SERIAL TRANSPLANTATION OF RABBIT PAPILLOMAS CAUSED BY THE SHOPE VIRUS

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The epidermal papillomas occurring under natural conditions on the skin of western cottontail rabbits were deemed neoplastic by Shope who discovered their virus cause (1), and a later point-by-point comparison showed them to possess all the immediate characters by which tumors are recognized (2). Furthermore it was found that cancers often derive from the growths as result of secondary changes in the virus-infected epithelium (3). Some of the cancers arising from the papillomas experimentally induced with the virus in domestic rabbits have been propagated by serial transplantation (4). In contrast, every attempt to transplant the papillomas themselves has failed, though they are often remarkably vigorous, indeed sometimes locally aggressive, and though the efforts to transfer them have been many, in this laboratory as well as elsewhere. The present paper reports serial propagation of two of them by the utilization of suckling rabbits. The papillomatous tissue flourished in the young animals, soon causing the death of those in which it grew most vigorously, and in others continuing to enlarge for some months after its hosts had reached maturity. Yet it had gained nothing in ability through its sojourn in them, regularly failing to grow when passed on to new adults.

Mongrel domestic rabbits bred in the Rockefeller Institute were used throughout save in two instances in which the groups of newborn animals were provided by pregnant does procured from elsewhere. The colors of the animals were mostly brown agouti or New Zealand Red, but included gray, white, and black, alone or in patchy combination.

The growths for transfer were obtained by broadcast inoculation of the skin of adult rabbits, which had previously been rendered more susceptible to infection by applications of turpentine. The virus material employed was a 5 per cent extract of glycerinated papillomas (paps.) found on cottontail W. R. 152 N when it was trapped. Within 13 days after the inoculations broad, slightly raised expanses of confluent or semiconfluent papillomatosis, showing as yet only traces of keratinization in the gross, appeared on the newly healed skin. Thin slices of them were shaved off, cut fine, suspended in a 1:20 mixture of rabbit serum with Locke's solution, and implanted in the muscles of the forelegs and anterior and posterior thighs of the rabbits carrying them, 1 cc. at each site. Nodules of pap. tissue rapidly formed and portions of these were hashed and suspended, and transfer was made to new hosts of various ages. Adults and weanlings (6 weeks old) received 1 cc. at each of the six sites just mentioned, whereas newborn animals got 0.3 cc., in one or both anterior thighs.
only. The skin was slit prior to insertion of the implanting needle to exclude the accidental carrying in of normal epithelium, perhaps to become infected with virus. The serum-Locke's solution used in the transfer to the first group of baby animals contained 25,000 units of crystalline penicillin G (Squibb) per cc., but none was employed subsequently.

Propagation of the Papillomas

Experiment 1 (Papilloma A).—

The primary host, of agouti hue, was killed 6 weeks after implantation and the intramuscular nodules were excised under aseptic conditions. They were irregularly spherical, 2 to 4.5 cm. in diameter, well encapsulated, and had the composition of those previously described (2), consisting that is to say of a peripheral layer of living pap. tissue enclosing a central mass of keratin. Growth had been mainly expansile, but the microscope showed that some invasive tongues of pap. epithelium had penetrated into the enveloping connective tissue.

The keratin was scraped away from the living tissue, this was hoshed in serum-Locke's containing penicillin, as already stated, and after about an hour at room temperature more serum-Locke's was added and implantation made into the anterior thigh muscles of two litters 1 day old, and into the left anterior thigh only of a third such litter, all of Institute stock.

1st Generation (see Chart 1).—The pap. “took” in all but one of nine surviving sucklings, and in nearly every instance grew as well as in its primary host or even better, rapidly forming more or less spherical masses which became huge in the animals that survived long,—measuring up to 15 cm. across after 5½ months. In the rabbit, set down as negative in the chart, tiny nodules could be felt, but they began to disappear after a month and may have consisted of reactive tissue.

Several of the rabbits were killed early to learn more of the growths or to transplant them. Those of the animal first sacrificed, 4 weeks after implantation, were 1.8 cm. across but proved to be completely keratinized. The growths of another animal killed a week later were a little larger, and while mostly keratinized had a peripheral zone of living tissue. A suspension of it was injected into a new group of rabbits (2nd Generation A). A third rabbit was killed in the following week and again fragments of the growths, now measuring 4 and 6 cm. across, were also transferred (2nd Generation B).

The suckling in which the pap. grew most rapidly was not sacrificed until 3½ months after implantation, that is to say after it had been for some while an adult. It had been implanted in one thigh only and the resulting huge mass, now 14 cm. across, was still alive at the periphery, and had the same general character as those already examined. Another animal of the same litter was killed after 5½ months. It too had been implanted in one thigh only, and bits of its growth, 15 cm. across,—the largest of any obtained during the work,—were transferred to new hosts (2nd Generation C). Two of the remaining animals died of intercurrent causes almost 6 months after implantation; both had big growths (Chart 1), but these had failed of late to enlarge significantly and autopsy showed those of one host to be wholly keratinized whereas those of the other still contained much living tissue. The longest lived animal died more than 6½ months after implantation, and by mischance no postmortem examination was made. Its implants measured only 5 and 3 cm. but had been enlarging slowly.

2nd Generation.—The first transfers attempted were made with pap. tissue in excellent condition, procured from a suckling in which it had grown for only 5 weeks. It was introduced in 18 young adults (2nd Generation A) at the usual six situations, but grew in none.

Three litters 1 to 2 days old were used for the next attempt (2nd Generation B). Again the donor was a suckling, a notably favorable host as shown by the size of the nodules it carried after 6 weeks (Chart 1). The implants “took” in all but one of 17 newborn animals, and in many of them did as well as those in the rabbit originally providing the pap. material.
One of the suckling hosts died after 5 weeks, another after 6, and five more succumbed after 2\(\frac{1}{2}\) to 2\(\frac{3}{4}\) months. These latter had growths measuring up to 10 cm. in diameter; they were still enlarging. Another animal was killed after 3\(\frac{1}{2}\) months for transfer purposes (3rd Generation A); it had growths 4 and 8 cm. across. At about the same time a rabbit with 8 cm. growths died, and one was killed whose growths had done well at first but later disappeared; only an intramuscular, paper-thin film, 1 to 2 cm. across, of yellowish, amorphous material remained where the nodules had been. A week later, that is to say after 3\(\frac{1}{2}\) months, yet another host with big growths died, and two more succumbed after 4 months. The paps. of one of these had begun to retrogress in recent weeks and they were stationary in the other. All of them were found to be wholly keratinized.

The remaining four hosts lived much longer, but their paps. ceased to enlarge after 4 months and dwindled gradually later. When they were killed after 7 to 10\(\frac{1}{2}\) months, sizable nodules still remained but of keratinized tissue only.

For a third transfer (2nd Generation C) tissue was procured from a growth which had existed almost 6 months, during which time its host had become a large adult. Implantations were made into a single day old litter (three individuals) and 15 weanlings. The paps. failed in the newborn animals but grew aggressively and with undiminished vigor in one weanling, masses 4 to 9 cm. across forming at the six implantation sites within 3 months, when transfer was made to the 3rd Generation B. No rabbit of the other groups constituting the 2nd Generation provided such vigorous tissue.

3rd Generation.—Three litters of newborn rabbits served for the 3rd Generation A. The pap. utilized for transfer had steadily grown larger after its host matured, and it “took” in eight out of twelve newborn hosts, proliferating in four with more vigor than in any previous animals, the growths becoming so large within a few months as to cause death. In four others the paps. did less well and ceased to enlarge after 3 months, disappearing in one instance and becoming wholly keratinized in two others, as autopsies showed 30 and 7 months after the implantation. The fourth animal had developed only small growths, some of which had retrogressed when it was killed after 3\(\frac{1}{2}\) months, while others were stationary. These latter provided living tissue for further transfer.

The 18 animals utilized for the 3rd Generation B were all adults when implanted and in none did the pap. grow, although it had been exceedingly vigorous in the weanling host of the 2nd Generation C from which the transfers were made, and although this weanling had become an adult by then and two of the implanted animals had borne litters in which the pap. had done well (3rd Generation A).

4th Generation.—The early death of most of the hosts of the previous generation, together with a local shortage of pregnant does, necessitated the use as hosts of sucklings from an outside source. Three litters of such animals 1 to 2 days old were implanted in one thigh with living tissue from the stationary and retrogressing growths, 3\(\frac{1}{2}\) months old, of the animal of the 3rd Generation A mentioned above. It grew in only one of the twelve sucklings receiving it, but vigorously in this, the pap. mass measuring 6 cm. across when its host was killed and transfers were made to the 5th Generation.

5th Generation.—Again the implanted rabbits were newborn and were derived from the outside stock providing those of the previous generations. It grew in none of the seven that survived implantation.

Chart 1 shows that Papilloma A fared as well in the first three groups of newborn animals successively receiving it as it did after implantation in its primary host, and in its 3rd Generation A grew even more vigorously. There is every reason to suppose that it would have been maintained had the progressively enlarging growths instead of dwindling ones been utilized for transfer.
Charts 1 and 2. Each implanted rabbit is indicated by a circle, save in the 4th and 5th Generations where an X denotes that the suckling came from an outside source. Every papilloma developing is recorded in black, the width of the black area telling the diameter of the growth and the length of its duration. Negative instances are recorded in black vertical lines. The lines connecting the generations lie outside the time scale, which is so arranged as to tell the total period during which the papilloma was maintained in the muscles of successive hosts. Unless otherwise indicated the growths contained living tissue when examined finally.
to the 4th Generation, and had animals of the original stock been available as hosts.

Experiment 2 (Papilloma B).—

A second pap. was established in newborn animals in the same way as the first. On the skin of the original host it had appeared somewhat less vigorous than Papilloma A, and was only semiconfluent when transferred to the leg muscles. It course on serial transplantation is shown in Chart 2. It grew in 17 of 20 newborn animals of the 1st Generation but in only two did as well as in its primary host. Four of the sucklings died from extraneous causes within the first 3 weeks after implantation, and three in which the pap. had done well were killed after 4 to 7 weeks,—one to learn what the growths were like, and two for transfer of them (2nd Generations A and B). The growths of all the remaining animals save one began to dwindle soon afterwards, either disappearing as time went on or becoming keratinized. The nodules of the exceptional one continued to enlarge, attaining diameters of 4 and 10 cm. after 5 months but subsequently becoming smaller; and when the animal died, almost 7 months after implantation, they were found to be wholly keratinized.

The pap. took in none of the 18 young adults constituting the 2nd Generation A, whereas it grew in both of two animals implanted when newborn (2nd Generation B), the sole survivors of three litters. But its growth was slow and the largest nodules were only 2.5 cm. across at most when transfer was made from them after 2½ months (3rd Generation). The nodules in the remaining animal were not so big at this time, but one kept on enlarging for nearly 6 months, only to disappear eventually, as did that in the other leg, which had not fared so well.

Three litters of newborn animals were implanted for the 3rd Generation. The pap. failed in two of them (six individuals), but flourished briefly in four out of six animals of the third, retrogressing later.

It will be seen (Chart 2) that Papilloma B showed little vigor during its propagation. Transfer to the 3rd Generation was made at a time when retrogression had commonly begun, which may have been too late. It seems likely that the growth could have been maintained had early transplantation been regularly done, but the pap. tissue did not have enough vigor to warrant the effort.

Cause for Death of the Implanted Animals.—The papillomatous tissue shaved off the skin of its primary host and implanted in its legs contained many bacteria, needless to say, and some persisted in all the growths subsequently obtained, producing inflammation round about them and obviously influencing their character, as will appear further on. Yet this infection was always mild, never purulent, and in the young hosts dying early other reasons for death were found. Papilloma A killed many sucklings of the 1st, 2nd, and 3rd Generations through its sheer bulk, replacing the muscles of their hind legs so largely that they dragged these, with result in maceration of the underlying skin, progressive emaciation following. Some adults with large tumors were not incapacitated in this way and were still well nourished at death. They had not seemed ill, and nothing of significance was found at autopsy.

1 The penicillin employed in the first transfer of Papilloma A could scarcely have penetrated throughout the fragments of tissue exposed to it.
Papilloma B killed none of its hosts, except perhaps the animal of the 1st Generation in which it had formed the biggest masses. This animal, long since become adult, was quite thin at autopsy, and again no sufficient reason for death was found. Most of the many early fatalities were due to intercurrent causes.

Course of the Papillomas on Propagation

Papilloma A, as observed on the skin of its original host, was considerably more vigorous than B after the same length of time; it was confluent, already covered with a thin layer of keratin, and almost twice as high as B, which was only semiconfluent, with keratinization of its surface just begun. A was pink, whereas B was gray with melanin. The differences in vigor found expression in the behavior of the growths on transfer. Papilloma A fared well as long as it was propagated in sucklings of Institute stock, and its rate of proliferation did not fall off, whereas B did badly after its first transfer and worse still in later ones. Both did best in the litters with highest frequency of takes, as might have been expected.

Enlargements of the growths virtually ceased in most instances when their hosts were 3 to 3½ months old, yet living papillomatous tissue was not infrequently present for a much longer time, as shown by the success of further transfers to sucklings (Chart 1, 2nd Generation C, 4th Generation; Chart 2, 3rd Generation).

In the end every growth of long standing, no matter how large a size it had reached, became stationary or smaller except the one furnishing material for the 2nd Generation C of Papilloma A; and though the tissue of this one was still capable of vigorous proliferation, as proved by its behavior in the sole host of the 2nd Generation C in which it succeeded, reasons exist to assume that it too would eventually have dwindled. Pressure factors affected the growths more and more adversely as they increased in size. Their enlargement was mainly expansive, through cell division at the periphery of the nodules with keratinization toward the center. As they grew bigger the conditions for cell activity progressively worsened, the connective tissue capsule of reactive tissue becoming thicker, fibrous, and inelastic, and hence offering a greater and greater barrier to enlargement, a barrier often reinforced furthermore by muscle aponeuroses against which the enlarging masses eventually pressed.

Not only did the local conditions work against continued success of the papillomas, but the maturation of their hosts did so as well. Adult rabbits regularly proved unfavorable when implanted with the growths; not a single take was obtained. And though those papillomas which had become established during the youth of their hosts often continued to proliferate for a considerable time after they had reached maturity, the eventual slowing up and retrogression of all but one of them make it seem likely that this too would have disappeared in the end.
Character of the Growths

The morphological findings were in the main like those noted after autoimplantation in the leg muscles (2). The fragments of papillomatous tissue rounded individually into small cysts, keratinizing toward the center, and these soon formed a single aggregate, though separated from one another by thin walls of reactive connective tissue. The general contour of the growth at this stage was somewhat lobulated. But as more and more keratin was laid down pressure conditions gradually cut off the blood supply to the interior of the mass with result that this consisted almost entirely of dead tissue by the time it had become large, only a shallow peripheral layer remained alive, and the mass had a sharply rounded contour. But from this in the most favorable animals, those of Papilloma A in its 2nd Generation C and 3rd Generation A, tongues of proliferating epithelium penetrated into the encapsulating connective tissue and rounded up into new cysts (Fig. 1). Not infrequently they advanced so rapidly as to extend through into the muscle, now and then replacing some of its fibers individually (Fig. 2), a happening already noted in autoimplants (2). The papilloma cells frequently entered lymph channels and even small venules; yet no metastases resulted, for not only did the epithelial cells adhere to one another after entering the vessels, but usually they became covered over by fibrinous clots which later underwent organization.

When growth was slow, the many small cysts composing the papilloma tended to coalesce, with result eventually in a more or less spherical mass of keratin and dead connective tissue which shelled out readily from a thin peripheral layer of proliferating epithelium, usually smooth but sometimes with blunt fimbriae on its surface.

Proliferation in the most favorable young animals was much more active and aggressive than that ordinarily witnessed after the autoimplantation of papillomatous tissue in adult rabbits, and so it sometimes continued to be for several months after its hosts had matured. The stimulating influence of bacterial infection probably had much to do with this. Papillomas A and B were both infected from the first and so remained throughout the period of their propagation; and it has been observed that under similar circumstances the papilloma cells of autoimplants may invade the surrounding muscle and replace its fibers individually (2) like the growths now under discussion. One other difference from the ordinary can also be ascribed to the influence of the bacteria, namely death here and there of the differentiating layer of papilloma cells before it had entirely keratinized (Fig. 3), a not infrequent happening. It took place in the growths of every generation, but never affected large areas and was no more pronounced in the last hosts than in the first. When the

\[ A \text{ similar penetration into blood vessels has occasionally been observed in primary papillomas, and invasion of the lymphatics is frequent (2).} \]
growths did badly scattered lymphocytes and polymorphonuclear leucocytes were found amidst the surrounding fibrous tissue, in greatest number where keratinization had just been completed. Retrogressing masses were surrounded and to a varying degree later invaded by macrophages; foreign body giant cells also appeared.

It may be recalled that originally Papilloma B was gray, as is often the case with growths resulting from the infection of pigmented skin with the Shope virus. In such instances the melanoblasts lying amidst the infected epithelium have not become neoplastic but have merely been stimulated to multiply actively and form melanin in quantity (5). The autoimplants of Papilloma B were also gray, and the growths of an albino rabbit of the first group to which transfers were made had dark gray areas several mm. across of living papillomatous tissue when the animal was killed 7 weeks after implantation, the microscope disclosing great numbers of melanoblasts loaded with pigment. Owing to the exceptional conditions provided by the papillomas, these highly specialized cells had not only survived transplantation, but had multiplied. In the growths of the later generations,—which were due to transplantation from the albino just mentioned,—none were found; but it is uncertain whether any of the pigmented tissue had been utilized in carrying the growth on.

DISCUSSION

The findings make plain that both of the papillomas now under consideration could almost certainly have been maintained indefinitely if they had been regularly passed from one suckling rabbit to another of the same stock which was newborn. But the aim of the work was to obtain growths that would flourish in a succession of mature animals, and the failure of Papilloma A to grow in any of 18 young adults of the 2nd Generation A after it had done well in the suckling from which passage was made showed that this was not readily to be accomplished. In the early days of cancer research when pure breeds of animals were not available, it was found that mouse tumors usually gained greatly in transplantability during their sojourn in the first host to which they were successfully transferred (6). Hence some of the rabbit papillomas now propagated in sucklings were purposely left undisturbed until long after their hosts had matured, on the assumption that they might then succeed in other adults. But nothing of the sort happened. Papilloma A, after growing in a host of the 1st Generation for more than 6 months, “took” in only one of the 15 weanlings (2nd Generation C) to which it was next transferred, and though it prospered in this animal, growing vigorously for a further 3 months, it failed in every one of 18 adults (3rd Generation A) which then received it (Chart 1). The delay in passing on Papilloma B from a matured host of the 2nd Generation did not enhance its ability to grow in sucklings (Chart 2), nor did the long
sojourn of Papilloma A in the animal of the 3rd Generation A from which it was transferred to newborn animals of an alien stock.

Very different has been our experience with the Vx7 carcinoma, an anaplastic cancer yet to be reported upon in detail, which derived from a virus papilloma and was for a while propagated serially in sucklings (4). It grew so slowly in some that they matured while still carrying it and transfers from them to other adults, though succeeding in but a small proportion of those first utilized, have been uniformly successful since.

Virus papillomas can be temporarily altered in appearance and behavior by extraneous influences, for example by the injection of Scharlach R which stimulates their proliferation and renders them briefly anaplastic (2); but always they revert to type as these influences wane. None of the many experimental efforts made in this laboratory to obtain papillomas differing from type has been successful. The spontaneous growths show a remarkable lack of variety; of the hundreds thus far studied from wild cottontail rabbits inhabiting a wide sweep of territory, not one has differed significantly from the rest. All have proved to be identical expressions of the narrow, peculiar, formative influence of the Shope virus, and their character shows that this latter has everywhere remained the same. True, the cancers eventually arising from papillomas due to the virus show much diversity and may conceivably be due to eventual variation of the latter, but if this be so it is truly singular that no benign growths expressive of lesser alteration have ever been encountered.

All of the papillomas resulting from the intramuscular transplantations of the present work were searched for cancerous changes such as commonly take place after some months in those situated on the skin. The growths of the later generations were scrutinized in special because they were the outcome of many months of proliferation all told; they were everywhere thinly sliced and every questionable area was examined microscopically yet nothing significant was found. The greater the activity of cutaneous papillomas, the earlier do carcinomas arise from them. Papilloma A had been growing in the muscles with notable vigor for 12 months when its host of the 4th Generation was killed, yet it was still entirely benign. So too were the six papillomatous masses in a favorable rabbit of the 2nd Generation after a total proliferation of 11 months, and those in a host of the 3rd Generation A after 10 months, and of three others after more than 9 months. Very different were the findings in 15 adult rabbits which carried cutaneous papillomas due to an extract of the same virus material which had yielded Papilloma A, similarly prepared and of the same strength. In eight out of 15 of these indubitable cancers appeared between the 6th and 10th month, and by the 11th had reached considerable size. But there were obvious reasons for the difference. Cutaneous papillomas are exposed to maceration, gnawing, and other forms of trauma which tend to hasten malignant change, whereas intramuscular growths are protected.
The carcinomas arising from virus papillomas do little better than these latter on direct transfer to adult rabbits, only two having established themselves to the present out of many transplanted to large groups of animals, one of which failed after only two passages. The mongrel character of rabbits, even after long inbreeding, seems to have been the decisive obstacle. Now this has been circumvented by the use of newborn rabbits as the hosts. At this writing, six cancers have been propagated in these, besides the papillomas here described. The success of these last adds yet another attribute to those demonstrating that in all immediate ways the cells of virus papillomas behave as if neoplastic in character.

SUMMARY

Two cutaneous papillomas produced with the Shope virus in adult domestic rabbits were transferred to their leg muscles and thence to those of several successive groups of rabbits of various ages. The growths failed to establish themselves in some newborn animals, but in many formed huge masses, immobilizing the legs with result in early death. Not infrequently they did well for months after their hosts had matured, yet nevertheless they failed regularly on further transfer in adults. They were eventually lost because left long prior to passage on the supposition that they might gain the ability to succeed in mature hosts.

The papillomas when growing actively sometimes penetrated the reactive tissue encapsulating them and replaced the adjacent muscle. They often entered lymph and blood vessels but never metastasized, nor did they undergo carcinomatous change though their total period of proliferation was as considerable as that elapsing in many instances before cancer appeared in papillomas produced by the same virus material and left on the skin.

Note Added in Proof.—In recent experiments the hashed and pooled skin of many rabbit fetuses, procured 4 or 5 days prior to birth, was implanted in the thigh muscles of numerous newborn animals of the mongrel agouti stock. The amount of skin implanted was four to five times that used for the transfer of papilloma tissue, and much of its corium had been removed by scraping. Only small flattened cysts, containing keratin and hair and lined with living epidermis, resulted after 2 to 3 months.

BIBLIOGRAPHY

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All of the sections were stained with eosin and methylene blue.

Fig. 1. One of the six intramuscular growths in the favorable host of the 2nd Generation C, Papilloma A. The animal was killed 3 months after implantation as a weanling. The growth consisted of numerous multilocular cysts lined with active papillomatous epithelium, mostly keratinizing but in some spots dying prior thereto. X 10.

Fig. 2. Periphery of another growth in the same animal, showing invasion of the encapsulating connective tissue by vigorously proliferating papillomatous epithelium. Mitotic figures were numerous. X 44.

Fig. 3. Higher power of an area in which the papilloma cells are dying prior to keratinization. The arrow points to a clump of bacteria. X 122.
(Rogers: Transplantation of virus papillomas)