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Human infection with monkeypox virus: laboratory investigation of six cases in West Africa *

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Between September 1970 and May 1971 six cases of human infection with monkeypox virus were identified in three West African countries—Liberia, Sierra Leone, and Nigeria. Four of the cases were confirmed by viral isolation, and two were diagnosed on epidemiological and serological evidence. Poxvirus strains isolated from the four cases were indistinguishable from reference monkeypox strains (Copenhagen and Utrecht), and all were easily differentiated from variola and vaccinia viruses. The isolated strains produced small necrotic haemorrhagic pocks on CAM, grew well at 39.0°C, formed large plaques in Vero cell cultures, showed markedly more virulence for chick embryos and mice than do variola strains, and produced large necrotic haemorrhagic local lesions with generalized illness and florid secondary exanthem when inoculated into rabbit skin.

The finding of smallpox-like illness in humans resulting from infection with a poxvirus of lower animal origin serves to emphasize the importance of thorough epidemiological and laboratory evaluation of all suspect smallpox cases occurring in areas where smallpox has been or is about to be eradicated.

Between September 1970 and May 1971 six cases of suspected smallpox were reported from areas of West Africa, although these areas were believed to have been smallpox free for more than a year before the onset of these illnesses. The clinical and epidemiological features of these cases have been presented separately (Foster et al., 1971). Poxviruses with the characteristics of monkeypox virus have been recovered from four of the six cases. An additional and similar human case of apparent monkeypox virus infection has been reported from the Democratic Republic of the Congo [6] (Ladnyi & Ziegler, 1971). The laboratory investigations related to the six cases from West Africa are the subject of this report.

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METHODS

Initial viral diagnostic tests

Lesion fluid and/or crust material from the patients was examined by negative stain electron microscopy (EM) (Cruickshank et al., 1966), by agar gel precipitation (AG) for poxvirus antigen (Dumbell & Nizamuddin, 1959), and by inoculation of the chorioallantoic membrane (CAM) of 12-day-old embryonated hen's eggs using standard methods (Noble et al., 1970; World Health Organization, 1969).

Poxvirus characterization tests

Poxvirus strains recovered from the patients were characterized and compared with known reference strains of variola, vaccinia, and monkeypox viruses by the following methods:

Pock morphology on CAM. Each poxvirus strain was propagated at 35°C in the CAM of 12-day-old embryonated hen's eggs, and the membranes were harvested after 72 h for examination of pock morphology.

Ceiling temperature test in CAM. The ability of the strains to replicate at 35.0°C, 38.2°C, 39.0°C, and

39.5°C was tested by the technique described previously by Bedson & Dumbell (1961). At each temperature the inoculum (0.1 ml) was adjusted to contain sufficient pock forming units to allow quantitative measurement of virus titre loss at the different incubation temperatures. The incubators were controlled to allow no more than ±0.1°C variation.

Tissue culture plaque morphology. The Vero line of stable Green Monkey kidney cells (Macfarlane & Sommerville, 1969) was used to compare the plaquing characteristics of these poxvirus strains. Monolayers of these cells were grown in tube cultures by standard methods. The viruses were diluted in Eagle's maintenance medium to contain 20–30 plaque forming units (PFU) per 0.1 ml of inoculum, and the dilution was inoculated directly into the tissue culture fluids. After incubation at 35°C for 3 days, the cell monolayers were stained by adding 0.2 ml of 0.13% solution of crystal violet in formol directly to the tissue culture fluid. The stain was decanted after 10–15 min and the plaque morphology was examined using a dissecting microscope.

Chick embryo lethality. The virulence of the strains in embryonated hen's eggs was measured by the method described by Bauer (1960), as modified by Bedson & Dumbell (1961). Titrations of each virus in half log intervals were made in 12-day-old embryonated hen's eggs; eight eggs were inoculated with each dilution. Eggs were examined for embryo viability every 12 h for the first 4 days and daily thereafter. From the results, the log dose of virus giving a mean survival time of 4 days (the D4 value) was calculated.

Mouse virulence. The neurovirulence of each virus strain for infant Swiss white mice was measured by the method reported by Schell (1960). Titrations of each strain were made by inoculating intracerebrally eight suckling mice with each serial 10-fold dilution of virus. All mice were observed daily, and the virus dose giving a mean survival time of 4 days (D4 value) was calculated. The virulence of each virus strain was also measured by inoculating the footpads of suckling mice and observing the litters daily for signs of local or systemic infection and for death.

Pathogenesis in rabbits. Adult white rabbits were inoculated intradermally with 0.1 ml of virus suspension per dilution for each poxvirus strain. Serial 10-fold dilutions of virus were inoculated into the shaved skin of the rabbit's back to determine the dermal infectivity titre of each strain, and to compare the appearance and severity of lesions caused by one strain with those of lesions caused by similar infectious doses of other strains.

Reference poxvirus strains

The following virus strains were used in the above tests for comparison with the virus strains isolated from the West African patients.

Variola major " Harvey " — WHO Regional Smallpox Laboratory reference strain
Variola minor " Butler " — WHO Regional Smallpox Laboratory reference strain
Variola " Niger 68-12 " — isolated from a smallpox patient in Niger during 1968
Variola (West African strains V68-18, V68-258, V69-5, V69-57, V70 I-48) — isolates from smallpox patients in West Africa from 1968–70
Monkeypox " Copenhagen " — isolated by Von Magnus (1959) from monkeys with vesicular disease
Monkeypox " Utrecht 65-32 " — isolate from a giant anteater during an epizootic of pox-like disease at Rotterdam (Peters, 1966)
Monkeypox " McConnell " — isolated from diseased monkey in Washington (McConnell et al., 1962)
Vaccinia " Wyeth " — strain used for vaccine production by Wyeth Laboratories, Philadelphia, Pa., USA

RESULTS

Virus diagnostic studies

Specimen material for virus isolation studies was received from patients no. 1 and no. 4 (Liberia), from patient no. 5 (Sierra Leone), and from patient no. 6 (Nigeria). No material for virological study was collected from the lesions of patients no. 2 or 3 (Liberia). The results of the initial viral diagnostic tests on these specimens are shown in Table 1. Eight separate poxvirus isolations were obtained from specimens submitted from these patients. A representative virus isolate strain from each patient was selected for further characterization and comparative studies: strain V70-I-187 (from patient no. 1), strain V70-I-199 (from patient no. 4), strain V70-I-266 (from patient no. 5), and strain V71-I-82 (from patient no. 6).

Pock appearance on CAM

The Liberian and Sierra Leone poxvirus isolates produced small pocks with central necrosis and haemorrhage when inoculated on to the chorioallantoic membrane. The lesions resembled those produced by the Utrecht, Copenhagen, and McConnell strains of monkeypox virus and differed from those produced by the Harvey strain of variola virus, the

Table 1. Poxvirus isolation results: West African patients, 1970–71

CDC no.	Patient no.	Country	Date of onset	Material[a]	Date collected	EM[b]	AG[c]	CAM[d]
V70–I–187	1	Liberia	13 Sept. 1970	skin	18 Sept. 1970	pox	pox	small haemorrhagic pocks
				VF	18 Sept. 1970	pox	ND[e]	small haemorrhagic pocks
				crusts	23 Sept. 1970	pox	pox	small haemorrhagic pocks
				VF	23 Sept. 1970	pox	ND[e]	small haemorrhagic pocks
	2	Liberia	12 Sept. 1970	—[f]				
	3	Liberia	13 Sept. 1970	—[f]				
V70–I–199	4	Liberia	2 Oct. 1970	crusts	7 Oct. 1970	—	—	negative
				VF	7 Oct. 1970	pox	ND[e]	small haemorrhagic pocks
				crusts	10 Oct. 1970	—	—	negative
V70–I–266	5	Sierra Leone	1 Dec. 1970	VF	10 Dec. 1970	pox	pox	small haemorrhagic pocks
				crusts	10 Dec. 1970	pox	pox	small haemorrhagic pocks
V71–I–82	6	Nigeria	4 April 1971	crusts	15 April 1971	pox	pox	small pocks with minimal haemorrhage

[a] VF — vesicular fluid.
[b] EM —electron microscopy, negative stain.
[c] AG — agar gel precipitation test for poxvirus antigen.
[d] CAM — 12-day-old embryonated hen eggs — chorioallantoic membrane route.
[e] ND — not done.
[f] No specimens obtained for viral isolation.

latter being slightly larger and having no central necrosis or haemorrhage. The poxvirus isolate from the Nigerian patient formed small white pocks on CAM without appreciable haemorrhage or necrosis, and the pocks resembled most closely the characteristic pocks of variola virus.

Ceiling temperature for growth in eggs

Table 2 summarizes the results of ceiling-temperature tests on the West African isolate strains and on the reference strains of variola, monkeypox, and vaccinia viruses. Unlike variola major, the isolate strains all grew well on CAM at 39°C. When compared with the pock titres of controls grown at 35°C, the titres of variola virus grown at 39°C were decreased by more than 3 logs, whereas titres of the isolate strains or reference monkeypox strains grown at 39°C were decreased by less than 1 log. Likewise a 3-log loss in titre at 39.5°C distinguished the West African isolates from vaccinia virus, which showed no decrease in titre when incubated at the higher temperature.

Table 2. Ceiling temperature for poxvirus growth in egg CAM: results with West African poxvirus isolates, 1970–71

Virus strain	Temperature for egg incubation (°C)[a]			
	35.0	38.2	39.0	39.5
vaccinia (Wyeth)	+	+	+	+
V70–I–187	+	+	+	–
V70–I–199	+	+	+	–
V70–I–266	+	+	+	–
V71–I–82	+	+	+	–
monkeypox (Utrecht)	+	+	+	–
variola (Harvey)	+	+	–	–
variola (Butler)	+	–	–	–

[a] + = growth of poxvirus and production of pocks on CAM
– = failure of poxvirus to replicate and form pocks on CAM.

Plaque morphology in Vero cell cultures

In Vero cell monolayers, the four isolate strains and the Utrecht and Copenhagen strains of monkeypox virus formed large plaques with crenelated borders and relatively clear centres crossed by occasional cytoplasmic strands (Fig. 1). There was some rounding of cells, and intracytoplasmic vacuoles are pro-

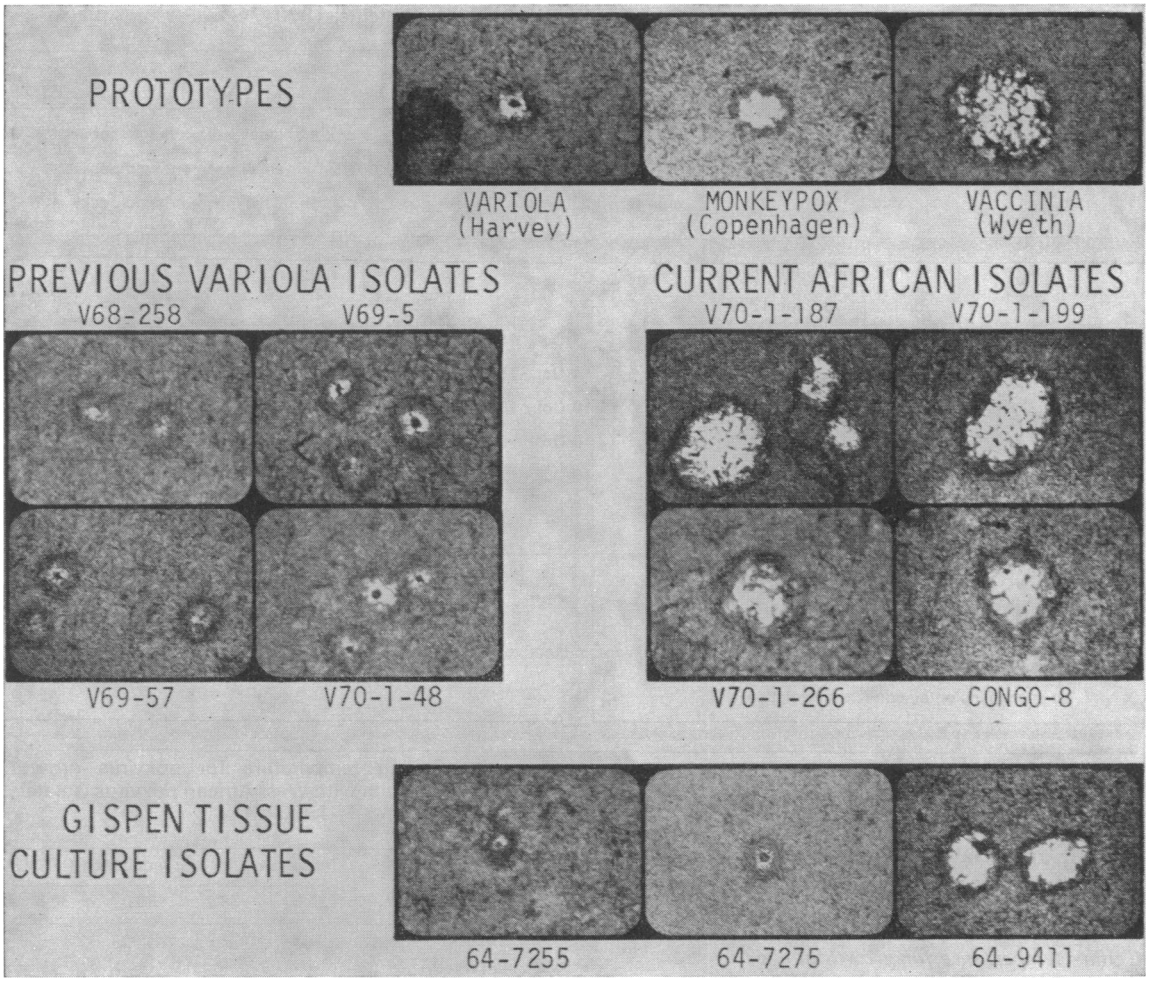

Fig. 1. Appearance of poxvirus plaques in Vero tissue culture.

minent. The plaquing characteristics of variola virus were quite different from those of monkeypox virus. Both the Harvey strain and the other variola strains isolated from previous smallpox patients in Africa cause hyperplastic clumping of cells, followed by giant cell syncytial formation; small plaques are formed as cells tear away from the syncytial clumps. The plaque appearance of the Liberian, Sierra Leone, and Nigerian isolates more closely resembled monkeypox and vaccinia viruses than variola.

Chick embrio lethality

The results of chick embryo inoculation with various doses of vaccinia, Harvey variola, and Utrecht monkeypox viruses and of isolates V70-I-187 and V70-I-266 are presented in Fig. 2. The data for V70-I-187 and V70-I-266 fall on the same line as those for the Utrecht monkeypox and vaccinia (D4 dose = 2.0–2.2 log virus/0.1 ml), and the lethality of these strains for the chick embryo is at least 1 000 times that of variola major virus (D4 dose = 5.0 log virus/0.1 ml). Strains V70-I-199 and V71-I-82 were also found to be substantially more virulent to the chick embryo than was Harvey variola.

Mouse virulence

One-day-old suckling mice inoculated intracerebrally with log dilutions of the poxvirus strains produced the following results. In the inoculated mice, death was usually preceded by cyanosis, wast-

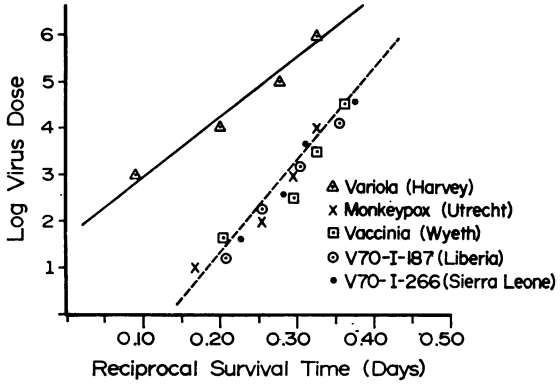

Fig. 2. Chick embryo lethality test: poxvirus isolates from Liberia and Sierra Leone.

ing, and twitching. The LD_{50} at 7 days was 6.4 log PFU/ml for Harvey variola and less than 2 log PFU/ml for Utrecht monkeypox, V70-I-187, and V70-I-266. When the reciprocal survival time is plotted against the infective dose (Fig. 3), the Liberian and Sierra Leone isolates are seen to be distinctly more lethal than the Harvey variola strain, and correspond very closely with the Utrecht strain of monkeypox virus. In addition, the inoculation of the footpads of 1-day-old mice with Utrecht monkeypox virus, V70-I-187, V70-I-199, V70-I-266, or V71-I-82 produced generalized infection and 100% mortality in the mice by day 7, whereas mouse-footpad inoculation with Harvey variola virus or Wyeth vaccinia virus produced only local infection of the limb and occasional runting but no mortality earlier than 12 days after inoculation.

Rabbit skin inoculation

All four isolate strains (V70-I-187, V70-I-199, V70-I-266, and V71-I-82) produced large, necrotic, haemorrhagic local lesions at the site of the intradermal inoculation; the infection progressed to generalized illness with florid secondary exanthem (Fig. 4). The virus could easily be recovered from the secondary lesions and could be passed into a fresh rabbit's skin. The Utrecht monkeypox virus also produced large, necrotic, haemorrhagic local lesions with generalized illness and secondary exanthem. Intradermal inoculation of Harvey variola virus, however, caused only minimal, pink, local lesions in the rabbit and the virus could not be passed more than once in rabbit skin. Vaccinia virus also produced only a local lesion with no systemic illness.

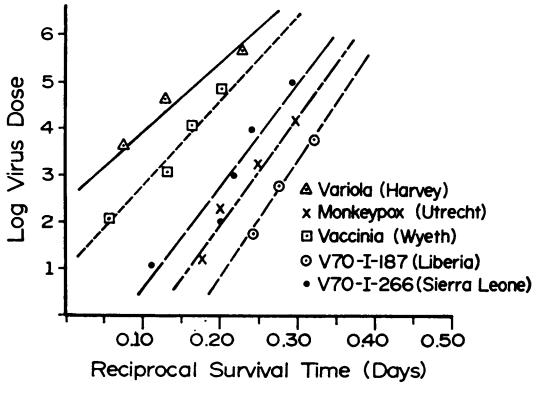

Fig. 3. Suckling mouse virulence test: poxvirus isolates from Liberia and Sierra Leone.

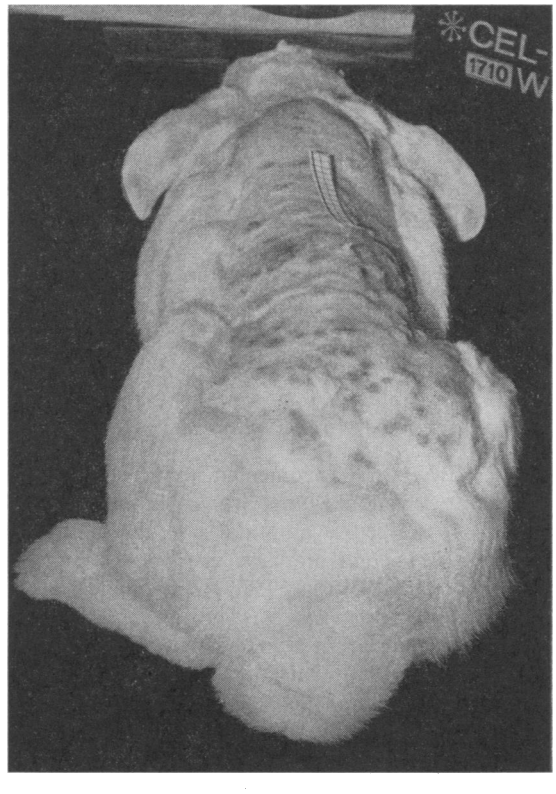

Fig. 4. Appearance of rabbit skin four days after inoculation with poxvirus strain V70-I-187.

Table 3. Comparison of ceiling temperatures of poxvirus isolates and reference strains with virulence for the chick embryo, suckling mouse, and rabbit

Strain	Ceiling temp.(°C)	D_4 value: chick	D_4 value: mice	Lesion in rabbit dermis [a]
V70–I–187	39.0	2.0	2.0	+++
V70–I–199	39.0	2.0	2.6	+++
V70–I–266	39.0	2.1	3.9	+++
V71–I–82	39.0	2.3	3.3	+++
monkeypox (Utrecht)	39.0	2.2	3.1	+++
vaccinia (Wyeth)	39.5	2.2	5.6	+
variola (Harvey Major)	38.2	5.0	6.2	±

[a] +++ = large, necrotic, haemorrhagic local lesion with generalized secondary vesicular exanthem
\+ = local vesicular lesion with no generalized illness
± = minimal local non-vesicular lesion.

Table 3 presents the results of the ceiling-temperature studies and of the tests of virulence for chick embryos, mice, and rabbits. The strain characteristics of the four West African isolate strains were strikingly similar to those of the reference monkeypox strain (Utrecht).

To confirm that these isolates from Liberia, Nigeria, and Sierra Leone are unique, 44 poxvirus strains isolated since 1968 from patients with clinical smallpox in West Africa were reexamined by inoculation into rabbit skin, embryonated eggs, and Vero cell cultures. All 44 of these earlier African isolates behaved like variola virus.

DISCUSSION

There were no documented reports of human infection with monkeypox virus prior to 1970. Von Magnus described a non-fatal pox disease occurring in *Cynomolgus* monkeys in Copenhagen (Von Magnus et al., 1959) in 1958. The etiological agent, named monkeypox virus, has since been identified in outbreaks of illness in monkeys or apes in the USA (Prier et al., 1960; McConnell et al., 1962), the Netherlands (Peters, 1966), and France (Milhaud et al., 1969). The strains of monkeypox virus isolated during these outbreaks were found to represent a uniform group differing little from the prototype Copenhagen strain. The biological characteristics of the monkeypox strains, however, were shown to be distinctly different from those of variola or vaccinia viruses.

The identification of poxviruses with the characteristics of the classical monkeypox virus from four patients with human smallpox-like illness in West Africa during 1970–71 has been presented in this report. Poxvirus isolates from each of the four cases have been intensively studied and compared with reference strains of variola, vaccinia, and monkeypox viruses by means of pock morphology, ceiling-temperature testing, plaque morphology, chick embryo lethality, suckling mouse lethality, and pathogenesis in rabbit dermis.

All four isolate strains closely resembled the reference monkeypox strains used in these studies. The Liberian (V70-I-187 and V70-I-199) and Sierra Leone (V70-I-266) isolates produce small pocks with central necrosis and haemorrhage when inoculated on to the CAM of 12-day-old embryonated hen's eggs. Unlike monkeypox virus, however, the Nigerian isolate (V71-I-82) produced pocks with minimal necrosis or haemorrhage. When inoculated into monkey kidney tissue culture (Vero cells), all four of these isolate strains produced large plaques similar to those of the monkeypox reference strains but distinctly different from those produced by strains of variola virus. Like monkeypox, the four isolate strains grew well at 39°C; variola virus does not. The isolate strains were much more lethal for chick embryos than variola virus, and their virulence for chick embryos and suckling mice was nearly identical to that produced by the monkeypox virus strains.

The most dramatic difference between the West African isolates and variola or vaccinia viruses is in their virulence for rabbits. Variola causes a minimal pink lesion when inoculated intradermally and cannot be successfully passed in rabbit skin. Vaccinia virus causes a small local lesion, but no systemic disease. All four West African isolate strains and the reference monkeypox strains produce large, necrotic, haemorrhagic local lesions and a generalized illness with florid secondary exanthems. The virus is easily recovered from the secondary lesions and passes easily in rabbit skin.

These studies identify a poxvirus that differs from either variola or vaccinia virus and that is indistinguishable from monkeypox virus. It is suggested that these human monkeypox cases are the result of chance infection of man with an animal virus. The antibody studies on monkey sera reported by Foster et al. (1971) indicate a very low level of poxvirus infections in wild monkeys in West Africa, and

suggest that such chance contacts of humans with infected monkeys would be rare. The recognition of six possible occurrences within an 8-month period is of considerable interest. Sources of the infection other than the monkey must still be considered.

Noble & Rich (1969) have demonstrated experimentally that variola virus can be maintained by direct monkey-to-monkey contact for at least several generations of infections. It is not known at present if variola (or vaccinia) virus, when passaged through numerous monkey passages, might alter its growth and virulence characteristics.

The poxvirus strains isolated from these patients in Liberia, Sierra Leone, and Nigeria fit into a homogeneous group indistinguishable from the reference monkeypox strains. These studies do not indicate a group of heterogeneous poxviruses with characteristics lying between variola and monkeypox. Such a group would be expected if chance modification of variola virus had occurred during multiple passages of variola virus in lower animal species. Evidence (Noble, 1970) does not support the hypothesis that a significant simian reservoir of smallpox exists, nor does monkeypox virus appear to be a product of " adaptation " of variola or vaccinia virus by passage in the monkey species.

The occurrence of six cases of clinical smallpox-like illness in areas of West Africa that had been free of smallpox for over a year has raised the question of the etiology and source of the infections. The identification of poxviruses with the characteristics of the classical monkeypox virus from these cases indicates that chance infection of man with poxviruses from other animals can occur and that thorough epidemiological and laboratory investigation of suspect pox disease in areas where smallpox eradication has been, or is about to be, achieved must be carried out.

RÉSUMÉ

INFECTION HUMAINE PAR LE VIRUS DU MONKEYPOX: ÉTUDE AU LABORATOIRE DE SIX CAS OBSERVÉS EN AFRIQUE OCCIDENTALE

De septembre 1970 à mai 1971, six cas d'infection humaine par le virus du monkeypox ont été découverts dans trois pays d'Afrique occidentale (Libéria, Nigéria et Sierra Leone). Pour quatre d'entre eux, le diagnostic a été confirmé par l'isolement du virus; les deux autres ont été identifiés après une enquête épidémiologique et sérologique.

Quatre des souches isolées ont été examinées au laboratoire. Elles se sont révélées très semblables aux souches de référence du virus du monkeypox (souches Copenhague et Utrecht) et ont pu être aisément différenciées du virus vaccinal et du virus variolique. Elles produisaient sur la membrane chorio-allantoïde de l'embryon de poulet des pustules de petite taille, avec nécrose et hémorragies centrales; étaient aisément cultivables à 39,0 °C; formaient de grandes plages en culture cellulaire de rein de singe; témoignaient d'une virulence pour l'embryon de poulet et la souris de loin supérieure à celle du virus variolique; et, inoculées par voie intradermique au lapin, provoquaient localement des lésions étendues, nécrotiques et hémorragiques, accompagnées de phénomènes généraux et d'exanthème secondaire.

La découverte de cas humains d'une affection ressemblant à la variole mais causée par un poxvirus d'origine animale montre la nécessité d'une étude poussée, épidémiologique et de laboratoire, de tous les cas suspects survenant dans des régions où la variole a été ou est sur le point d'être éradiquée.

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