissibles, dont la teinte allait du rouge au brun. L'aspect variable de ces lésions peut s'expliquer par l'inaptitude des souches « blanches » sauvages à croître chez le lapin.

Cette étude confirme l'hétérogénéité des lésions produites sur MCA par les souches de virus du monkeypox. La différenciation entre les souches « blanches » sauvages et la souche « blanche » mutante est possible grâce à l'étude de trois propriétés: formation de plages en culture cellulaire RK 13, croissance sur cette même culture et croissance dans la peau du lapin. A l'opposé de la souche mutante, les souches « blanches » sauvages sont incapables de croître sur culture cellulaire RK 13 et ne provoquent un foyer d'hyperplasie que lors des premiers passages. De même, elles ne produisent une lésion locale chez le lapin que lors de la 1re inoculation et ne peuvent être transmises à d'autres animaux.

Sous le rapport du pouvoir pathogène pour l'embryon de poulet et de la température optimale de croissance, les souches « blanches » sauvages isolées chez des singes sains sont proches du virus variolique, tandis que la souche « blanche » mutante se comporte comme le virus du monkeypox.

REFERENCES

Arita, I. & Henderson, D. A. (1968) *Bull. Wld Hlth Org.*, **39**, 277

Baxby, D. (1969) *J. Hyg. (Lond.)*, **67**, 637

Gispen, R. & Brand-Saathof, B. (1967) *Archiv. ges. Virusforsch.*, **21**, 334

Gispen, R. & Kapsenberg, J. G. (1967) *Vers. Volksgezondh.*, **140**

Gispen, R., Verlinde, J. D. & Zwart, P. (1967) *Archiv ges. Virusforsch.*, **21**, 205

Magnus, P. von, Krag Andersen, E., Petersen, K. B. & Birch-Andersen, A. (1959) *Acta path. microbiol. scand.*, **46**, 156

Westwood, J. C. N., Phipps, P. H. & Boulter, E. A. (1969) *J. Hyg. (Lond.)*, **55**, 123