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Yaba Tumor Virus. I. Studies on Pathogenesis and Immunity* (33368)

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Subcutaneous noncapsulated tumors of histiocytic origin (histiocytomas) were first observed in 1957 among rhesus monkeys kept in open air cages at Yaba, Nigeria (1). In subsequent studies, the etiological agent was found to be a member of the poxvirus group (2) and inoculation of human volunteers with the virus produced similar tumors (3).

Yaba tumors, whether occurring naturally or induced experimentally in monkeys regress spontaneously within 1–2 months; regression is apparently due to an *in vivo* cytopathic effect of the causative agent (2, 4). While the tumors are progressing, specific antibodies develop and appear to have little if any effect on established tumors (5). Cross resistance experiments indicated no immunogenic relationship between Yaba tumor virus on one hand, and vaccinia, monkey pox, and orf viruses on the other (2, 6).

In the present experiments we studied pathogenic and immunologic responses of rhesus monkeys to Yaba tumor virus; the antigenic relationship between Yaba, vaccinia, and monkey pox viruses; and the development of viral antigen in tumor cells as monitored by the complement fixation and fluorescent antibody techniques.

Materials and Methods. Viruses. Yaba tumor virus[2] was obtained from Dr. David S. Yohn, Roswell Park Memorial Institute, Buffalo, New York. The virus was passed twice in rhesus monkeys by the subcutaneous route of inoculation and a large stock of tumors in 5- to 10-gram quantities were stored frozen at —70°. Monkey pox virus[2], as infected cell culture fluid, was obtained from Dr. Preben von Magnus, Staten Seruminstitut, Copenhagen. The vaccinia virus was derived from a commercial (Wyeth) smallpox vaccine preparation.

Monkeys. Young adult rhesus or cynomolgus monkeys lacking preexisting Yaba virus antibodies, as determined by the CF test, were used for all Yaba virus infections. Monkeys were inoculated with Yaba virus either by subcutaneous (s.c.) or intravenous (i.v.) routes. Inoculated monkeys were kept

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in individual cages except when sentinel monkeys were included; a single sentinel animal was housed in a cage along with an infected monkey.

Serologic tests.[3] The microtiter system was used for complement fixation (CF) and hemagglutination (HA) and hemagglutination inhibition (HI) tests. Yaba CF antigen consisted of clarified 20% tumor suspension extracted twice in equal amounts with Genetron 113. Monkey pox and vaccinia CF and HA antigens were clarified fluids from infected cell cultures (primary rabbit kidney for monkey pox, and primary rhesus kidney for vaccinia). Sera were inactivated at 56° for 30 min for CF tests; they were treated with kaolin and absorbed with chicken red cells for HI tests. The CF tests were performed using 2 units of antigen and 5 $C'H_{50}$ units of complement with overnight fixation. Four units of HA antigen were used in HI tests; the antigen–antiserum mixtures were incubated at 25° for 1–2 hr before addition of chicken erythrocytes.

Fluorescent antibody (FA) technique. Gamma globulin prepared from the serum of a rhesus monkey that developed specific CF antibodies (1:256) after Yaba tumor development was conjugated with fluorescein isothiocyanate (7). This conjugate was used to stain frozen Yaba tumor sections. The specificity of the reagents was established by (a) blocking of the reaction by preincubation with unlabeled specific antiserum, and (b) failure of other conjugates prepared against monkey pox, respiratory syncytial, and rubella viruses to show specific fluorescence with frozen Yaba tumor sections.

Results. End points: Infection and tumor-production. Yaba virus tumors, harvested from a rhesus monkey on day 34, measuring between 1.0 and 3.0 cm in diameter, upon trituration in phosphate buffered saline (PBS) and clarification by low speed centrifugation, comprised the stock used in titration and challenge studies. The titration results obtained in antibody-free monkeys are summarized in Tables I and II. These studies indicate that the titer of the stock virus was $\sim 10^{7.9}$/ml (calculated on the basis that 0.2 ml of 10^{-7} virus produced tumors at 6 of the 8 subcutaneous sites). The titer by the intravenous route exceeded $10^{5.0}$ ID_{50}/ml.

Monkeys were observed daily for 30 days; two dimensional measurements of subcutaneous tumors were obtained at weekly intervals. Measurements are approximation of size, but ranged widely enough to point up several aspects of differing rates of growth in rhesus and cynomolgus monkeys. The times of gross appearance of tumors in each species (Tables I and II) inoculated with graded doses of virus were quite uniform; however once tumorigenesis had begun much larger tumors ultimately developed in rhesus than in cynomolgus monkeys. The tumors developing in rhesus monkeys at 10^{-6} virus dilution attained sizes similar to those inoculated with a 1:10 dilution (24.1 ± 2.5 cm^2). This general rule was not absolute; infrequently only small tumors developed in rhesus, and occasionally, as in D623 (Table II) a large tumor developed in cynomolgus monkeys. Another matter of interest relates to the conspicuous development of larger tumors in MPV convalescent monkeys than in companion controls inoculated at the same time with the same dilutions of Yaba tumor virus.

The pathological pattern observed after i.v. inoculation was notable in most monkeys with respect to the development of tumor nodules on the face, arms and extremities, and localization deep in skeletal muscles and along perivenous connective tissues. Isolated segments of typical tumor cells (with large cytoplasmic inclusions) were found in the lungs of monkeys; these small foci developed just underneath the visceral pleura (4).

Specific CF antibodies were raised in monkeys developing tumors. Such antibodies developed slightly slower in cynomolgus than in rhesus monkeys (by about 7–10 days), but by day 42 equivalent titer values were obtained in both species. In addition CF antibodies appeared slightly earlier in rhesus monkeys inoculated s.c. than among those inoculated i.v.

The sentinel monkeys (Table I) kept in

[3] The skillful technical assistance of Mrs. Shloe Barrick and Miss Isobel Gray in the serological studies is noted with appreciation.

TABLE I. Tumor Development and CF Antibody Response: Monkeys Inoculated with Various Dilutions of Yaba Virus.[a]

Rhesus monkey no.	Inoculum			Development of tumors		Antibody titers (CF)[e] Days									
	Dilution	Route[b]	Site[b]	No. of takes[c]	Time of appearance (days)	Pre-	7	14	21	28	35	42	49	57	≤69
D615	1.6×10^{-1}	s.c.	8	8/8	7	<8	16	64	128	128	128	128	128	128	
	10^{-2}	s.c.	8	8/8	7										
D613	10^{-3}	s.c.	8	8/8	12	<8	<8	32	128	128	128	S[d]			
	10^{-4}	s.c.	8	8/8	14										
D616	10^{-5}	s.c.	8	8/8	14	<8	<8	16	256	256	512	512	S[d]		
	10^{-6}	s.c.	8	8/8	14										
D617	1.0×10^{-1}	i.v.	—	3+	12	<8	<8	32	64	256	256	128	S[d]		
D618	10^{-2}	i.v.	—	3+	21	<8	<8	16	16	128	128	256	S[d]		
D619	10^{-4}	i.v.	—	3+	14	<8	<8	<8	16	64	64	128	128	S[d]	
D620	10^{-5}	i.v.	—	4+	28	<8	<8	<8	<8	32	64	128	64	128	
Sentinel monkeys															
D621[f]	none	—	—	none	none	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8
D622	none	—	—	none	none	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8

[a] The Yaba tumor virus was a 10% tumor extract from a rhesus monkey (D612).

[b] 0.2 ml of virus dilution inoculated subcutaneously (s.c.) at each of 8 sites on the right and left sides of back, each 3 cm apart; 1.0 ml of virus dilution inoculated into the right saphenous vein.

[c] 4+ = tumor nodules on arms, trunk and face, along with multiple clusters in skeletal muscle of extremities and in perivenous connective tissue; 3+ = the same, except none found in perivenous tissues.

[d] Monekys sacrificed at interval indicated; 3 of these animals had miliary tuberculosis.

[e] CF, complement fixation test.

[f] Monkey D621, sacrificed on day 62 was caged with monkey 615; monkey 622, sacrificed on day 69 was caged with monkey 617.

direct contact with infected monkeys neither developed tumors nor produced antibodies during an observation period varying from 62 to 69 days.

Antigenic differences between Yaba, monkey pox, and vaccinia viruses. (a) In vivo *studies.* In studies done in 1966 we found monkey pox not to convey any resistance in monkeys challenged with Yaba tumor virus. However, in those studies, involving 29 monkeys a 10% Yaba tumor suspension was used; hence, partial immunity might have been missed. Recently opportunity arose to repeat the study, using graded doses of Yaba virus. The results of this test involving 13 monkeys are summarized in Table II. The data illustrate lack of resistance to Yaba virus among monkeys recently recovered from monkey pox. None of the monkeys was immune to as little as 10 ID_{50} of Yaba virus. All monkeys inoculated with Yaba virus developed specific antibodies; none of the normal monkeys developed MPV antibodies, and none of the MP monkeys developed a significant rise in MPV antibodies.

Following infection from vaccinia virus, monkeys were also fully susceptible to Yaba tumor virus. Previous studies (8) indicate solid resistance of MP convalescent monkeys to vaccinia virus.

(b) In vitro *studies.* The results of the *in vivo* studies were augmented by *in vitro* cross serologic tests. Specific antisera against monkey pox and vaccinia viruses were prepared in rhesus monkeys by intramuscular (monkey pox) or intradermal (vaccinia) route of inoculation. Sera were collected prior to, and about 8 weeks after inoculation. The Yaba

TABLE II. Tumor Development and Antibody Response: Monkeys Convalescent from MPV and Challenged with Yaba Virus.[a]

Class	Yaba tumor virus: Cynomolgus monkeys no.	Yaba tumor virus: Inoculum (s.c.) (ml)	Development of tumors: No. of takes	Development of tumors: First appearance[b] (days)	Development of tumors: Maximal size[c] (cm²)	Development of tumors: Maximal size[c] (days)	Antibody titers (geometric mean): MPV (HI) Pre[d]	MPV (HI) Post	Yaba (CF) Pre	Yaba (CF) Post
MPV convalescent monkeys	2	1.6×10^{-2}	16/16	7	3.16 ± 1.2	(~35)	80	28	<8	200
		10^{-3}	15/16	10.5	4.66 ± 2.5	(~35)	50	20	<8	90
		10^{-4}	16/16	14	5.72 ± 1.7	(~35)	50	20	<8	128
		10^{-5}	15/16	21	3.34 ± 2.0	(~42)	50	80	<8	200
		10^{-6}	16/16	21	1.55 ± 1.0	(~42)	50	20	<8	128
Normal seronegative monkeys	1	1.6×10^{-3}	8/8	14	3.73 ± 0.7	(~35)	<10	<10	<8	128
		10^{-4}	8/8	21	1.76 ± 1.0	(~42)				
	1	10^{-5}	8/8	21	0.64 ± 0.2	(~21)	<10	<10	<8	>32
		10^{-6}	8/8	21	0.64 ± 0.2	(~21)				
	1	10^{-7}	6/8	28	0.36 ± 0.1	(~42)	<10	<10	<8	128
		10^{-8}	0/8	—	—	—				

[a] The Yaba tumor virus was a 10% tumor extract from a rhesus monkey (D612). See legend of Table I for pattern of inoculations.

[b] Determined initially by palpation; measurements were obtained using a centimeter ruler.

[c] The time of maximal size is given for the week when tumors reached such size; no extrapolations were determined although maximal size may have been attained early in the weekly interval. In the maximal size (cm²), the standard deviations (±) are corrected for small samples.

[d] The convalescent monkeys were challenged 96 days after infection with MPV. The post-bloods listed were obtained 69 days after challenge with Yaba virus. Additional sera collected on days 14 and 28 yielded similar HI values. None of these monkeys had tuberculosis, on histologic study.

virus antiserum was obtained from an s.c. inoculated rhesus monkey that showed a homologous CF titer of <1:8 prior to infection and of 1:128, 3–4 weeks postinfection. Results of the cross CF tests are presented in Table II. While there is a close antigenic relationship between vaccinia and monkey pox viruses, an antigenic relationship between these two viruses and Yaba virus was not found. Since, so far, we have been unable to prepare a specific HA antigen for Yaba virus, one-way cross HI tests with Yaba antisera and two-way cross HI tests with monkey pox and vaccinia were performed. Results are presented in Table III. Again the close relationship between vaccinia and monkey pox virus, and a lack of antigenic relationship between these two viruses and Yaba virus was clearly indicated.

Development of viral antigen(s) in tumor cells. The development of Yaba viral antigen(s) in tumors during their growth in rhesus monkeys was studied by the direct FA technique. Frozen sections were prepared from tumors removed 14, 21, 28, 32, 42, and 49 days after inoculation; sections were stained with serum conjugates as described above. Portions of the same tumors removed on days 14, 28, 42, and 49 were used in preparing CF antigens and tested for CF activity with known positive and negative Yaba virus antisera. Scattered fluorescent foci were found throughout the cytoplasm of

TABLE III. Cross CF Tests with Yaba, Monkey Pox, and Vaccinia Antigens and Antisera.[a]

Antigens	Yaba Pre	Yaba Post	Monkey pox Pre	Monkey pox Post	Vaccinia Pre	Vaccinia Post
Yaba	<8	256	<8	<8	<8	<8
Monkey pox	<8	<8	<8	16	<8	16
Vaccinia	<8	<8	<8	16	<8	16

[a] See text for description of antigens and antisera.

TABLE IV. HI Tests with Yaba Antisera and Monkey Pox and Vaccinia Antigens and Antisera.[a]

	Antisera					
	Yaba		Monkey pox		Vaccinia	
Antigen	Pre	Post	Pre	Post	Pre	Post
Vaccinia	<10	<10	<10	80	<10	40
Monkey pox	<10	<10	<10	160	<10	40

[a] See text for description of antigens and antisera.

tumor cells excised 14 days after inoculation. By day 21 there was increased intensity of staining; by days 28, 35, and 42 contiguous foci of fluorescence were found. The antigen labeled by FA was found in the cytoplasm of tumor cells. Tumors removed on day 49 or later were only slightly fluorescent. Similarly, CF activity was barely detected in tumors removed after 14 days but was fully developed in tumors removed 28 and 42 days after infection. Tumors removed later than 42 days postinfection did not show greater CF activity. These results are summarized in Table IV.

Discussion. Yaba virus has been included in the unclassified subgroup of the pox virus group on the basis of its physical structure and chemical composition (2, 9). However, it possesses a tumorigenic characteristic that is not shared by other members of the pox virus group. The histiocytomas produced by Yaba virus in man and monkeys differ from rabbit and deer fibromas, produced by other pox viruses, in that collagen fiber formation is not observed in Yaba virus induced tumors (9). Others, from their *in vitro* (5, 6, 10) and *in vivo* (2, 6) studies have noted the immunologic independency of Yaba from monkey pox, vaccinia, and orf viruses. Our data obtained by CF and HI methods confirm, and extend these earlier findings. The *in vivo* data in respect to Yaba and MP viruses provide quantitative evidence of immunologic independence of these two viruses. Thus, in addition to its unique pathogenicity Yaba tumor virus is also antigenically distinct from several pox viruses which are infectious for the same host.

The antibody response of monkeys to Yaba virus infection is both rapid and specific. Both s.c. and i.v. inoculations appear equally effective for antibody production. However, higher dilutions of virus inoculated either s.c. or i.v. show a delay in both tumor formation and antibody production but the final titers (as detected by the CF test) eventually attained by rhesus monkeys inoculated with various dilutions of virus fall essentially into the same range (128–512). Infected monkeys tested as long as 96 days after inoculation did not show a significant decline in CF antibody titer. A decline in antibody titer after regression of tumors has been reported by other workers (5).

Detection of viral antigen(s) in tumor cells removed at weekly intervals from infected monkeys by the FA technique and CF test indicated that while the former could readily detect the formation of viral antigens 14 days post infection, the latter was barely capable of such detection at that stage. Tumors removed after 21 days showed much greater amounts of cytoplasmic Yaba fluorescence but full cytoplasmic immunofluorescence was observed in tumor cells removed 35 and 42

TABLE V. Immunofluorescence and CF Antigen Titers of Tumor Cells Removed from Rhesus Monkeys Inoculated s.c. with Yaba Virus.

	Tumor cells removed after					
Test (days):	14	21	28	35	42	49
Immunofluorescence[a]	2+	3+	4+	4+	4+	+
CF titer[b]	8	16	64	64	64	8

[a] Immunofluorescence as detected by a known positive fluorescent antibody; + to 4+ represent 25–100% of cells showing specific fluorescence.

[b] CF titers of antigens (prepared from various tumors) as determined by box titrations with known positive and negative antisera.

days after infection. Similarly tumor cells removed on days 35 and 42 postinfection showed the greatest CF activity. The amounts of both immunofluorescence and CF activity showed a significant decrease in tumors removed on or after 49 days postinfection.

The variations noted in tumor development in rhesus and cynomolgus monkeys are not readily explained. Small doses of virus were not associated with lesser tumor development in rhesus, although they were in cynomolgus monkeys. Thus, the phenomenon is not entirely virus-dose dependent. All monkeys were apparently heretofore unexposed to Yaba virus, since antibodies were not found just prior to their inoculation. This assumption may be questioned, but infection occurring within 3 months prior to entry in the laboratory should have left its serological imprint (see Table II). The factors relating to this growth inhibition in normal cynomolgus monkeys is unknown, just as is apparent potentiation of tumor growth in MPV convalescent monkeys. The variations may be chance biological effect in small groups of animals. Further observations should define whether the effect is real or spurious.

Finally, our experiences indicate that Yaba tumor virus is not highly contagious, inasmuch as normal sentinel monkeys failed to develop clinical or serological evidence of infection up to 69 days, even when exposed to tumor ulceration. Our hope to follow these sentinel monkeys for 6 months was interrupted by the appearance of tuberculosis in this group of rhesus monkeys.

Summary. Tumor development and CF antibody responses of monkeys inoculated s.c. and i.v. with various dilutions of Yaba tumor virus were investigated. Tumors developed at different times and at different sites with CF antibodies appearing soon after the development of tumors and attaining final CF titers comparable in all inoculated animals. No antigenic relationship between Yaba virus and monkey pox viruses was found by *in vivo* challenge, or between Yaba, monkey pox, and vaccinia virus by cross CF and HI tests. The development of viral antigen(s) in tumor cells removed from infected monkeys at weekly intervals was monitored by the FA technique and CF test. Although the FA technique detected the viral antigen earlier, both procedures were equally effective for detection of antigen in tumors removed 4 or more weeks postinfection.

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