Yaba Tumor Virus
II. Studies on Inactivation and Reactivation (34300)

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(Introduced by H. A. Wenner)

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Yaba tumor virus is one of the ungrouped members of the poxviruses. This virus produces benign histiocytomas in monkeys and in man. Monkeys previously infected with vaccinia or monkey pox viruses are fully susceptible to Yaba tumor virus. No serologic relationship between Yaba and these other viruses has been disclosed during *in vivo* studies by hemagglutination-inhibition (HI) or complement- fixation (CF) tests (1).

Hybridization between two antigenically unrelated poxviruses has been deemed an unlikely possibility (2). The results of the experiments described below *suggest* the possibility that a hybrid virion bearing certain antigens of both Yaba and vaccinia viruses might be obtained when a mixture of UV-inactivated Yaba and active vaccinia viruses is injected into susceptible monkeys.

Materials and Methods. Viruses. Yaba tumor virus was originally obtained from Dr. David Yohn, Roswell Park Memorial Institute, Buffalo, N.Y. Large stocks of tumors were prepared at this laboratory by subcutaneous inoculation of rhesus monkey. These stocks have been stored frozen at —70°. For the present studies, a 10% tumor homogenate was made in chilled phosphate buffered saline by homogenization in a Servall omnimixer for 3 periods of 2 min each at full speed. This homogenate was spun at 2000 rpm for 30 min at 4°, the supernatant was spun again at 4000 rpm for 1 hr at 4°. The supernatant fluid was dispensed in 2- and 5-ml amounts and stored at —70°. The tumor inducing titer of this stock virus determined by the subcutaneous inoculation of the antibody-free rhesus monkey with serial 10-fold virus dilutions each at 4 sites was approximately $10^{6.5}$/0.2 ml. The titer was calculated according to the Reed-Muench method on the basis of tumor production at the end of 6 weeks of observation period. The vaccinia virus was clarified fluids from primary rhesus kidney cells infected with Wyeth smallpox vaccine. Similarly, the monkey pox virus (1) was obtained from clarified fluid overlays harvested from infected primary rabbit kidney cells.

Monkeys. Adult rhesus monkeys were used in all experiments. They were seronegative for Yaba CF and vaccinia HI antibodies. Animals were injected subcutaneously with 0.2 ml of serially diluted inocula. Three to four inoculations were made at each of the medial and lateral aspects of the arms and at each of the gluteal and gastrocnemius regions of the leg. Inoculations into the thoracic and abdominal regions or into the back of the animals were generally unsatisfactory.

Ultraviolet (UV) inactivation. A General Electric germicidal lamp (No. 15 T8, 15 W, delivering 3100 ergs/cm^2) placed vertically at a distance of 20 cm above a shaker rotating 60 times/min was used. Virus suspensions as 2-ml amounts in 60 × 15-mm petri dishes were placed on the shaker. All operations, namely irradiation, mixing, and serial dilutions were performed in dim incandescent light; tubes containing irradiated viruses were wrapped in aluminum foil from whence they were inoculated into monkeys (Yaba) or cell cultures (vaccinia and monkey pox) as soon as possible after 10-fold serial dilutions. These precautions were taken in order to avoid possible photo-inactivation. The viability of the UV-treated Yaba virus was determined by titration in monkeys while those

TABLE I. Titer of Yaba Tumor Virus after Heating at Different Temperatures for Various Periods.[a]

Control (unheated)	37°			45°			56°		
	0.5 hr	1 hr	2 hr	0.5 hr	1 hr	2 hr	0.5 hr	1 hr	2 hr
$\geq 10^{6.0}$	$\geq 10^{6.0}$	$\geq 10^{6.0}$	$\geq 10^{6.0}$	$\geq 10^{4.0}$	$\geq 10^{4.0}$	$10^{0.5}$	$10^{0.5}$	0	0

[a] Titer values for 0.2 ml of serially (10-fold) diluted virus inocula injected subcutaneously into rhesus monkeys.

of UV-treated vaccinia and monkey pox viruses were determined by titration in primary rhesus kidney and primary rabbit kidney cell cultures, respectively.

Serological tests. The microtiter system was used in complement fixation (CF), hemagglutination (HA) and hemagglutination-inhibition (HI) tests. Yaba CF antigen was clarified (as described above) 10% tumor homogenate extracted twice in equal amounts in Genetron 113; vaccinia CF and HA antigens were clarified fluid from infected cell cultures. Sera were inactivated at 56° for 30 min for CF tests; they were treated with kaolin and absorbed with chicken red cells for the HI test. The CF tests were performed using 2 units of antigen and 5′CH_{50} units of complement with overnight fixation. Four units of HA antigen were used in HI tests; the antigen–antisera mixtures were incubated at 25° for 1–2 hr before addition of chicken erythrocytes.

Results. A. Effect of heat, pH, and UV light on Yaba tumor virus. Sensitivity of Yaba virus to heat. Three 1-ml samples, in stoppered tubes, of stock Yaba virus were placed in each of three waterbaths at 37, 45, and 56°. One sample from each of the three waterbaths was removed after 0.5, 1, and 2 hr. The samples were placed immediately in ice water, diluted in cold phosphate buffered saline and inoculated into monkeys. The results are recorded in Table I. Although heating at 45° for 2 hr did not completely inactivate the tumorigenic capacity of Yaba virus, heating at 56° for 1 hr resulted in complete inactivation. Temperature of 37° did not affect the virus up to 2 hr.

Sensitivity of Yaba virus to pH. Samples of Yaba virus were adjusted with appropriate buffers (sodium citrate–Tris–HCl and basic Tris) to pH values of 3, 5, 7, and 9. The samples were kept at room temperature for 1 hr and then immediately readjusted to pH 7 with appropriate buffers. They were then further diluted serially and inoculated into monkeys; animals were observed for tumor formation for a period of 6 weeks. The results are presented in Table II. While pH 3 completely inactivated the virus, pH 5, 7, and 9 had no demonstrable deleterious effect.

Inactivation of Yaba tumor virus with UV light. Samples of Yaba, vaccinia and monkey pox viruses were irradiated with UV light under identical conditions in the manner described above. The results showing the sensitivity of these viruses to UV light are presented in Table III. Although the test systems for the detection of viable virus varied, they appeared the most sensitive under the described conditions and hence deemed satisfactory for comparison purposes. As shown, UV light inactivated the Yaba virus in less than 30 sec. Both vaccinia and monkey pox viruses appeared to be slightly more resistant than Yaba to UV light.

B. Reactivation of Yaba virus. Tumor formation by mixture of UV-inactivated Yaba and active vaccinia virus. Stock Yaba virus was irradiated with UV light for 1 min and then mixed with 10^{-2} and 10^{-3} dilutions (approx 5×10^2 and 5×10^3 TCD_{50}) of active vaccinia virus. The two mixtures were

TABLE II. Titer of Yaba Tumor Virus after Exposure to Various pH Values for 1 hr at 25°.[a]

pH:	3	5	7	9
	0	$\geq 10^{6.0}$	$\geq 10^{6.0}$	$\geq 10^{6.0}$

[a] Titer values are for 0.2 ml of serially diluted inocula injected subcutaneously into rhesus monkeys.

TABLE III. Titers of Yaba Tumor, Monkey Pox and Vaccinia Viruses after Exposure to UV Light for Various Periods.[a]

Yaba						Vaccinia				Monkey pox			
	(sec)			(min)				(min)				(min)	
Control (unexposed)	10	20	30	1	2	Control (unexposed)	30 sec	1	2	Control (unexposed)	30 sec	1	2
$10^{6.25}$	$10^{0.25}$	0	0	0	0	$10^{4.5}$	$10^{0.5}$	0	0	$10^{5.0}$	$10^{0.25}$	0	0

[a] Titer values are for 0.2 ml of serially diluted Yaba virus in monkeys and 0.1 ml of monkey pox and vaccinia viruses in cell cultures. See text for methods of testing for virus viability.

then inoculated each at three sites into one monkey. The same monkey was inoculated at distant sites with control UV-irradiated Yaba virus alone. Tumors developed at all 6 sites inoculated with inactivated Yaba-active vaccinia mixtures. No tumor developed at the control site. Ten percent homogenates (prepared as described for the stock Yaba virus) of these tumors were passaged again in monkeys and tumors arising from second passage were homogenized and passaged a third time. Histologic studies of tumors arising during each of the three passages showed histiocytomas entirely like those found in monkeys inoculated with active Yaba virus. The antigenic properties of these tumors were also studied by the CF method using standard specific Yaba and vaccinia antisera. The results are presented in Table IV. As shown, tumor extracts from all three passages fixed complement with both anti-Yaba and antivaccinia sera. Stock Yaba and vaccinia viruses reacted only with their respective antisera. Moreover tumor homogenates derived from all three passages were inoculated into primary monkey kidney cell cultures. No vaccinia virus and no HA activity were detected after two additional passages of each homogenate in cell cultures.

Monkeys given Yaba–vaccinia virus mixtures and second and third tumor homogenates were studied serologically for CF and HI antibodies to Yaba and vaccinia viruses. The results are presented in Table V. CF antibodies against Yaba were readily detected in all monkeys. Vaccinia CF antibodies appeared at a level which was barely detectable in the first passage and continued as such in the following two passages. The vaccinia HI antibodies, however, were clearly demonstrable in the first passage but dropped to low levels in the following passages. Since viable vaccinia virus was contained in the Yaba–vaccinia mixture injected into monkeys in the first passage, an antibody response to the vaccinia antigen was anticipated. However, neither from the first passage tumor nor from the two subsequent *in vivo* passage tumors, could infectious vaccinia virus be isolated. Although the tumors from the second and third passages reacted with both Yaba and vaccinia antisera and presumably contained both antigens, the reason for the low level of antivaccinia CF and HI antibodies in the second and third passage monkeys is not yet entirely explanable. Perhaps antigenicity as detected *in vitro* by the CF test in these experiments is different from *in vivo* antibody-inducing property (see "Discussion").

In the next experiment tumor homogenates from the first, second, and third passages diluted to contain approximately 100 tumor-

TABLE IV. CF Reactions of First, Second, and Third Passage Tumors of UV-Inactivated Yaba Tumor Virus and Vaccinia Virus Mixture.[a]

	Standard antisera	
CF antigen	Vaccinia	Yaba
Tumor passage 1	64	512
2	128	256
3	64	256
Vaccinia (control)	128	<8
Yaba (control)	<8	512

[a] See text for procedure. Monkeys inoculated IM with vaccinia virus did not develop disease; antibodies appeared within 3 weeks. Monkeys, inoculated ID, developed dermal vaccinal lesions; antibodies also developed in 10 to 20 days (9).

TABLE V. Serologic Responses of Monkeys to Yaba–Vaccinia Mixture and to Second and Third Passage Tumors.[a]

Test	Antigen	Passage	Antisera: Days of bleeding after inoculation						
			pre	7	14	21	28	35	48
CF	Yaba	1	<8	<8	64	64	128	128	512
		2	<8	<8	16	64	128	128	256
		3	<8	<8	16	64	128	128	512
	Vaccinia	1	<8	<8	<8	8	8	8	8
		2	<8	<8	<8	8	8	8	8
		3	<8	<8	<8	8	8	8	8
HI	Vaccinia	1	<10	<10	20	80	40	20	20
		2	<10	<10	10	10	10	10	10
		3	<10	<10	10	10	10	10	10

[a] See text for procedure.

inducing doses were mixed with 1:16 dilutions of specific Yaba and with specific vaccinia antisera, incubated at room temperature for 1 hr, and then inoculated, each mixture at three sites, into monkeys. The animals were observed 6 weeks for tumor formation. The results appear in Table VI. As shown, tumor formation by homogenates of the first and second passages were only partially inhibited by the Yaba antiserum. In contrast, homogenates of the third passage as well as the stock Yaba virus were completely neutralized by the Yaba antiserum. Although the difference in the antigenicity of tumors from the three passages, as determined by neutralization test, is not sharply defined, the antigenic heterogeneity among these tumors is clearly demonstrated. Vaccinia antiserum showed no inhibitory effect in this experiment.

TABLE VI. Effects of Vaccinia and Yaba Antisera on the Tumorigenesis of First, Second, and Third Passage Tumors.[a]

Tumor	Antisera[b]	Tumor formation
1st passage	Vaccinia	3/3
	Yaba	2/3
2nd passage	Vaccinia	3/3
	Yaba	2/3
3rd passage	Vaccinia	3/3
	Yaba	0/3
Stock Yaba virus[c] (control)	Vaccinia	3/3
	Yaba	0/3

[a] See text for procedure.

[b] Both antisera were diluted 1:16 before mixing with tumor homogenates.

[c] Sera from a monkey inoculated with unmodified Yaba tumor virus derived from a developing tumor.

Discussion. Our data confirm and extend earlier observations that Yaba tumor virus is perhaps slightly more sensitive than vaccinia and monkey pox viruses to heat, pH, and UV irradiation (3). The reactivation experiments suggest that UV-inactivated Yaba virus was activated by vaccinia virus resulting in the formation of a tumorigenic virus which apparently retained, at least for three passages, certain antigenic components of both viruses, as detected by the property of fixing complement with specific antisera to the parent types. The immunologic responses of monkeys infected with second and third passage tumors indicate the dominance of the Yaba viral antigens. Similarly, results of the serum neutralization tests also suggest antigenic heterogeneity in these tumors which drifted toward Yaba antigen dominance in subsequent passages.

Two possibilities are suggested. First assuming that Yaba tumor and vaccinia viruses are not closely related in antigenic structure, then the results might suggest formation of a tumorigenic hybrid virus. In the process of repairing the UV-damaged genome of the Yaba parent which resulted in the formation

of a hybrid virus, a portion of the vaccinia genome was incorporated into the genome of the resultant hybrid and henceforth coded for certain structural core protein antigens of the hybrid that are synthesized early in the viral replication cycle and have a low molecular weight and hence may be nonantigenic or poorly antigenic (4, 5, 6), but like the NP antigen of poxviruses are capable of reacting with antisera to poxviruses belonging to various subgroups (7). The results of our CF tests with the tumor antigens may reflect such a recombination.

Secondly, viruses in the vaccinia subgroup and Yaba virus may be more closely related antigenically than heretofore believed (8). The process of repair noted above might still apply but for another reason—namely that Yaba virus is reconstituted by synthesis of certain common protein antigens actually shared between the two viruses. Once the primary defect in Yaba virus is repaired, then assembly of new Yaba-specific surface antigens would eventually cover related vaccinia antigen(s).

Although the experimental data are clearly definable the interpretations are difficult. As yet we have no direct evidence that reactivation of Yaba tumor virus was genetically mediated.

Summary. A study of the effects of heat, pH, and UV light on Yaba tumor virus indicated that heating at 56° for 1 hr, pH 3 at room temperature for 1 hr, and irradiation for less than 30 sec completely inactivated the virus. Inoculation of a mixture of UV-inactivated Yaba virus and active vaccinia virus into monkeys resulted in the formation of a tumorigenic presumptive hybrid virus which retained, for at least three passages, certain antigenic components of both Yaba and vaccinia viruses. A possible mechanism of the hybridization is discussed.

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