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Comparison of Tanapox virus and Yaba-like viruses causing epidemic disease in monkeys

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SUMMARY

The virus of Tanapox isolated from the lesions of patients during an outbreak of mild disease in Africa has been found to be indistinguishable in its biological and serological properties from a virus isolated from outbreaks of a pox virus infection in monkeys in primate centres in America. The natural hosts of this virus are believed to be African monkeys and 'Tanapox virus' is proposed as a suitable designation for the virus.

INTRODUCTION

Tanapox is a mild human infection with one or sometimes two pock-like lesions in the skin, observed only in the Tana River valley of Kenya in 1957 and 1962 and fully described by Downie *et al.* (1971). The causative agent is undoubtedly a member of the pox virus group in general biological characters and electron-microscopic appearance, but has no serological relationship to other pox viruses pathogenic for man. In the laboratory Tanapox virus grows only in cultures of tissue derived from man or monkey and produces experimental infection only in monkeys. Because of these biological peculiarities of the virus and on general epidemiological grounds it was thought that Tanapox virus had a monkey reservoir from which the natives of the Tana River valley were occasionally infected. However, it has not been possible to prove this by examination of wild monkeys in the area. Thus it was of especial interest when in 1965 and 1966 there occurred outbreaks of pox virus infection in monkey colonies in California (España, 1971), in Oregon (Hall & McNulty, 1967; Nicholas & McNulty, 1968) and in Texas (Casey, Woodruff & Butcher, 1967; Crandell, Casey & Brumlow, 1969), particularly when the infection spread to men in contact with these animals with clinical and histological features very similar to natural Tanapox infection. The disease in monkeys in California was labelled Yaba-like Disease (Y.L.D.) because of its clinical similarity to the pox virus infection observed in rhesus monkeys kept at a research station in Africa (Bearcroft & Jamieson, 1958; Niven *et al.* 1961). Moreover, the virus from the monkey outbreaks in U.S.A. was shown to have the same restricted pathogenicity for animals and cytopathic effects on tissue culture as Tanapox virus (España, 1966; Hull, 1968; Schmidt, 1970) and was serologically unrelated to

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vaccinia. It was now of obvious importance to determine if these Yaba-like agents were related to Tanapox virus so that the monkey reservoir hypothesis could be tested. The present paper presents the results of reciprocal studies of the viruses with antisera derived from monkeys with natural infection or deliberately immunized or artificially infected with the two agents. The results show that the two viruses are immunologically identical, and support our view that Tanapox of man is essentially a zoonosis.

MATERIALS AND METHODS

Virus strains

The Tanapox virus strain isolated in 1962 had been passed three times in tissue culture of human thyroid, four times in human amnion, nine times in W.I. cells (a continuous line derived from human embryo lung) and three times in Vero cells before being used in the tests described below. The strain of Y.L.D. virus was received by A.W.D. in April 1969 and had been passed 29 times in vervet monkey kidney cell cultures and three times in BSC-1 cells. In this paper it is referred to as the 'California' strain. The strain of '1211' was received from Dr McNulty of the Oregon Primate Centre in June 1969 and is referred to below as the 'Oregon' strain.

For much of the work detailed below, virus suspensions to be used in neutralization tests were prepared from bottle cultures of Vero or BSC-1 cells maintained in 199 medium containing 1 % foetal calf serum or in Eagle's MEM or L-15 medium containing 2 % inactivated foetal calf serum. For the immunization of monkeys, however, the virus was grown in the same tissue cultures with 1 % vervet monkey serum in place of calf serum. When the cell sheets showed lesions throughout (after 8–12 days incubation at 35° C.) the cells were scraped into the medium and the suspension spun at 2000 rev./min. for 20 min. The supernatant was discarded, the cell deposit suspended in 10 ml. of buffered distilled water and sonicated for 40 sec. The suspension was then centrifuged at 2000 rev./min. for 15 min. and the supernatant kept. Two more extractions of the sonicated deposit were made in the same way. The three extracts, containing most of the virus, were then spun in a refrigerated Spinco angle centrifuge for 30 min. at 15,000 rev./min. The supernatant fluid from the first extract was kept as antigen to be used in complement fixation (C.F.) and precipitation tests and the deposited virus from the three extracts was suspended in buffered distilled water, pooled and kept frozen at $-70°$ C. as stock virus suspensions. For immunization of monkeys virus from Vero cells grown in vervet serum + '199' was prepared in the same way. But the crude virus suspension prepared as described was then spun through 35 % sucrose and the virus deposit washed once by high speed centrifugation in buffered distilled water and the deposit resuspended and kept for immunizing monkeys by intravenous injection.

Preparation of antisera

Sera were collected from monkeys affected by the outbreak of disease in the Primate Centre in California and at different periods after experimental infection of these animals with the California virus. In Liverpool four animals were immun-

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ized with the three strains of virus; one rhesus and one vervet monkey with Tanapox virus, one vervet with Oregon virus and one rhesus with California virus. These animals were bled before immunization (serum 1). They were then infected intradermally with 0·1 ml. of stock virus suspension at each of 12 sites. Three weeks later when the lesions resulting from the injections had disappeared the animals were bled (serum 2) and given an intravenous injection of 2 ml. of the appropriate purified virus suspension. These intravenous injections were repeated 6 and 12 days later and the animals bled (serum 3) 6 days after the third injection.

Complement-fixation tests

In Liverpool the antigen was prepared from the extract of infected sonicated tissue culture cells as described above. In the Primate Centre in California the viruses were grown in bottle cultures of BSC-1 or vervet monkey kidney cells. After 6–8 days when most of the cell sheets showed specific lesions the maintenance medium was replaced by Earle's balanced salt solution with 0·5 % lactalbumin hydrolysate. The cells were scraped into this, the suspension was frozen and thawed three times and the cell debris removed by centrifugation at 5000 rev./min. for 20 min. The supernatant constituted the antigen. Residual virus was inactivated by incubation at 37° C. for 2 hr. after the addition of Beta-propiolactone to make 0·025 %. Control antigens were prepared in the same way from uninfected tissue cultures. In both laboratories antigens were titrated in doubling dilutions against doubling dilutions of antisera. The dilution chosen for further tests was the highest that gave maximum serum titre. The technique of the tests was similar in both laboratories. Mixtures of serum dilution, complement (2–2½ MHD) and antigen, 0·1 ml. of each, were held at 4° C. overnight and next morning kept at 37° C. for ½ hr. after adding the haemolytic system (España & Hammon, 1948; Downie *et al.* 1971).

Neutralization tests

All sera were inactivated before testing. Equal volumes of serum dilutions were mixed with virus suspension diluted to contain 50–100 f.f.u. (focus forming units) in 0·1 ml. and incubated at 37° C. for 2 hr.; 0·2 ml. of each mixture was then inoculated into each of 3–5 tissue culture tubes of Vero or BSC-1 cells and 1·0 ml. of maintenance medium added to each tube. A mixture of virus with dilutions of antibody-free serum was included in each test to provide the base line lesion count. After incubation of the culture tubes for 7–12 days the lesions in each tube were counted using a × 10 binocular microscope. (The tubes were examined daily from the fifth day onwards and the final count made when foci were well developed and a further daily increase in the number of foci in control tubes had ceased.) From the results the dilution of serum effecting a 50 % reduction in the mean count was estimated. In the Primate Centre in California the antibody content of sera was sometimes estimated by titrating tenfold dilutions of virus in the presence of a constant dilution of serum. From the results the neutralization index was determined by subtracting the logarithm of the virus titre in the test serum from the logarithm of the control virus titre.

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Precipitation tests

These were made in 1·0% or 0·85% agar in layers 1·0 mm. thick on ordinary microscope slides. The wells in the agar layer were 4 mm. in diameter with the centres of neighbouring wells 5·5 mm. apart. Sera and antigens in neighbouring wells were allowed to react for 24 or 48 hr. at room temperature before the results were read. In some instances sera and antigens were concentrated by freeze drying and re-solution in one-fifth of the original volume of distilled water to intensify the reactions. Treatment of the agar with 1·0% tannic acid solution (Alpert, Monroe & Schur, 1970) served to accentuate lines of precipitation before photography.

RESULTS

Active immunity experiments

It had been noted in the original outbreaks of infection in the American Primate Centres that recovered animals were immune to re-infection by virus inoculated intradermally. Three monkeys that had been immunized in Liverpool by intravenous injections of virus for the preparation of antisera were tested 2 days after their final bleeding along with a normal control vervet monkey by injecting each of the three virus strains intradermally into each animal; the titre of the virus suspensions used was approximately 10^7 f.f.u. per ml. The animals were sedated on the 4th and 7th days after injection for careful examination of their lesions.

The control vervet monkey after 4 days showed raised thickened lesions about 1·5 cm. in diameter at the sites of injection of all three viruses. After 7 days the lesions had not increased much in size but showed central necrosis. The lesions thereafter regressed and showed only slight residual scabbing after 14 days. These lesions were similar in size and course of evolution to those exhibited by the monkeys when first infected intradermally with Oregon and California viruses. The vervet monkey used to prepare antisera against Tana virus had shown only small papular lesions when given its first intradermal injections of Tana virus. It was subsequently discovered that this monkey had a significant titre of neutralizing antibody to Tanapox virus before injection.

All three immunized monkeys after challenge showed only tiny papular lesions after 4 days and these had practically gone by the 7th day. Although the vervet monkey immunized with Tana virus apparently had some degree of immunity before being used in these experiments, the results indicate that the three viruses could not be differentiated by these tests.

Complement-fixation tests

The sera of monkeys which had recovered from contact or experimental infection with California virus in the Primate Centre were tested against antigens prepared from Tana and California infected tissue cultures. The results are shown in Tables 1 and 2. The titres shown in Table 2 represent the average from six monkeys which had been inoculated intradermally with a suspension of California virus prepared from the 30th serial passage in vervet monkey kidney cell cultures. The findings

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recorded in Tables 1 and 2 strongly suggest that the complement-fixing antigens of Tanapox and California viruses are identical.

The sera collected before (1), during (2) and after (3) immunization of monkeys in Liverpool with the three viruses were similarly tested against tissue culture antigens. The results are shown in Table 3.

The Oregon antigen and Oregon antisera would appear to be weaker than those of the other two viruses and the Tanapox antiserum had a somewhat higher titre

Table 1. *Complement-fixing titres against California and Tana tissue culture antigens of sera from* Macaca mulatta *with spontaneous Yaba-like disease*

Monkey sera (*M. mulatta*)	C.F. titre of sera *vs.* antigens	
	Tanapox	California
3201	64*	64
3206	8	16
3211	64	128
3213	32	32
3216	16	32
3223	128	128
3226	32	32
3228	16	16
3242	32	64
3243	16	16
3246	128	128
3252	16	16
3257	16	16
3281	64	64
3292	128	128
3515	4	8
3693	64	32
3986	32	64
4090	16	16
4209	16	32

* Reciprocal of highest serum dilution giving a 2+ or better fixation of complement.

Table 2. *Complement-fixing titres against California and Tanapox tissue culture antigens of sera from* M. mulatta *inoculated with California virus*

Days after inoculation	C.F. titre of sera *vs.* antigens	
	California	Tanapox
0	0*	0
13	8	4
26	32	32
39	128	64
53	64	64
67	32	32
82	16	16
95	16	16
116	8	8
124	8	8

* Reciprocal of serum dilutions. Average titre of six monkeys.

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than the California antiserum. However, the results indicate that there is no qualitative difference in the antigens from the three viruses.

Neutralization tests

Sera from *Macaca mulatta* with spontaneous Y.L.D. lesions which were beginning to regress neutralized the cytopathic effect induced by either Tanapox or California viruses. Table 4 shows that the neutralizing indices of four Y.L.D. monkey convalescent sera were similar against either virus.

M. mulatta inoculated intradermally with the California virus developed neutralizing antibodies within 7–10 days after inoculation. The antibody levels increased as the lesions developed, reaching a maximum between 18 and 22 days. Titres remained at this level until the lesions regressed. After regression, neutralizing antibodies decreased sharply and were barely detectable six months after inoculation. The results of representative experiments in which sera from serial bleedings of two infected monkeys were tested for their neutralizing activity against both California and Tanapox viruses are shown in Table 5.

The results of the studies presented in Tables 4 and 5 indicated that, as in the case of complement-fixation tests, Tanapox and California viruses cannot be distinguished by neutralization tests. The sera from monkeys, convalescent from and immunized against California virus infection, neutralized both viruses to a high

Table 3. *Complement-fixing titre of sera from three monkeys immunized with Tanapox, California and Oregon viruses*

	Titre of sera against antigens of		
Monkey sera	Tanapox	Oregon	California
Tanapox 1	<10	<10	<10
2	40	40	40
3	160	40	160
Oregon 1	<10	<10	<10
2	10	10	10
3	40	20	40
California 1	<10	<10	<10
2	40	40	40
3	80	40	80

Table 4. *Neutralizing indices of Y.L.D. convalescent monkey sera* (M. mulatta) *against California and Tanapox viruses*

	Infectivity titre of virus		Neutralization index	
Monkey serum (1/20)	California	Tanapox	California	Tanapox
982	1·3	1·0	4·3	5·0
1269	1·6	1·5	4·0	4·5
1270	2·0	2·0	3·6	4·0
1285	2·2	2·5	3·4	3·5
Normal monkey	5·0	5·3	0·6	0·7
None	5·6	6·0	—	—

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titre and the neutralization index was essentially the same for both Tanapox and Y.L.D. viruses.

The sera of monkeys immunized against the three viruses in Liverpool were tested in threefold dilutions against all three viruses: the results are shown in Table 6. These tests show that all three sera neutralize all three viruses to high titre. The slight difference in titre with individual sera and viruses does not appear to be significant. The pre-immunization serum of the Tanapox monkey had low titre antibody to all three viruses and as noted above this may have determined the smallness of the lesions produced in this animal by the first intradermal injections of Tanapox virus.

Table 5. *Neutralizing activity against California and Tanapox viruses of sera from two* M. mulatta *inoculated with California virus*

		% reduction of lesion count compared with control			
		Monkey no. 3203		Monkey no. 3247	
Days after inoculation	Serum diln.	California virus	Tanapox virus	California virus	Tanapox virus
1	1/10	3·0	3·1	6·0	6·1
7	1/10	14·8	24·3	26·5	24·3
	1/40	8·8	12·2	8·8	6·1
19	1/10	91·2	87·9	73·6	75·8
	1/40	86·8	84·9	47·1	42·5
	1/160	73·6	69·7	N.T.	N.T.
44	1/10	97·0	95·5	86·8	87·9
	1/40	86·8	87·9	58·9	60·7
	1/160	75·0	74·3	50·0	51·6
183	1/10	26·5	12·2	3·0	3·1
No. of f.f.u. in virus controls		68	66		

Table 6. *Neutralization tests against Tanapox, California and Oregon viruses with the sera of monkeys immunized against the three viruses*

	Neutralizing titres of sera against		
Monkey sera	Tanapox virus	Oregon virus	California virus
Tanapox 1	60	60	30
2	600	135*	135*
3	6000	6000	6000
Oregon 1	< 5	< 5	< 5
2	500	135*	135*
3	4000	2500	2000
California 1	< 5	< 5	< 5
2	200	200	100
3	4000	2500	3000

* = Not tested at higher serum dilutions.

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Precipitation tests

For these tests the third sera from the monkeys immunized against Tanapox, Oregon and California viruses were used. These sera did not produce very strong lines of precipitation when tested against antigen prepared from tissue culture infected with the three viruses. Concentration of antigen and antisera by freeze drying enhanced the reactions. An immune rabbit serum (1211), kindly supplied by Dr McNulty, had been prepared by immunization of a rabbit with Oregon virus which had been purified by sucrose density gradient centrifugation. The precipitation reactions between these immune sera with each of the three virus antigens are shown in Pl. 1, fig. 1. Allowing for differences in the precipitating potency of the sera and antigens the results seem to show reactions of identity with all three antigens tested against the four antisera.

In Davis, tests were carried out with immune animal sera (mouse, monkey, guinea pig) against Tanapox and California viruses. All sera tested reacted in the same manner against either antigen. There were no differences in the line patterns produced by either California or Tana antigens, indicating identical antigenic make-up in the two viruses.

In tests illustrated by Pl. 1, fig. 2 the position of the reagents was reversed and each antigen was tested against the four antisera placed in the surrounding cups. In this test the California and Oregon antisera and the California antigen show weak reactivity but again there seems to be identity of reactions between each antigen and the three immune monkey sera. The precipitation lines resulting from the interaction of the antigens with the immune rabbit serum '1211' do not show identity with the reactions shown by the immune monkey sera. This seems especially clear of the reactions with Oregon antigen. The immune rabbit serum did not precipitate with the control antigen from uninfected tissue culture, but this control antigen was not concentrated as were the virus antigens. The lack of identity of reactions with the immune rabbit serum when compared with those given by the immune monkey sera suggests that there were antibodies in the immune rabbit serum which were not present in the immune monkey sera. These antibodies may have been directed against antigens in normal monkey tissue cells. The observation that this immune rabbit serum fixed complement to a considerable titre with 'antigen' from uninfected tissue culture cells support this suggestion. However, the precipitation reactions between the immune monkey sera and the three virus antigens again indicate that with these reagents the antigens of the three viruses are identical.

DISCUSSION

The viruses isolated from Tanapox in man, from the natural epizootic among monkeys in the U.S.A. and from human contacts infected from them have been shown to have similar biological properties (Downie *et al.* 1971; España, 1966; Hull, 1968; McNulty *et al.* 1968). The lesions of Tanapox and those in naturally infected monkeys were histologically identical and recent electron-microscopic studies have shown that the changes produced in infected tissue culture cells by

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Tanapox and California viruses are the same (España, Brayton & Ruebner, 1971). Because of the similarity of the clinical disease caused by Tanapox virus and by the virus from the epizootics in monkeys it seemed reasonable to find out whether the same virus was responsible. The results reported in this paper have shown that the viruses are identical by the various serological tests used. Nicholas has also found that an antiserum prepared in rabbits against Oregon virus neutralized Tanapox and Oregon viruses to the same titre (personal communication). The name 'OrTeCapox virus' has been suggested for the monkey virus studied in laboratories in Oregon, Texas and California (Nicholas, 1970). However, as this virus is the same as Tanapox virus, which was isolated from African patients in 1962 and recorded in 1963 (Allison, 1965), it would appear that 'Tanapox' virus might be the more appropriate designation for this member of the pox group.

The demonstration that the same virus seems to have been responsible for the outbreaks of infection among macaque monkeys in America and the epidemics of Tanapox in Kenyans has an important bearing on the epidemiology of the disease in the Tana River valley. The infected monkeys in the Primate Centres in America had been supplied by the same importer on whose overcrowded premises African and Asiatic monkeys had been housed in the same building. Because the disease affected mostly Asiatic monkeys of the genus *macaca* while African green monkeys were apparently insusceptible, it has been suggested that the virus may have spread to the macaques from inapparent infection in the African monkeys (Schmidt, 1970). The outbreaks of Tanapox observed in 1957 and 1962 occurred at times of severe flooding when the human population together with their domestic and wild animals were crowded on small islands among the flood waters (Downie *et al.* 1971). It seems likely that the human population acquired their infection from wild monkeys – in other words the native population in the Tana River valley, like the Asiatic monkeys in the Primate Centres in America, served as the indicators of latent infection with Tanapox virus in African monkeys.

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EXPLANATION OF PLATE

Rabbit serum '1211' was prepared against purified Oregon virus. Tan = Tanapox; Ore = Oregon; Cal = California; Con = control; A = antigen; S = serum.

Fig. 1. Precipitation test in agar gel in which each of four antisera were tested against antigens of Tanapox, Oregon and California viruses.

Fig. 2. Precipitation test in agar gel in which four antigens were tested against Tanapox, Oregon, California and control antisera.

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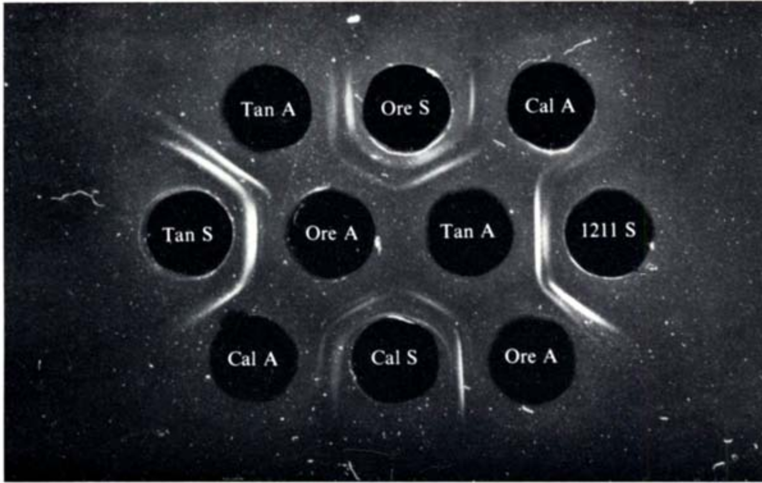

Fig. 1.

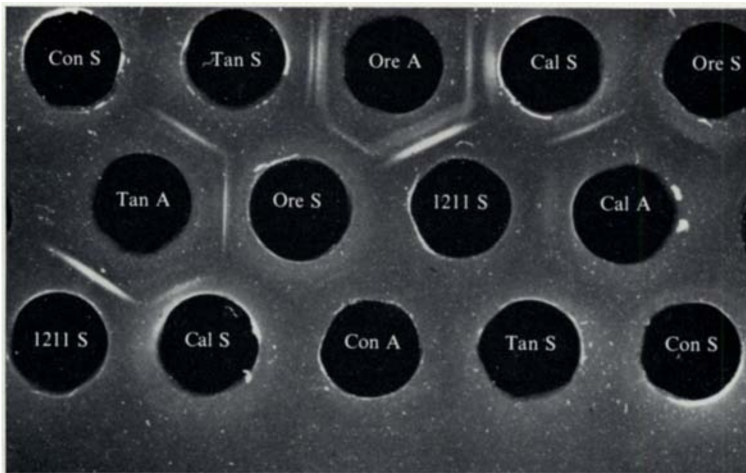

Fig. 2.

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