EXPERIMENTS ON THE CAUSE OF THE RABBIT CARCINOMAS DERIVED FROM VIRUS-INDUCED PAPILLOMAS*

II. Loss by the Vx2 Carcinoma of the Power to Immunize Hosts against the Papilloma Virus

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A squamous-cell carcinoma resulting from secondary malignant change in a papilloma induced with the Shope virus in a domestic rabbit was transplanted to other rabbits in the spring of 1938 (1), and is now in its 73rd Tumor Generation. During the first years of its propagation all of the animals in which it grew progressively became resistant to the virus, their blood serum regularly acquiring the ability to neutralize it *in vitro* and to fix complement in mixture with it (1); even after serial propagation in 22 groups of rabbits, including five in succession which had been hyperimmunized against the virus by Shope's method (2), the tumor still elaborated an antigen resembling the virus immunologically, as shown by the character of the antibody elicited (3). When the last test demonstrating this fact was made, the tumor had been maintained for 3 years and 7 months, and everything seemed to indicate that it would continue to produce the antigen. For this and other reasons further tests were not carried out until the cancer had been propagated for 4½ years more, when a check on the state of affairs seemed in order, and accordingly the skin of a number of rabbits carrying the Vx2, then in its 46th Generation, was inoculated with the papilloma virus, as also that of normal controls. The resulting growths proved the tumor hosts to be as susceptible to infection as these latter. Furthermore, additional tests showed that the tumor in its 47th, 48th, and 50th Generations no longer elicited a complement-fixing antibody against the Shope virus, and direct inoculation of rabbits carrying the cancer in its 50th again made plain that the growths did not stimulate any resistance to the Shope virus. These facts and their meaning will be dealt with here.

The designation, V2, was given to the carcinoma prior to World War II,

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but during it came to have other significance. Hence the tumor will be called the Vx2 from now on.

Results of Inoculating the Papilloma Virus into Rabbits Carrying the Vx2 in Its 46th Generation

The most delicate method of disclosing resistance to the papilloma virus is by means of direct inoculation, neutralization *in vitro* with blood serum coming next in sensitivity, and complement fixation some way behind (4). Inoculation was employed in the following test.

In January, 1946, that is to say nearly 8 years after first transfer of the Vx2, tissue from a growth of the 45th Gen. was cut fine, suspended in Locke's solution, and injected into the anterior muscles of each thigh of 15 adult rabbits,[1] approximately the same amount at every site; and 62 days later 10 of these animals were inoculated with the Shope virus. For this purpose two materials known to yield widely different amounts of virus were utilized, the glycerinated papillomas (paps.) from cottontail rabbits W. R. 6–31 and 1–52, respectively. A 10 per cent extract of each was made in Locke's solution by grinding with sand; spinning was done to remove particles; the supernatant fluids were centrifuged again until they had become clear; and portions were diluted to 1 per cent. The four virus-containing fluids thus obtained were rubbed in equal quantity into sandpapered areas measuring about 4 by 7 cm. on the sides of the 10 animals above mentioned. Four had big Vx2 carcinomas at the time; in two others the growth had recently retrogressed after forming nodules several centimeters across; and in the remaining four no tumors had ever appeared. Five normal rabbits, of the same breed and size as the tumor hosts, were similarly inoculated. The areas receiving the virus,—separated from one another by strips of fur,—were dried in a current of warm air, and covered individually with a layer of paraffined gauze, followed by an enveloping binder of gauze, with a many-tailed bandage over all. The number of paps. appearing was recorded as usual (5): ± = 1 pap.; ± = 2, 3, or 4 discrete paps.; + = 5 to 15 paps.; ++ = many discrete paps.; +++ = semiconfluent paps.; ++++ = confluent papillomatosis.

The findings given in Table 1 show the inocula to have been well graded in titer.

No comparison of the papillomas appearing in the test rabbits was possible until the 14th day after inoculation, because healing was markedly delayed in those carrying large tumors. As always when broadcast inoculation is done, the virus had been strewn on raw corium, epidermal infection taking place in the main only as cells from the severed hair follicles extended laterally under an overlying scab to cover the denuded surface (6). Ordinarily epithelial repair is completed between the 4th and the 8th day, the scab coming away then, but it proved so slow in most of the rabbits with tumors that some or all of the inoculated areas were still covered with tenacious scab after 2 weeks or more, those with the biggest growths remaining scabbed longest. They were already thin when inoculated and emaciated rapidly afterwards as the tumors continued to enlarge, and when at last the scabs came away the healed areas were found to be covered with an exceedingly thin, glazed epithelium and were sometimes scarred as well. No such delayed coverage with atrophic epithelium takes place after the inoculation of normal rabbits, though long scabbing may result and scarring as well if the corium has been scraped too deep,—a fact exemplified now and again by some of the control animals of the present test.

An *s* has been inserted in Table 1 to indicate where the scab remained long; and it will

[1] Mongrel agouti rabbits from the breeding stock of the Institute were used throughout the work.

TABLE 1

Results of Inoculation of the Papilloma Virus into Rabbits Implanted with the Vx2 Carcinoma 62 Days Previously (46th Generation)

Group	Rabbit No.	Size of tumors	Inoculum:	14 days				16 days				19 days			
				Virus 631		Virus 152		Virus 631		Virus 152		Virus 631		Virus 152	
				10%	1%	10%	1%	10%	1%	10%	1%	10%	1%	10%	1%
		cm.													
Normal animals	1			++++	++±	++±	++±	++++	+++	+++	++±	++++	+++	+++±	++±
	2			+++±	++±	++	+	++++	+++	++±	++	++++	+++	++±	++
	3			+++±	++	++	+	++++	++	++±	+±	++++	++±	+++	++
	4			+++	++	s±	±	++++	+++	++	+	++++	+++	++	+
	5			+++	++	s+	±	+++	++	+	±	+++±	+++	++	+
Negative after implantation	45			+++	++	s+	++	+++±	+++	++±	+±	+++±	+++	++±	++
	40			+++	++	+	0	+++	++±	s+	±	+++±	++±	++±	+±
	44			+++	0	+	0	+++	+	++	+	+++±	+	++	+
	39			++	±	s0	0	++±	++	s0	+	++±	++	++	++
With growing or regressing cancers	46	0* - 4.		+++±	+++	+++	++	++++	+++	+++	++±	++++	+++	+++	++±
	48	7. - 7.		++±	++±	s+	s±	+++±	+++	+	+	+++±	+++	++±	++±
	47	10. - 6.		s++	++	+	±	s+++	+++	++±	+	s+++	+++±	++±	+
	49	6. - 8.		s++±	++	s±	±	+++	++	+±	+	+++	++±	+++	+±
	41	0‡ - 0§		s++	±	s0	±	+++	++	±	+±	+++	++	+±	++
	38	12. - 7.		s0	s++±	s0	s0	s±	s++±	s0	s+	s+	s++	s0	s+†

s = scabbed area. * Previously 2.5 cm. in diam. ‡ Previously 4.0 cm. in diam. § Previously 3.0 cm. in diam. † died on 19th day.

be seen that some papillomas arose even on the animal with the slowest healing, the greatly emaciated rabbit 38, which died of its tumors on the 19th day while its inoculated areas were still almost entirely scabbed. The papillomas appeared on a zone just within the borders of the inoculated expanses, where the scab had begun to separate, doing so by good chance on the areas where the fluids containing least virus had been put (1 per cent suspensions of materials 6-31 and 1-52, respectively). Here as many papillomas arose as on spots of similar size on some of the control animals and those in which the Vx2 had failed to grow. This similarity is the more significant because delayed epidermal regeneration lessens the opportunity for infection with free virus,—which can no longer be recovered from the site of inoculation after 48 hours (7),—and also because papillomatous proliferation slows whenever the host animal is rapidly losing weight for any reason, as we have had repeated occasion to note. This latter factor, as affecting the later findings in Table 1, need scarcely be invoked since these were essentially the same from group to group of animals, save for the special instance of rabbit 38.

With due allowance made for the complications just described, it becomes obvious (Table 1) that the rabbits carrying large Vx2 cancers, or in which smaller ones had retrogressed, manifested no immunity whatever on inoculation with the papilloma virus. One might suppose from the findings on the 14th day that the animals implanted unsuccessfully with the cancer showed some slight resistance to the virus were it not known that the Vx2 in its early generations, during the period that is to say when it regularly induced such resistance as it grew, elicited none that was demonstrable until it had reached a considerable size (1). Normal rabbits differ somewhat in susceptibility to infection with the papilloma virus (see Table 3; results with a saline suspension), and the findings now in question can be put down to this circumstance and to the variables inherent in the crude technique of test inoculation.

Results of Complement Fixation Tests with the Sera of Rabbits Carrying Tumors of the 47th and 48th Generations

Specimens of serum from the next two groups of animals implanted with the cancer were subjected to complement fixation tests with the papilloma virus (Tables 2 and 3). As already stated, such tests had regularly yielded strongly positive results in the case of every rabbit carrying large tumors in the first 22 generations (1).

Blood was taken in June, 1946, from 12 rabbits of the 47th Gen., each with two big Vx2 cancers due to implantation 85 days previously, and from five normal animals as well and six with large paps. due to broadcast inoculation with virus. All the cancers were huge, as much as 18 cm. across. The sera were examined as "unknowns" by J. G. K. according to a standard technique (4), with two reliable Shope virus materials as antigens. The specimens from the animals with paps. fixed complement strongly, whereas none from the other rabbits did so at all (Table 2).

Sera for a second similar test were procured in the following September from 11 rabbits, each with a single huge Vx2 carcinoma 87 days old, of the 48th Gen. Again several of the tumors were 18 cm. across. Serum specimens from five normal rabbits and from five with well established paps. served as controls. Again the sera from these last fixed complement strongly,

TABLE 2

Complement Fixation Tests with Serum of Rabbits Carrying Vx2 Carcinomas (47th Generation)

Source of serum		Duration of growths	Size of cancers	Complement fixation tests* Antigen W. R. 21, 1:100 Serum dilutions 1:2	1:4	1:8	1:16	1:32	Antigen W. R. 29, 1:100 Serum dilutions 1:2	1:4	1:8	1:16	1:32
Rabbit No.		*days*	*cm.*										
Rabbits with cancers (47th generation)	2-67	85	14.0-11.0	0	0	0	0	0	0	0	0	0	0
	2-68	"	10.0-13.0	0	0	0	0	0	0	0	0	0	0
	2-70	"	6.0- 7.0	0	0	0	0	0	0	0	0	0	0
	2-72	"	12.0-14.0	0	0	0	0	0	0	0	0	0	0
	2-73	"	12.0-12.0	0	0	0	0	0	0	0	0	0	0
	2-74	"	16.0-16.0	0	0	0	0	0	0	0	0	0	0
	2-75	"	12.0- 5.0	0	0	0	0	0	0	0	0	0	0
	2-76	"	10.0- 6.0	0	0	0	0	0	0	0	0	0	0
	2-77	"	11.0-12.0	0	0	0	0	0	0	0	0	0	0
	2-79	"	12.0-16.0	0	0	0	0	0	0	0	0	0	0
	2-80	"	6.0-12.0	0	0	0	0	0	0	0	0	0	0
	2-81	"	7.7- 7.7	0	0	0	0	0	0	0	0	0	0
Rabbits with benign, virus-induced papillomas	2-53	121		++++	++++	++++	++++	0	++++	++++	++++	+++±	0
	2-61	"		++++	++++	++++	++++	0	++++	++++	++++	+	0
	2-58	"		++++	++++	++++	+++±	0	++++	++++	++++	±	0
	2-54	"		++++	++++	++++	0	0	++++	++++	++±	0	0
	2-60	"		++++	++++	+++±	0	0	++++	++++	+++	0	0
	2-55	"		++++	++++	+++±	0	0	++++	++++	+	0	0

* The complement fixation tests were done by a standardized method previously described (4).

++++ = complete fixation of 2 units of complement (no hemolysis); 0 = no fixation (complete hemolysis). The antigens were not anticomplementary in concurrent tests, nor were any of the serum specimens.

In a concurrent test the sera from five normal control rabbits failed to fix complement in dilutions 1:2-1:64 in mixture with each of the two antigens.

TABLE 3

Complement Fixation Tests with Serum of Rabbits Carrying Vx2 Carcinomas (48th Generation)

Source of serum		Duration of growths	Size of cancers	Complement fixation tests* Antigen W. R. 21, 1:100 Serum dilutions 1:2	1:4	1:8	1:16	1:32	Antigen W. R. 29, 1:100 Serum dilutions 1:2	1:4	1:8	1:16	1:32
Rabbit No.		*days*	*cm.*										
Rabbits with cancers (48th generation)	2-04	87	8.0	0	0	0	0	0	0	0	0	0	0
	2-05	"	14.0	0	0	0	0	0	0	0	0	0	0
	2-07	"	8.0	0	0	0	0	0	0	0	0	0	0
	2-08	"	12.0	0	0	0	0	0	0	0	0	0	0
	2-09	"	14.0	0	0	0	0	0	0	0	0	0	0
	2-10	"	15.0	0	0	0	0	0	0	0	0	0	0
	2-11	"	6.0	0	0	0	0	0	0	0	0	0	0
	2-14	"	9.0	0	0	0	0	0	0	0	0	0	0
	2-03	"	10.0	0	0	0	0	0‡	0	0	0	0	0
	2-15	"	6.0	+±	+	+	±	0‡	+±	+±	0	0	0‡
	2-01	"	8.0	+++±	+±	±	0	0‡	+±	±	0	0	0‡
Rabbits with virus-induced papillomas	2-81	77		++++	++++	++++	++++	+++	++++	++++	++++	++++	+++±
	2-82	"		++++	++++	++++	+++±	+	++++	++++	++++	++++	±
	2-78	"		++++	++++	++++	++++	±	++++	++++	++++	++++	±
	2-45	"		++++	++++	++++	+±	0	++++	++++	++++	++++	±
	2-22	"		++++	++++	+	0	0	++++	++++	+	0	0

* See footnote of Table 2 for method. Serum from five normal control rabbits, tested concurrently, failed to fix complement in mixture with the two antigens.

‡ Anomalous result. A second complement fixation test with the sera of the two rabbits (2-15, 2-01) yielded similar findings, but neutralization tests gave little or no indication that the sera contained even a trace of antiviral antibody.

whereas the control sera and those from nine of the cancer-bearing rabbits failed to do so at all. The specimens from the other two animals carrying the Vx2 yielded anomalous findings (Table 3).

Attempts to Infect the Vx2 Carcinoma with the Papilloma Virus

The evidence seemed now to justify the conclusion that the antigen resembling the papilloma virus immunologically, which had been present in the Vx2 carcinoma throughout the early years of its propagation, was gone from the tumor, and the question arose whether the latter could be experi-

Attempts to Infect the Vx2

49th Tumor Generation

No. of rabbits	Initial treatment	Group and No.	Days 8	Days 18	No. developing tumors	Days 63
30	*20* implanted with Vx2 tissue in Locke's solution	I *10* kept as controls			8	All killed. Rate growth of the tum had been similar f group to group, they proved ide in morphology. Portions of the la tumors of each gr were pooled and planted in a group (50th Tu Generation).
		II *10*	5 injected with virus intravenously*		4	
				Other 5 similarly injected, but with different virus material‡	5	
	10 similarly implanted but with tissue in Locke's containing virus*	III *10*			7	
5 larger animals	Implanted in legs of one side with Vx2 tissue exposed to virus *in vitro*, and in those of other side with unexposed tissue*	IV *5*			2	Killed Vx2's similar in ri and left legs. *Discarded*

* Two were inoculated with the virus on the side as well, papillomas resulting.
‡ Three " " " " " " " " " " " " "

mentally infected with the virus. The papillomas and carcinomas induced by tarring rabbit skin had been readily infected, remarkable changes often resulting both in their character and behavior (8, 9). In most of the successful instances the virus had been injected into the blood stream of animals carrying the tar tumors and had localized in these, but in some cases bits of the growths had been exposed to the virus *in vitro* and reimplanted in their hosts. Both methods of infection were utilized in the Vx2, and, after the growths due to proliferation of the cells exposed to virus had become big, fragments

th the Papilloma Virus

50th Tumor Generation			
w e .o.	Days 239	41	Results of serum tests (Table 5)
	Big tumors in all		Negative save for one dubious fixation of complement
	Big tumors in 3, tiny in 1, 2 neg.		No fixation of complement but sera from hosts with big tumors neutralized the virus slightly
	Big tumors in all		One serum fixed complement dubiously; several slightly neutralized the virus
	Big tumors in 4, small in 3		One serum fixed complement dubiously; several from the hosts with big tumors slightly neutralized the virus
	All bled for complement fixation and neutralization tests (Table 5)	Virus inoculated into skin of all that seemed likely to live several weeks—see text.	
Unimplanted controls (furnished sera for test)			Wholly negative

of them were transferred to new hosts, and when these in turn had developed large tumors they were tested for resistance to the virus. The successive steps in the experiment are outlined in Table 4.

Infection of tar tumors with the Shope virus often causes them to grow with unprecedented activity, yet without change in morphology. The present experiment was so designed as to cover this possibility.

The neoplastic material for the implantations came from a single huge growth from an animal of the 48th Gen. whose blood had wholly failed to fix complement with the Shope virus (like that of the others of its group). 9 gm. of tumor was used to obtain 150 cc. of suspension. This was made by forcing the malignant tissue through a monel metal sieve (16 meshes per cm.) by means of a pestle, adding from time to time a 1 in 20 mixture of normal rabbit serum and Locke's solution, and pipetting the suspension away. It contained many small tissue fragments and free cells and cell clumps in great number.

Thirty-five rabbits were injected with the suspension, each at six sites (muscles of the forelegs, anterior, and posterior thighs). The animals had been separated into four groups on the basis of their weights,—which ranged from 3200 to 4500 gm. Groups I, II, and III, of 10 animals each, were closely comparable in this respect, and Group IV consisted of five heavier rabbits. For Groups I and II the tumor suspension was mixed with an equal amount of serum-Locke's solution, let stand an hour at room temperature, with frequent stirring, and then injected, 1 cc. at each site. For Group III the suspension was mixed with an equal amount of a 10 per cent Berkefeld-filtered extract of the paps. of W. R. 6-31 in serum-Locke's solution.[2] Again the mixture was let stand before injection, and this time the skin was slit prior to insertion of the implanting needle, to exclude the possibility that it might carry in cells from the epidermis, perhaps to become converted into virus pap. cells, thus confusing the outcome. Also two animals of the group were inoculated with the virus suspension into the skin of the side, as control to its pathogenicity. Paps. developed there within 10 days.

The outsize rabbits of Group IV were implanted in the legs of one side with tumor tissue that had been exposed to virus *in vitro* and at corresponding sites in the opposite legs with unexposed tissue. For this purpose a portion of the suspension of cancerous tissue mixed with virus-containing fluid, made up as for Group III, was thrown down with the centrifuge after 20 minutes at room temperature, the supernatant fluid was withdrawn as completely as possible and replaced with serum-Locke's, the tissue resuspended, and injection done through skin slits. The skin of two of the animals was inoculated with the supernatant fluid, obtained as just stated, in order to learn whether it still held any virus, or all had become fixed on the tissue, as might well have happened (10).[3] After a wait of some minutes, to allow for the removal from the blood stream of such virus as might have entered it from the injected material or from the directly inoculated areas of skin (8, 9), the opposite legs of the animals were implanted with tumor suspension made up as for Groups I and II but now treated in all ways like that mixed with virus-containing fluid save that serum-Locke's had been used instead of this latter.

It will be recalled that Groups I and II, of 10 rabbits each, had been implanted with Vx2 tissue unexposed to virus. An effort was made to infect this tissue *in situ* after 8 days, a time sufficient for vascularization of it to have occurred. Five animals of Group II were injected intravenously with 10 cc. each of a freshly made 5 per cent suspension of virus W. R. 6–31, filtered as previously; and two of the five were inoculated with it on the skin of the sides

[2] Virus material W. R. 6–31 had been chosen as notably pathogenic, producing paps. sooner and in greater number than any other available.

[3] It still contained a great deal of virus as shown by the numerous skin paps. developing.

in addition, to test its pathogenicity,—with profuse papillomatosis as result. After another 10 days a similar attempt at infection *in situ* was made with the five remaining animals of Group II, which were then injected intravenously with 10 cc. of a mixture in equal parts of 6 per cent Berkefeld filtrates of two pap. materials not previously employed (W. R. 6–33 and 5–97), three receiving it into the skin as well,—with result in paps.

Tumors soon appeared at every implantation site in 8 of the 10 animals of Group I, which had received tissue unexposed to virus, in 4 of the 5 of Group II, injected intravenously with virus 8 days after implantation with such tissue, in 5 of 5 receiving a similar injection after 18 days, in 7 out of 10 of Group III, which had been implanted with tumor tissue suspended in virus-containing fluid, and in 2 of the 5 of Group IV, which had received tissue exposed and unexposed to virus *in vitro* at corresponding situations.

On the 21st day, caliper measurements and outline drawings were made of all of the growths and this was done again weekly until central necrosis of the neoplastic tissue, with secondary cyst formation, rendered their size undependable. Until then they had an almost symmetrical football shape; and their rates of enlargement from group to group proved practically identical. Wide differences were noted in this respect from host to host, but none that could be referred to the influence of the virus, and the morphology of the tumors was unaffected by the latter, as sections ultimately showed, all of the growths remaining wholly similar, anaplastic Vx2 carcinomas.

The rabbits were killed 63 days after implantation, and blocks from the cancers, fixed in acid Zenker's solution, were sectioned, and stained with methylene blue and eosin. No transplantations were made from Group IV, but portions of the tumors of the two or three rabbits with the largest growths in each of the other groups were pooled in equal quantity, and 5 per cent suspensions made in serum-Locke's solution. Nine normal rabbits (Group B) were implanted at the usual six sites with the suspension made from the tumor material provided by the multiplication of cells unexposed to virus, seven (Group C) with that from growths due to cells which had been introduced together with virus-containing fluid, while six each (Groups D and E) received cells from the tumors of animals injected intravenously with virus 8 and 18 days respectively after implantation. The resulting growths were measured at intervals and drawn in outline as before.

Tumors resulted in most instances, and grew at nearly the same rate in the majority (Table 5). In one rabbit of Group C however, with implants from growths due to neoplastic tissue suspended in virus-containing fluid, only a single nodule appeared and this enlarged very slowly; another had four tumors, also slow-growing; while in a third the six growths arising remained under the average in size. Two animals of Group D, which had received neoplastic material from hosts injected with virus 8 days after implantation, developed no tumors at all, and in a third mere tiny, transient nodules formed. The growths which had provided the suspension for this group came from two animals only, and in one they had fared ill of late as shown by their almost gristly consistency, due to heavy encapsulation with a reactive tissue containing many round cells. These exceptional instances yielded significant data, as will appear (Table 5).

After 39 days the tumors were from 4 to 7 cm. in longest diameter in most of the new hosts. All the rabbits were bled then for complement fixation and neutralization tests. Two days later those animals that seemed likely to survive for a few more weeks were inoculated with 5 and 0.5 per cent suspensions of virus W. R. 1-27, into scarified squares on the skin of one side, and with similar preparations of W. R. 6-31 on the other side,— this to learn whether they had any resistance to the virus. Six normal animals (Group A, Table 2) of the same approximate age and weight as the tumor hosts were inoculated also. They had been bled for the complement fixation test just previously, as had three rabbits carrying large virus

Neutralization Tests with the ... of 50th Generati...

Source of serum	Rabbit	Diameter of tumors	Test rabbits	14 days								
				d	a	g	f	h	b	c	e	d
	No.	*cm.*										
Saline control				0	±	±	+	++	+++	+++	++++	+±
Group	66			±			++			+++	±	++
A	69			+			+++			+++	+++	+±
	70			+			++			0	+++	+±
Normal rabbits	67				++	0		0	++			
	68				0	++		0	++			
	71				+++	++		+++±	+			
B	40	4.0 - 4.3 - 4.3 - 6.0 - 4.0 - 4.0		*±	+	0	++	++	+++	++	++++	+
	17	4.5 - 4.3 - 5.5 - 4.5 - 3.2 - 3.0		+			+			++++	++	++±
	15	4.0 - 0 - 4.5 - 5.0 - 3.5 - 3.2		+	±				±	0		++
Rabbits with implants from tumors not exposed to virus	13	4.3 - 4.3 - 6.0 - 4.0 - 5.0 - 4.0		++	0				+++	±		++
	26	4.3 - 3.5 - 3.5 - 3.8 - 3.0 - 3.2		+++	0				++	0		+++
	30	4.0 - 4.0 - 4.3 - 4.0 - 3.3 - 3.3				0	±	±			++	
	19	4.0 - 3.0 - 4.3 - 6.0 - 3.8 - 3.3				±	0	0			++	
	34	5.0 - 3.5 - 5.5 - 5.0 - 3.5 - 3.0			0	++		0	++			
	37	4.5 - 4.2 - 7.0 - 7.0 - 4.0 - 3.2				++	0	±			++	
C	22	0 - 0 - 0 - 1 - 0 - 0		++			++			++	++±±	+++
With implants from growths due to tumor cells exposed to virus in suspension	18	0.6 - 1.6 - 1.7 - 1.5 - 0 - 0			±	++		±	+++			
	27	3.2 - 3.2 - 1.8 - 2.7 - 1.3 - 2.0			0	+++		+++	++++			
	25	4.5 - 4.5 - 4.5 - 5.0 - 4.0 - 4.0		±			0			+	0	+±
	21	5.0 - 4.5 - 6.5 - 6.0 - 4.5 - 3.0		‡0	+±	0	±	±	++	0	+++	±
	38	5.0 - 5.5 - 5.5 - 5.5 - 3.5 - 4.0		±	0				0	0		+
	24	5.5 - 6.0 - 10.0 - 7.0 - 3.5 - 6.0				+	+	0			++	
D	39	None		+±	±				++	+		+±
With implants from growths 8 days old when host injected with virus	35	"				++	+				++++	
	36	Tiny—now gone			0	+++		++	++++±			
	20	4.5 - 4.5 - 4.5 - 3.5 - 3.5 - 3.0		0	0				++	+++		+
	23	5.0 - 3.5 - 5.0 - 4.0 - 3.5 - 3.0				±	0	±			++	
	12	4.5 - 5.3 - 4.3 - 4.5 - 3.5 - 3.5			0	0		±	±			
E	33	5.0 - 5.0 - 5.0 - 6.5 - 6.5 - 6.0		§0	0	0	0	0	0	0	+	0
With implants from growths 18 days old when host injected with virus	31	5.0 - 5.5 - 5.0 - 4.0 - 3.5 - 3.5		0	0				+++	+		±
	41	5.3 - 6.5 - 6.5 - 5.0 - 6.0 - 6.0		0			++			0	0	±
	32	6.5 - 5.5 - 5.5 - 6.0 - 4.0 - 5.0		0	0				+++	+++±		+
	16	4.0 - 5.0 - 5.0 - 5.0 - 3.8 - 3.5				0	+	±			±	
	28	5.5 - 5.5 - 6.5 - 6.0 - 5.5 - 6.0				±	+	+			0	
With virus papillomas	50				0	0		0	0			
	98			0			0			0	0	0
	59			0	0	0	0	0	0	0	0	0

* Dubious complement fixation; numerous papillomas on later inoculation. ‡ Dubious complement fixation; died soon after

ls Bearing the Vx2 Carcinoma

s 39 days old

19 days						22 days							
g	f	h	b	c	e	d	a	g	f	h	b	c	e
+	+±	++±	+++±	+++±	++++	++	++	+	+++	+++	++++	++++	++++
	++			+++	+±	+±			+++			++++	++
	+++±			+++	+++	++			++++			++++±	+++±
	+++			++	+++±	+++±			+++±			+++	++++
		+±	+++				+++	+		++±	+++++		
+±		+	+++				++	+++		+±	+++		
±		+++±	+++				++++	+++		++++++++±			
+	++	++±	+++±	+++	++++	+±	+++±	+±	+++±	+++	++++	+++±	++++
	++			++++	++±	+++±			+++			++++	+++
			+±	+±		++	+±				+±	+±	
			+++	++		+++	+±				+++	++	
			++±	+±		+++	++				+++	+±	
	++	++			+++			+	+++	++			+++±
	+	±			+++			±	+±	+			+++±
+±		+±	++				+±	++±		++	++±		
+±	+	+±			++			++±	+±	+±			++
	++±			+++	+++±	+++±			++±			+++±	+++±
+		+±	+++±				+++	++±		++	++++		
r±		+++	++++				+++	++++		++++±	++++		
	+			+±	+	++			+±			++	+
	+	+±	+++	+	+++	+	+++	+	+±	++	+++±	+±	+++
			++	+±		+±	++				+++±	++	
+	+±	+			+++			+±	++±	++			+++±
			+++±	++		++	++				++++	+++	
-+	+±				++++			+++	+++				++++
-±		++	+++±				+±	++++		+++	++++		
			++±	+++		++	+±				+++±	+++±	
-±	+	+±			++			++	++	++			++
		+	++				++	+		++	++±		
	+	+	+	+	+±	±	±	+	++	+±	+±	+±	++
			+++	++		+	+++				+++±	+++	
	++±			+±	+±	+			+++			++	++
			+++	+++±		+±	+±				+++±	++++	
+	++±	+			+			+	+++	+±			++
	++±	+±			+±			+	+++	++			++±
0		0	0				0	0		0	±		
	0			0	0	0			0			0	±
0	0	±	±	0	0	0	0	±	0	±	±	±	±

n. § Dubious complement fixation; inoculation test marred by scabbing.

paps. on their skin. The sera from these last fixed complement strongly with the virus, whereas none of the others did so at all, save three in which the findings were dubious. On repetition of the test, with another antigen in addition to the two previously employed, the same three again yielded dubious fixation (see footnote to Table 5).

A neutralization test was done after the sera had been stored frozen for 23 days. Part of each specimen was mixed with an equal amount of a 1 per cent extract of pap. material W. R. 6-31, incubated for 2 hours, and inoculated into scarified squares on some or all of eight large normal rabbits. The mixtures with sera that had given dubious complement fixation previously were inoculated into all eight test animals, but, owing to the inclusion in the test of specimens from other experiments, the rest could be inoculated into four animals only, not in every case the same four. This has rendered comparison of the findings difficult, and the more so since the differences were not great.

Normal rabbits yield sera affecting the Shope virus very slightly when at all, as already mentioned, though the susceptibility of their epidermis to infection with it varies not inconsiderably, as also remarked, a fact manifest in the differing number of paps. produced by the same dilute virus suspension. The test rabbits of Table 5 varied more than usual in this latter respect, as the results with the control mixture of virus with saline made plain. Hence the results have been tabulated in order (from left to right) of the increasing susceptibility to infection thus disclosed; and the individual results with the test mixtures from the tumor hosts of each group have been so ranked that the results with those inoculated into the same test rabbit fall in the same vertical line.

It will be seen (Table 5) that the sera from the control animals carrying paps. due to the virus neutralized it almost or quite completely, whereas those from the normal controls (previously negative in the complement fixation tests) had at most only the slightest inhibiting effect. The specimens from the animals of Group B, all carrying large Vx2 tumors due to the proliferation of cells unexposed to virus, were also practically devoid of neutralizing power. In Group C however, rabbits implanted with tissue from tumors due to the proliferation of cells directly exposed to virus, differences of note were obtained. The sera from individuals with small tumors, and fewer of them than usual, caused no neutralization, whereas it was perceptible (14th day) in the case of those with large ones. Group D provides a similar contrast, while in Group E, consisting entirely of rabbits having large growths, there appeared to be some evidence of a general neutralizing power. The serum specimen which disclosed this most definitely (that of rabbit 33) had given dubious complement fixation previously and hence had been inoculated into all eight test rabbits. So too had the serum of No. 40 for the same reason, and that of No. 21; but neither displayed neutralizing power. This was so inconsiderable even in the case of No. 33 as scarcely to be discernible any longer by the 22nd day after inoculation of the test mixture.

The findings of Table 5 fall in with previous experience that the neutralization test is more sensitive than complement fixation as an indicator of resistance to the papilloma virus. It appeared to disclose some slight immunity to the Shope virus in practically all of the rabbits of the 50th Generation which had developed big Vx2 carcinomas after implantation with tissue procured from those tumors of the 49th Generation resulting from the multiplication of cells exposed to virus *in vitro* or *in vivo*. The animals in which small growths only had appeared, or none at all, proved devoid of immunity, a fact excluding the possibility that it was due to antibodies carried over with the neoplastic tissue from the rabbits of the previous generation into which the Shope virus had been introduced in one way or another.[4]

[4] The total quantity of neoplastic tissue implanted in each rabbit of the 50th Generation was about 0.3 gm.

The neutralization test is transcended in sensitivity, as has been stated, by the response to direct inoculation with the virus. For this reason all of the rabbits of Table 5, except those with papillomas and those which would soon die of the Vx2, were inoculated with virus two days after they had been bled for the neutralization test just discussed.

Four clarified suspensions (5 and 0.5 per cent strength of viruses 1-27 and 6-31) were rubbed into large squares on the sides of each rabbit. The majority of those carrying big Vx2 growths died before enough time had elapsed after inoculation for papillomas to appear; the others wasted rapidly, like those of Experiment 1 (Table 1), and the expanses which had received the virus remained scabbed in most instances for 2 weeks or longer though the current of air used for drying them had been but slightly warmed. No such wasting or scabbing of the inoculated controls took place, or of the animals having small growths or none: papillomas appeared promptly on all these, whereas they were considerably delayed in the case of the few rabbits with big tumors that survived for a sufficient time to develop them. The delay was longer and the papillomas were somewhat less numerous in the rabbits carrying growths derived from tumor cells exposed to virus; but for reasons already stated one cannot be sure that this was because of induced immunity and not consequent on the physical state of the animals,—by then at the extreme of emaciation.

No further transplantations of the growths were made.

DISCUSSION

The findings here recorded differ diametrically from those obtained with the Vx2 during the first years of its propagation. They have since been extended by complement fixation tests, done at intervals up to this writing, with sera from many rabbits of the 60th to the 73rd Tumor Generations inclusive, said sera regularly failing to fix complement with the papilloma virus. Almost 6 years have now elapsed since inoculation of animals of the 46th Generation first disclosed the fact that the Vx2 cancer no longer stimulates resistance to the virus.

The antiviral immunity manifested by rabbits carrying the Vx2 in its first 22 generations only gradually developed as the tumor enlarged, so one might ask whether the growth had been present long enough to have induced immunity in the animals of the experiments now reported. The data on this point are conclusive. The hosts of the carcinoma in its 1st Generation yielded sera, when bled 39 days after implantation, that had a definite neutralizing effect on the papilloma virus (1), although the tumors they then carried were small in comparison with those of the 50th Generation, Group B, Table 5, tested after the same interval with wholly negative results.[5] Strongly positive complement fixations were obtained with all of many sera from 5th Generation rabbits bled 41 days after implantation, when their growths were still quite small; and so too with specimens from 6th Generation hosts with tu-

[5] The sera from hosts of the 7th, 9th, 10th, and 19th Generations neutralized the virus markedly (1), but their tumors had been present for somewhat longer periods (59, 47, 67, and 55 days respectively), and hence the conditions are not strictly comparable.

mors 41 to 61 days old,—this though the total bulk of neoplastic tissue they carried individually was frequently less and never more than that of the 47th and 48th Generation rabbits which, when bled 85 to 88 days respectively after implantation, yielded sera negative on test.

None of the hosts of the Vx2 in its early generations were directly inoculated with the papilloma virus to learn whether immunity to this agent had developed, because the serological findings sufficiently demonstrated this fact.

It should be pointed out that those animals of the 50th Generation to which the Vx2 was transferred some weeks after experimental exposure of its cells to virus (Table 5, Groups C, D, and E), seemed to have acquired only an inconsiderable immunity to this latter as compared with that manifested by the hosts of the cancer in its first 22 generations. None of the 50th Generation sera fixed complement definitely, not even the specimens obtained from rabbits in which the tumors had become huge. But it may be that only a small proportion of the Vx2 cells exposed to the virus in the 49th Generation had become infected under the conditions experimentally provided; for the tissue suspension which was mixed with virus just prior to implantation contained many cell clumps into which it may not have penetrated, and after intravenous injection it may not have reached every cell. The virus-infected tumors of Group IV, Table 4, enlarged at the same rate as the control growths in the opposite legs, so no reason exists to suppose that the cells with which the virus became associated outstripped their fellows and came to preponderate as time went on.

Many extraneous viruses, some of them highly incongruous (*e.g.*, that of yellow fever) can go along in tumors as "passengers" (in Andrewes' phrase), effecting no cellular alterations or only injurious ones. Yet numerous facts have seemed to indicate that the papilloma virus is more than a mere passenger in the carcinomas ultimately resulting from its action. What would appear to be its peculiar formative influence is often plainly visible in the cancers (11); indeed one of them, the Vx4, can perhaps best be described as a virus papilloma turned malignant (12). Yet the Vx2 appeared no different in the 46th Generation, when it failed to immunize, from what it was in the 22nd, when it regularly did so. Nor did attempted infection with the virus in the 49th Generation result in any morphological changes: the growth remained the same completely anaplastic, epidermal carcinoma as before. But one may recall that similar anaplastic carcinomas, induced by tarring and subsequently infected with the Shope virus, often show no sign of the latter's presence other than enhanced activity (8–10).

The immunological findings with the Vx2 in its first years of propagation gave grounds for the assumption that the papilloma virus or a nearly related variant was permanently established in the growth, and its later failure to call forth the antiviral antibody was wholly unexpected. But the virus is

known to be exigent in its demands, failing to infect the epithelium of lips, gums, or tongue, indeed even conjunctival epithelium rendered keratinizing beforehand by deprivation of vitamin A (13). Perhaps its rate of increase in the Vx2 carcinoma, if it was really present, gradually fell behind that of the tumor cells with which it was associated during their long propagation, these eventually getting free from it, just as rapidly dividing paramecia ultimately free themselves from the entity known as kappa (14).

Many facts have seemed to indicate that the carcinomas deriving from rabbit papillomas cannot be due to the Shope virus as such but perhaps to variants of it, some of them expressive of such slight alterations that its morphological influence still dominates in the malignant growths they cause; *e.g.*, the Vx4 tumor already mentioned. Wider variations from type would account for those other carcinomas which show fewer cytological signs of the presence of virus or none. All this seemed likely during the period when the Vx2 carcinoma elaborated an antigen indistinguishable immunologically from the virus. But now that the immunological findings are negative,—while the carcinoma itself appears unaltered,—what can be the cause of the growth? If it is due to a variant of the Shope virus, the variation has been so great as to place the resulting cancer-producing agent beyond the antigenic range of the latter. No alteration of such magnitude has yet been observed in any of the other pathogenic viruses followed in laboratories,—though the experience to date can scarcely be regarded as final. Several serologically distinct viruses cause the naturally occurring disease known as influenza, and until just now (15) the same has appeared to hold true of poliomyelitis. But here one must remember that similarities in obvious effect do not necessarily imply similarities in origin; convergences in effect may take place as well as divergences in cause. The viruses responsible for what seems a single clinical state may perhaps sometimes be of widely different origin.

At this uncertain point the problem of the cause for the Vx2 carcinoma must perforce be left. But in one respect a small gain can perhaps be registered. For now that the cancer no longer stimulates immunity against the papilloma virus, it has come to resemble the classical malignant tumors in giving no recognizable sign of the nature of its actuating cause. Hence it may commend itself as an experimental material.

SUMMARY

Tests were made to learn whether an anaplastic, epidermal carcinoma, the Vx2, which had originated more than 8 years previously from a virus papilloma in a domestic rabbit, still rendered its hosts immune to the virus. It had done so in the first 22 successive groups of animals to which it was transferred during a period of 3½ years, its growth regularly eliciting a blood antibody that neutralized the Shope virus and fixed complement in mixture

with it; and on the assumption that this would continue to be the case no further observations were made for nearly 4½ years more. Then direct inoculation of animals carrying the tumor in its 46th Generation showed them to be as susceptible to the virus as normal rabbits; and sera procured from hosts of the 46th, 47th, 48th, and 50th Generations failed to neutralize the virus or fix complement with it. Tests of this last sort, repeated at intervals since,—most recently with sera from animals carrying the tumor in its 73rd Generation,—have yielded consistently negative findings.

Loss of the power to immunize against the papilloma virus was not attended by any perceptible change in the Vx2 carcinoma. Manifestly the antigen responsible for the immunity cannot, as such, have been the actuating cause of the tumor.

Attempts were made to infect the cells providing 48th Generation cancers, by mixing them with a suspension of the papilloma virus at time of implantation, or by injecting this agent into the blood stream of rabbits in which the tumog had already begun to proliferate. Its morphology and rate of growth remained unaltered; but tests of the animals to which transfers were next made yielded what appeared to be evidence of some slight immunity to the virus.

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