A TRANSPLANTABLE RABBIT CARCINOMA ORIGINATING IN A VIRUS-INDUCED PAPILLOMA AND CONTAINING THE VIRUS IN MASKED OR ALTERED FORM

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Carcinomas occasionally arise from the naturally occurring, cutaneous papillomas of cottontail rabbits (1), growths due to a virus discovered by Shope (2). This virus causes similar papillomas on inoculation into domestic, snowshoe, and jack rabbits (3), and in these foreign hosts its action results in malignancy far more often (4) than in cottontails. The cancers derive from the epidermal cells of the papillomas, whatever the host species (4), from elements that is to say which are proliferating as result of infection with the virus. These facts and others attest to the direct responsibility of the virus for the cancers, though whether it is their actuating principle is still uncertain.

Domestic rabbits would seem best suited to experiments on the relation of the virus to the malignant growths, since these are rare in cottontails, and jack and snowshoe rabbits do badly in captivity. But the virus strains which engender vigorous papillomas in domestic rabbits,—and it is only from such growths that cancer is likely to arise,—cannot ordinarily be recovered from the proliferating tissue (2).[1] None of our many attempts to procure virus from papillomas in which cancer had begun to appear at one spot or another has been successful, while results with the malignant growths themselves have also been negative. However, an antibody capable of neutralizing the virus *in vitro* appears sooner or later in the blood of nearly every animal carrying papillomas (5), and increases in amount as they enlarge. Its action upon the virus is specific, and it is absent from

[1] By testing many cottontail papillomas Shope procured some virus strains which could be passaged in domestic rabbits (*Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 830), but the papillomas produced on the successive inoculations appeared late, and enlarged slowly. The growths seldom became malignant and only after a long time, in our experience with them, and the virus was no longer recoverable from them when cancer appeared, nor could it be got from the cancers themselves (Kidd, J. G., and Rous, P., *J. Exp. Med.*, 1940, **71**, 469).

the blood of normal rabbits and those carrying tar tumors or the Brown-Pearce rabbit carcinoma (6). By serum tests one should be able to tell whether the virus—or perhaps an agent related to it antigenically,—exists and increases in the cancers.

A necessary first step in the undertaking would be to transplant the cancers, since their first hosts will have antibody in the blood as result of the preliminary virus papillomatosis. The transplantation of one cancer has already been reported (6) but the growth was lost on second transfer. The blood of two previously normal individuals in which it had grown was found to have a neutralizing effect on the virus, whereas that of controls was innocuous; but the neutralization was much less considerable than in the case of rabbits carrying papillomas. The present paper deals with another cancer which has been transplanted to fourteen successive groups of animals and now grows with great vigor. Like the previous tumor it has none of the morphological features of the virus-induced papilloma from which it came; yet the antibody which specifically neutralizes the virus appears in the blood of every individual in which the tumor progressively enlarges, and reaches a titer as great or greater than that attained in rabbits which have long carried large papillomas. These facts and their implications will be considered.

Material and Methods

The proliferation of virus papillomas is especially active in Dutch belted rabbits, and cancer follows with corresponding frequency (7). Hence rabbits of this variety were chosen for the work. They had been bought in open market for our previous efforts at transplantation, and only a single cancer grew after implantation in them out of six transferred to a total of 96 animals, and this one in but 2 of 17 individuals,—a finding not strange in view of the poor results with mouse tumors implanted in hosts of mixed stock, and the notorious difficulty of propagating squamous cell carcinomas. For present purposes a colony was developed from a single pair of Dutch "show" rabbits, and after many blood-related animals had become available some were inoculated with papilloma virus, and the cancers eventually arising from the growths thus produced were transferred to other individuals. To exclude any possibility that papilloma cells might be carried along together with the malignant elements, glandular metastases were utilized as material, and to increase the amount of it pieces of them were implanted in the leg muscles of the host. Growth in the muscles was usually rapid, nodules of cancerous tissue soon forming. These were cut fine in a mixture of Tyrode and host serum, additional Tyrode was added, and 1 cc. of the resulting suspension was injected into the upper extensor muscles of the forelegs and the anterior and posterior thigh muscles of new animals. The skin was slit prior to introduction of the injecting needle, to ensure that epidermal cells were not carried in on its point, perhaps to be infected with virus. Sometimes an injection was made also into one of the testicles, or a tumor fragment was placed in the anterior chamber of one eye, according to the technique used

by Greene (8). To ascertain whether active virus was present in the cancer which forms the subject of the present paper, most of the rabbits of the first ten tumor generations were inoculated with a 10 per cent Tyrode extract of part of the tumor hash, by rubbing the fluid into an area about 5 cm. square, freshly scarified with sandpaper, on the side of the body.

Several pairs of Dutch belted rabbits with typical markings and guaranteed to be pure bred were procured to start the colony. But the variations exhibited by their first offspring made clear that most were of mixed origin. Consequently all were discarded except a pair which had given litters with the characteristic markings described by Castle (9), and only those individuals were selected for the later matings which appeared typical. Their descendants had a pronounced heterogeneity nevertheless, some being spotted with gray or fawn, or having wall-eyes. Continued selection of individuals with typical markings has failed to exclude these variations, and the underlying genetic differences have been only too evident in the frequent failure of the transplantable cancer now under consideration to grow in some animals of a litter while it did so in others. Pieces of the metastases of ten primary cancers have been implanted in a total of 131 individuals, with only one success, and this was for a time precarious, as will be shown.

History of the Transplantations

The Primary Cancer.—The rabbit providing the tumor was a male, the offspring of F 5-82 and M 4, the progenitors of the colony. When it was 7½ months old, 10 per cent extracts of four different virus materials were inoculated into as many scarified spots, 4 by 6 cm. square, on each side of the body. Confluent papillomatosis developed at every situation and 9 months later one of the growths had begun to ulcerate and was extending deep. Three more underwent similar changes within the next month and a half, by which time the growth first mentioned had become a discoid, fleshy mass, 7.5 cm. across, ulcerated and foul. A firm nodule, 2 cm. in diameter, had recently appeared in the subcutaneous tissue about 4 cm. away from the nearest ulcerated tumor, in the direction of the axilla. It was excised and found to be encapsulated like a lymph node, and to consist of grayish-pink tissue with scattered, serpiginous necroses and a few small, well localized abscesses. A piece of the nodule was hashed and implanted in the forelegs and posterior thighs of the host. Thereafter the animal was twice transfused with large quantities of normal blood, but it was tottery on the 14th day after implantation and hence was killed. No other regional metastases existed, but several minute, glistening, gray nodules on the surface of the lungs proved microscopically to be secondaries. There was a fusiform nodule of malignant tissue, 2 cm. long, at one of the implantation sites, with smaller growths at the others.

Microscopic sections across the eight tumor masses on the sides of the rabbit showed that all had become malignant, and that in four the papilloma had been almost replaced by cancerous tissue. The axillary metastasis showed the same sort of cancer and so too did the implantation nodules in the legs.

First Tumor Generation.—The tissue of two of the largest leg nodules was hashed and implanted in the legs of 20 Dutch rabbits, and in an eye of 10 of them. All were of the first and second filial generations from F 5-82 and M 4, and siblings or cousins of the cancerous individual. During the next month a firm nodule 4 mm. across formed in the leg of rabbit 5-52 and three of the same size in the legs of 5-44; but they disappeared

within a few weeks. Rabbit 5-42 had at the end of the 5th week two spherical masses, 1.6 and 1.7 cm. across, in the forelegs and one of 0.7 cm. in a hind leg. They had not enlarged since the 4th week and for this reason the two largest were almost entirely removed for transplantation purposes. They grated slightly under the knife, projected beyond the muscle surface, and appeared to consist of finely striated tissue with scattered serpiginous necroses, surrounded by a thick zone of connective tissue, blurred at the margin. The microscope showed a carcinoma like the original but with numerous lymphocytes round about the epithelial islands and penetrating between their cells which in places appeared unhealthy. Everything indicated that retrogression of the tumors had begun, and the changes after operation substantiated this impression. The pieces left *in situ* rapidly disappeared and so too did the growth in the hind leg. The eye graft never grew and was gradually resorbed. The animal was subsequently used for breeding and it is still alive. The other 17 rabbits of the 1st Tumor Gen. remained negative.

Second Tumor Generation.—The tumors from D. R. 5-42 were hashed together and implanted in the legs of seven rabbits of the first and second filial generations from F 5-82 and M 4, and into F 5-82 herself as well as two non-related Dutch rabbits. All received also a tumor fragment in the anterior chamber of one eye, and the four males of the group were injected into one testicle. A single animal developed growths, namely, F 5-82 from which the colony had stemmed. She was by now 4 years and 3 months old. No tumors had appeared when she was examined 2 months after implantation, but when 2 more had elapsed a football-shaped mass, $6 \times 4 \times 4$ cm., was found in the muscle of the right, posterior thigh. Though fairly firm it seemed to fluctuate, and proved cystic on incision, much glairy, turbid fluid flowing forth from a large central cavity, followed by lumps of yellow, grumous material. The wall of the cyst was ragged, 1 to 3 mm. thick except toward the upper pole where was a solid boss projecting 5 mm. A slice was taken here for section. Microscopically it was found to consist almost wholly of connective tissue but with islands of carcinoma cells, showing many mitoses, along the wall of the cyst.

The cyst was left open but the skin was brought together with interrupted sutures. These became infected and the wound gaped yet healed almost completely in 2 weeks, no extension of the tumor into the subcutaneous tissue taking place. After another week the growth was larger than before, with a radish-shaped prolongation toward the hip. It was again cut into and the cyst was found to have reconstituted itself, once more containing glairy fluid and grumous, necrotic tissue, with a thicker wall. There was no sign at this time of bacterial infection. A large piece of the wall was cut away for transplantation and the skin brought together as before. Incidentally to the new transfers, rabbit 5-82 was injected with the suspension of tumor material it had itself provided, this time in the subcutaneous tissue of the groins and in the leg muscles at all five situations where the previous grafts had yielded no growths. A fragment of the tumor was also placed in the eye not previously utilized. The first intraocular graft had been almost completely resorbed.

Within the next month nodules appeared at four of the five intramuscular sites and the new intraocular graft had begun to grow. After a month and a half the original tumor mass was 6 cm. across, again a reconstituted cyst but with several solid nodules about it; and three of the new growths had much increased in size, the largest being 4 cm. across, spherical and fluctuating. The recently implanted anterior chamber of the

eye was half full of neoplastic tissue. During subsequent weeks the main tumor enlarged until it reached nearly from knee to hip, and by the 80th day after the second implantation the animal was thin and ill. It was transfused and the main tumor cut into, with the evacuation of much glairy fluid mixed with pus. Cultures showed a Gram-positive diphtheroid bacillus in the pus. The wound was left open to drain. The masses in the other legs continued to enlarge, reaching 6 cm. in diameter, though the growth in the eye looked less solid, as if resorbing. The animal weakened gradually despite a second transfusion, cachexia became marked, and death occurred 19 days after the last operation and 209 days after the first implantation. The axillary and iliac lymph nodes were devoid of metastases, but a large tumor embolus was found in one lung. It had lodged at an arterial fork, and the cells appeared in good condition and had almost penetrated the vessel wall. The growths in the legs were cystic and contained glairy fluid and opaque, yellow gouts. No tumors were found at the implantation sites in the groins.

Third Tumor Generation.—The material got at second operation from F 5-82 was injected into 28 of her descendants of the first, second, and third filial generations. She had been mated several times with M 4 and the offspring had been interbred or back-crossed. Six of the individuals now utilized had previously been implanted unsuccessfully with the original cancerous material, and in them the new implantations produced no growths. Progressively enlarging tumors were obtained, however, in six of the other 22 animals. Bits of the neoplastic tissue had been placed in an eye of twelve, with negative results, though four developed leg tumors while the intraocular grafts were disappearing.

Fourth Tumor Generation.—The 25 animals next employed were implanted at separate situations with tumor materials from two rabbits of the third generation, in order to spread the chances of success. These materials grew or failed in the same individuals.

Nineteen of the animals were of the first, second, and third generations from F 5-82 while the other six were of the gray brown (agouti) variety and had been bought at random. Two of the former and one of the latter developed tumors, thus providing opportunity for further transfers in both breeds. The cancer appeared at more situations in the agouti rabbit and enlarged faster.

Fifth Tumor Generation, Series A and B.—The growths provided by the blood-related, Dutch belted rabbits and the agouti individual just mentioned were transferred to 19 and 20 animals of these sorts respectively. Tumors developed in eight of the Dutch animals, with one retrogression subsequently, and in twelve of the agouti breed, with two retrogressions. Again the tumors appeared earlier and grew much faster in the agouti breed.

Sixth, Seventh, and Eighth Tumor Generations, Series A and B.—The cancer was by now well established in two breeds of rabbits, and it was transferred to three successive groups of each sort. It grew in more than half of the individuals implanted, appearing in 8 of the 10 agoutis of the 6th Gen. Animals of this sort (series A) regularly proved the more favorable, the cancer growing much faster in them though not in a greater number of individuals. Nine of the 10 animals comprising the 6th Gen. A were inoculated into a testicle, with negative results. For several of the transplantations the tumors of two individuals were employed, either together or separately. Those injected separately usually "took" in the same individuals, but the resulting growths often enlarged at differing rates.

Ninth to Thirteenth Generations.—Agouti animals have been relied upon entirely since the 8th Gen., and the tumor has continued to flourish. All of the 10 rabbits of the 9th Gen. and the 15 of the 11th Gen. developed growths, which retrogressed however after a few weeks in 6 and 2 individuals, respectively.

General Findings on Transplantation

The propagation of the cancer was uncertain in the beginning but it has gradually become sure. The growths providing material for the 2nd Tumor Gen. had begun to retrogress when used. In the next few groups of animals the cancer "took" in only an occasional individual of the large numbers to which it was transferred, and in favorable hosts it grew at only one or two of the six implantation sites. The differing character of the individual tumor fragments,—which often consisted almost wholly of reactive connective tissue,—doubtless had much to do with this. Later, as the proportion of cancer cells in the grafts increased, the results became less irregular, and of late tumors have regularly appeared wherever implantation was done in the leg muscles of favorable animals. Generally, though not always, the tumors in one individual behaved in the same way, growing or retrogressing together. A few animals were reimplanted with the tissue of their own enlarging tumors but in only one, the female progenitor of the colony (F 5-82), did new nodules result. Probably the host develops some secondary resistance to the cancer like that so often called forth by transplanted rat and mouse tumors. The tumor does badly in the subcutaneous connective tissue and in the anterior chamber of the eye,—where it has grown only once (again in F 5-82),—and a "take" has never occurred in the testicle.

The most rapidly enlarging cancers have regularly been chosen for transfer, and so swiftly have they grown of late as to cut down greatly the interval between transplantations. The tumor of the 1st Gen. was not utilized until the 139th day and those of the next few generations did not get big enough to provide material until 2 months or more had elapsed; but after the 5th Gen. large cancers were available within a few weeks, notably, in agouti animals, and the time between transfers has ranged between 17 and 40 days. The growth would not have killed until later but our aim was to pass the cancer through many successive hosts. Now growth is so rapid that frequently zeppelin-shaped nodules 2 cm. in diameter develop within 3 weeks. There is a critical period toward the end of this time when retrogression may occur,—the growths dwindling in one-third or more of the animals, so fast sometimes that in another 2 weeks they are gone. Retrogression is a rare event later, the cancers as a rule enlarging steadily. In the first tumor generations, when growth at single situations was frequent, the resulting cysts sometimes reached a diameter of 15 cm. or more and interfered with locomotion. Now, with tumors at all six implantation sites, death usually occurs by the time they are 8 to 10 cm. across. In one instance, of a half grown animal, the cancer killed in less than 6 weeks, and in an adult it did so in 2 months and a week. 10 to 15 weeks is the usual duration of life. Cachexia is very marked, owing doubtless to absorption from the large expanses of necrotic tissue on the walls of the cysts.

The original cancer rabbit had secondary growths in the lungs as well as the metastasis in a regional lymph node with which the transplantations were started; and F 5-82 of the 2nd Tumor Gen., an unusually favorable host, had a large embolus in a lung vessel, with cancer cells about to invade the alveolar tissue. No other animals showed

secondary growths until the 5th Gen. was reached, when nodules were found in the axillary and iliac glands of a few individuals. Since then metastases have been fairly frequent at these situations. In a Dutch belted rabbit of the 8th Gen. there were small pulmonary growths as well.

Many of the animals utilized for transplantation were newly weaned, Dutch belted rabbits, and some were half grown. They were selected because many transplantable tumors succeed best in young animals. Those we used were as nearly related to the original tumor animal as the adults implanted at the same time, yet the tumor grew in no greater proportion of them, though killing earlier.

It seems probable that the utilization of blood-related animals was responsible for the maintenance of the cancer in the first few groups of rabbits, but the results as a whole are like those ordinarily obtained when a tumor is propagated in animals of mixed genetic constitution, with selection for successive transfer of the growths that have done best. The gradual acquisition of the ability to proliferate in all hosts, even those of a different breed, the quickened rate of enlargement, and the gain in malignancy evidenced by earlier death and an increased frequency of metastasis formation, are all familiar phenomena to workers with transplanted growths.

In a recent experiment the neoplastic cells remained capable of forming growths after 24 hours at −22°C. though not at −70°. Some successful transplantations have been carried out by means of suspensions of the cancer cells, made by forcing the tissue through a fine sieve of monel metal and adding Tyrode solution.

Character of the Transplantable Tumor

As already mentioned, malignant changes occurred in all of the eight virus-induced papillomas of the animal from which the cancer came. It was impossible to tell from which tumor mass the metastasis had derived that was utilized as transplantation material, since all contained cancers of the same sort; but the likelihood narrowed to two of them. All were studied in cross-section; Fig. 1 illustrates the general findings. The progression there pictured from benign papilloma to squamous cell carcinoma by way of malignant papillomatosis has been the subject of several previous papers (7, 10, 4). It will be noted that the squamous cell carcinoma at the center of the tumor mass has penetrated deep, amidst much reactive connective tissue, and that small cysts are forming as result of necrosis of the cells prior to keratinization. The axillary metastasis utilized for transfer showed a cancer having the same traits (Fig. 2), and so too did the tiny pulmonary nodules though these latter were not yet cystic. In the leg tumors cysts were just beginning to form (Fig. 3). Mitoses were very numerous, and the growths were markedly desmoplastic.

The cancer has since retained its initial character (compare Figs. 3 and 4). As viewed in cross-section it consists of big cells which never keratinize, though they usually enlarge before they are overtaken by necrosis, and sometimes begin an abortive differentiation. The nuclei are also big, with much marginated chromatin, and the numerous mitoses are often pathological. The cells form expanses more or less widely separated by a profuse, new-formed connective tissue that obviously acts to limit their aggressive activities. When they are liberated from this, as when the tumor is cut up and transplanted, they often invade actively, extending in broad tongues between individual muscle fibers, and sometimes applying themselves directly to these and replacing their

substance, though never penetrating into them and along their interior as the most malignant of tumors do. Active invasion and destruction of this sort lasts no more than a few days, because a profuse reactive connective tissue proliferation soon walls off the tumor cells. While the wall is forming there may be edema outside it, as if from irritation (Fig. 4). The desmoplastic influence of the cancer is so great that however rapidly it burrows through the reactive tissue this still forms ahead, with result that the malignant cells seldom regain contact with normal structures, and then briefly and locally. On such occasions it may again extend between the muscle fibers and attack them. Ordinarily the layer of reactive tissue is 2 to 3 mm. thick and the cancer enlarges by continually invading it. In the absence of such aggression it becomes scar tissue, a tough, sharply defined capsule, and any tumor islands lying within it are compressed and usually die.

The extending carcinoma has a coarse pattern (Fig. 5): it grows out in blunt tongues which may broaden into large expanses of undifferentiated cells. There are scattered capillaries amidst these, each in a wisp of connective tissue (Fig. 6), and the neoplastic cells next the little vessels are often elongated and radial because of crowding. As proliferation continues those furthest away die and in consequence cysts form containing debris. The primary cancer had become cystic in this way, and so too had the glandular metastasis and the implantation nodules. When small the cysts may be forked or ramifying, according to the shape of the epithelial expanses in which they lie (Fig. 5). They enlarge progressively by death of the cells along their walls, and fluid soon begins to accumulate in them in addition to necrotic material. It gathers under considerable tension, as later palpation discloses, and hence the cysts become spherical and tend to coalesce with result in a large central cavity. If the cancer is very cellular the cavity may have a ragged lining. Until it reaches a diameter of several centimeters this lining may consist of living papillae (Fig. 7) having an origin diametrically different from that of the papillae which make up tar and virus papillomas. For they are the outcome of destructive not constructive activities,—mere remnants left by ischemic necrosis, their cores the blood vessels which had nourished the malignant cells, and their covering such of these latter as have survived by reason of a position near the blood stream. Occasional broader cores there are too, made up of the connective tissue which formerly lay between epithelial expanses now almost wholly dead. As the cysts enlarge further, the papillae become shorter, dying at the ends, and at length they are reduced to blunt protrusions by interior pressure (Fig. 8). Usually the cancer in the wall of the cyst continues to invade and break down, sometimes extending within lymphatics; and hence the cyst grows. In occasional instances the neoplastic epithelium becomes less and less able to penetrate the enveloping connective tissue and at length merely lines the cyst, and ultimately dies, with result that only encapsulated debris is left.

The intramuscular cancer of F 5-82 (2nd Tumor Gen.), as procured at first and second operation, had precisely the character of the implantation growths in the animal in which the tumor arose. It was a carcinoma with almost no tendency to differentiate, the cells dying early with result in cyst formation (Fig. 8). After the second operation, when the growth became infected with bacteria, its morphology changed. Its proliferating cells were no longer nearly alike in size and aspect but instead many were huge, multinucleate, or with giant nuclei (Fig. 17), while others were unusually small with no more than a rim of cytoplasm. Differences in tinctorial capacity were also great, some cells taking a deep color, others almost none. While these features were most marked

in the tumor subjected to operation they were present also at some spots in the other intramuscular growths, several of which contained pus as well as glairy and grumous material. There were fairly numerous polymorphonuclear leucocytes scattered amidst the reactive connective tissue. The intraocular tumor had a similar, unusual cytology, was partly dead, and obviously in process of resorption.

The changes in the cancer in this instance were doubtless due to the bacterial infection which became established in the tumor after the second operation; for similar pictures have been encountered now and again in tumors of the later generations in which bacteria were demonstrable. But the cellular abnormalities were not wholly confined to such growths; they have been found, affecting individual cells or small aggregates, even in those cancers which appear "healthiest."

In a considerable proportion of hosts, as already stated, the tumor retrogresses after proliferating rapidly for a few weeks. It ceases to invade, lymphocytes and macrophages accumulate about it, and some of the latter penetrate between its cells. Soon these begin to die and very swiftly all succumb, leaving islands of debris surrounded by foreign body giant cells amidst granulation tissue containing scattered polymorphonuclear leucocytes. Resorption of the dead material is rapid. The succession of histological events differs in no significant respect from that encountered with other transplantable tumors.

When proliferating in lymph glands the carcinoma exhibits its ordinary character, and becomes cystic on reaching a diameter of only a few millimeters (Figs. 9, 10). So long as its invasion is confined to the gland parenchyma no connective tissue reaction occurs, but this becomes pronounced once it has extended outside. The pulmonary metastases recently encountered have been minute (Fig. 11).

The way in which the tumor enlarges and breaks down brings singular consequences in the gross. When the cystic change is just beginning the neoplastic tissue appears solid on section, firm, close-textured, with scattered, yellow, serpiginous necroses and dots (Fig. 12). But by the time the growth has reached a diameter of 3 to 4 cm. a central cyst forms (Fig. 13), soon comprising much of its bulk (Fig. 14), and this cyst is a dominating feature thereafter (Figs. 15, 16). While still solid, the tumor has a fusiform, football, or radish shape, with its long axis in the direction of the muscle fibers, but later it becomes approximately spherical. If several nodules have arisen at one implantation site, as occasionally happens, each becomes cystic and they and the cysts they contain may coalesce if the animal lives long enough. At most situations though, a solitary cyst forms which in due course becomes large. Its wall of cancerous tissue is from 2 to 10 mm. thick, so firm sometimes as to grate under the knife, and variegated with serpiginous necroses or small cavities containing soft, necrotic material. The lining of the cyst appears ragged and partly necrotic at first, for reasons already given, but later it is studded with what look like giant pink granulations, these being actually covered with a thick layer of proliferating carcinoma cells, as the microscope shows. The contents of the cyst is peculiar, a clear, glairy fluid like thinned white of egg, with necrotic, yellow masses lying amidst it. It accumulates under a pressure which keeps the cyst tense. Sometimes the fluid is thickened and rendered turbid by finely dispersed, dead cells, and it may then have the ground-glass aspect of solidifying candle grease; or it may be light brown, or pink or chocolate-colored with pigment from the blood of papillae that have undergone necrosis. Usually though, the fluid is pellucid and colorless. The necrotic material has ordinarily the form of gouts or yellow, cus-

tardy lumps but occasionally it is clayey or pultaceous. The pressure within the cysts is so great that when the tumor extends through the muscle aponeurosis, as occasionally happens, herniation promptly follows and a dissecting cyst forms in the subcutaneous tissue. This is always flaccid and its wall is thin at first; for as yet there has been no extension outwards of the tumor but only a gushing forth of dead material. Later the cyst may acquire a carcinomatous lining. As a rule the cancer does badly in the subcutaneous tissue, seldom extending into this after incisions to procure material; and the skin has never been invaded despite the numerous opportunities that biopsies provided. On the one occasion when the cancer established itself after implantation in the anterior chamber of the eye, it eventually retrogressed. In this connection it may be recalled that two highly malignant cottontail cancers of a previous paper (4) also failed to grow after transfer to the anterior chamber.

In a few animals of recent tumor generations the cancer has assumed a new aspect, though only locally. At some spots it no longer grows in thick, blunt tongues but its invading cells extend through the connective tissue in narrow strands and die before multiplying into masses (Fig. 18). The ultimate in anaplasia would seem to have been achieved in this change. It was first noted in a tumor of the third generation which had grown for many months, and it has now become frequent. Sometimes it is obviously determined by inflammation of the supporting tissue, but in most cases it exists side by side with cancer of the ordinary type, apparently under the same conditions, as if it were the outcome of a discontinuous alteration in the neoplastic cells. Efforts to procure completely anaplastic tumors by selective transplantation are under way.

The first good-sized cancer obtained by transplantation (rabbit F 5-82 of the 2nd Gen.) would have been mistaken for an old abscess had not this error been made with the carcinoma previously propagated (6), which likewise gave rise to a cystic growth containing glairy fluid mixed with yellow, necrotic debris. This material and the profuse, reactive connective tissue were suggestive of bacterial infection, but none was demonstrable by cultures or stains. Our repeated attempts to procure bacteria from the tumor now under consideration have had no better success, save in a few cases in which there was pus in the cyst in addition to the ordinary contents. A Gram-positive diphtheroid was found in one of these, and unidentified Gram-negative rods in two others. The search for bacteria has involved taking cultures on a variety of media, including broth and agar to which rabbit's serum had been added, and repeated examination of the sediment of tumor extracts, as obtained by centrifugation and colored with methylene blue, Gram, or the Giemsa stain.

Tests for the Presence of Virus

The four virus materials inoculated into the rabbit providing the tumor for transplantation had been employed in previous experiments and were known to cause vigorous papillomas from which no virus was recoverable. Nor has it been got from the transplanted cancer. Every animal of the first ten tumor generations, except those of the 7th Gen. series B and the 9th Gen., was inoculated into the scarified skin with a 10 per cent extract of a part of the hashed tissue utilized for implantation. In none did skin growths arise.

In a further effort at direct demonstration of the virus a 5 per cent Tyrode extract was made of a cancer of the 11th Tumor Gen. and inoculated into the ears of nine rab-

bits, after it had been centrifuged to remove gross particles. The ears had been tarred over the inner surface twice weekly for 17 weeks and tar papillomas had appeared there in greater or less number. The organs were thus prepared because experiment had shown that when active papilloma virus is injected into animals thus treated it localizes abundantly in the tarred epidermis, causing some of the benign papillomas already present there to become cancers, and engendering both benign and malignant tumors where none were previously visible (11). In the present instance it seemed possible that a "masked" virus might be extracted from the cancerous tissue which would prove capable of infecting cells prepared by tarring, though powerless with normal elements. To ensure that the presumptive virus reached the epidermis, one ear of each animal was infiltrated with 10 cc. of the extract by way of a marginal vein, after the circulation had been shut off at the base with a rubber band, and the other ear was infiltrated under like circumstances with the same amount of Tyrode solution. Both organs were tarred once immediately after. When active papilloma virus is introduced under the conditions given innumerable growths soon appear (12), but in the experiment now described none arose that could be attributed to the injected material.

Several indirect methods were available to test for the presence of the papilloma virus in the cancers,—inoculation of the cancer animals with it to learn whether they had acquired any resistance, examination of their blood for the presence of specific antiviral antibody by means of the neutralization (5) and the complement fixation (13) tests respectively. Previous experiment had shown inoculation to be the most delicate of the three methods, disclosing very slight amounts of resistance; but use of it as routine would have defeated our aim to remove the cancer far from possible contamination with the virus. The neutralization test comes next in sensitiveness (14) and hence was largely relied upon. It gave clear cut results (Figs. 19, 20).

The *neutralization test* has been used in much previous work (5, 4). The serum of each rabbit examined in the present relation was mixed with an equal amount of a Berkefeld filtrate of a 5 per cent Tyrode extract of glycerinated, cottontail papilloma tissue; and after 2 hours at 37°C. the mixtures were rubbed into freshly scarified squares on the belly or side of each of three domestic rabbits. A control mixture with Tyrode instead of serum was also inoculated. The extracts were all capable of promptly producing confluent papillomatosis as the control tests proved. They were made from the papillomas of several cottontails in order to broaden the conditions. The squares, checkering the belly and sides of the rabbits, were dried in a blast of warm air and covered individually with sterilized gauze until healing had occurred, to exclude any possibility of transfer of the inocula. All specimens that were to be directly compared were rubbed into the same animals. After healing had taken place the squares were examined every 3 to 5 days, until no more growths appeared, that is to say, until the 36th to 45th days.

The neutralizing power of the sera tested showed itself not only in an absence of papillomas, or reduction in their number on the inoculated areas, but also in a delay in their appearance. Two records of the findings are presented in each of the tables (Tables I to VII), one obtained early, while the growths were appearing, and the final reading of their total number. As controls to each test of the tumor animals of Dutch belted stock, serum specimens were included which had come from rabbits of the same implantation which had failed to develop growths, as also specimens from normal rabbits which were their sibs or cousins. In the later experiments with agouti animals only normal controls

were available because the implantations regularly resulted in growths. To learn how the resistance of the cancer animals compared with that induced by virus papillomas, serum specimens from individuals carrying such growths were included in several of the experiments.

The complement fixation test involves the same antigen as the neutralization test, namely the virus (14), but it does not disclose the presence of antibody in quite as small amount. It gives immediate, quantitative results though, and hence was employed as

TABLE I

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers

(First Tumor Generation)

Source of serum	Rabbit No.	Number of tumors carried	Diameters of tumors	Growths from mixtures of serum and virus W. R. 54 in rabbits A, B, C					
				21st day			45th day		
				A	B	C	A	B	C
Rabbits			*cm.*						
With cancers (implanted 39 days)	5-42	Three	1.7 - 1.6 - 0.7 (T)	0	0	±	0	0	±
	5-44	Three	0.4 - 0.4 - 0.4*	0	0	0	++±	+	±
	5-52	One	0.4*	+++	+++	++	+++	+++	+++
Implanted but negative	5-38			+++	+++	++	++++	+++	+++
	5-46			+++	+++	++	++++	+++	++++
	5-50			+++	+++	++	++++	+++	++++
Normal rabbits of same stock	5-88			+++	+++	++	++++	+++	++++
	5-86			++±	++	++	++++	+++	++++
	F 5-82			+++	+++	++	++++	++±	++++
Tyrode control				+++	+++	++±	++++	++±	++++

(T) = the tumors of D. R. 5-42 were used for transplantation on the 40th day.
++++ = confluent papillomatosis.
+++ = semiconfluent papillomatosis.
++ = many discrete papillomas.
+ = 5 to 10 " "
± = 2 to 4 " "
± = solitary discrete papilloma.
* Retrogressed later.

routine with all of the animals except those of the first two generations, both as a check upon the neutralization tests and whenever the latter seemed unnecessary. The technique has already been described and the reliability of the method demonstrated. The results obtained with it in the work now under discussion were essentially corroboratory to the neutralization test, and hence they will not be tabulated save in the case of the animals of the 5th and 6th Gen. B, for which no neutralization data are available.

Table I gives the outcome of the neutralization tests with the animals of the 1st Tumor Gen. It will be seen that the serum of rabbit 5-42, which carried large tumors, did away almost completely with the pathogenicity of the virus mixed with it, that the specimen from rabbit 5-44 with three very small growths had less effect, while that from

rabbit 5-52 with one such growth had none at all. The sera of the normal controls and of the implanted animals which failed to develop tumors were likewise devoid of effect (Fig. 20).

Only one animal of the ten inoculated for the 2nd Tumor Gen. developed a growth; this was rabbit F 5-82 which figured in the comparison of Table I. Immediately after it was bled as a control in this comparison it was implanted with the cancer of rabbit 5-42. A single growth resulted and from time to time as this enlarged tests were made of the host's blood. Table II is concerned entirely with the changes they disclosed. The serum specimen procured on the 118th day, when the tumor was 5 cm. across, was kept in the cold until the 139th day and tested with serum taken then; for preservation during many weeks does not diminish the antibody titer, as tests directed to the point have shown. Both sera were now found to have moderate neutralizing power in contrast to the ineffectiveness of the previous specimen. Control sera from two normal, blood-related rabbits and two implanted with the same material as 5-82 but with negative results, proved devoid of effect on the virus. These findings have not been included in the table.

On the 140th day rabbit F 5-82 was reimplanted with its own growth, with result in four new nodules 14 days later. The primary growth was still below its size prior to the excision of material. The blood now had a pronounced neutralizing power (154th day), and so too on the 202nd day. One of the new nodules had then disappeared but the others had enlarged further. In these later tests, three and five normal animals were included, respectively, as well as two on each occasion that had been implanted when F 5-82 first was, with negative results. None of the sera of these controls had the slightest neutralizing influence, and they have been omitted from the table.

In the 3rd Tumor Gen., six animals developed growths, but one died before the 63rd day when blood tests were carried out (Table III). The three that had several large tumors all yielded sera with marked neutralizing capacity, as did rabbit 6-92 in which only one growth was present and this only 6 mm. across. The blood of rabbit 6-91, on the other hand, contained no demonstrable antibody although it had a growth 1.8 cm. in diameter; but the antibody appeared later, as Table IV will show. The sera from the controls proved wholly devoid of effect. Among them were two animals (5-36, 5-48) which had been implanted for the 1st Tumor Gen. with negative results. (They had not been amongst those from which serum was taken for the test recorded in Table I.) To extend the comparison sera from five agouti rabbits, each with several papillomas amounting together to a large mass, were introduced into the experiment. The neutralizing capacity of their bloods proved to be no greater than that of the animals with big cancers.

Table IV summarizes the comparison carried out with animals of the 4th Tumor Gen. Incidentally the serum of rabbit 6-91, of the 3rd Gen., was again tested. Its cancer was now, on the 165th day, 3 cm. across, and its blood, previously without neutralizing effect, exerted this pronouncedly. The other results of the table corroborate and extend the previous findings. Cancer rabbit 7-90 was an agouti, the first of its kind in which the tumor had been propagated. The others, including the animals with papillomas, were from the Dutch belted colony. It will be seen that four of the five cancer animals of the 4th Gen. had blood with definite neutralizing capacity, but that this was lacking in the case of the fifth (7-73) which had had for a brief time a single small nodule which disappeared about 2 weeks before the test was made. One of the

papilloma animals (5-48), with four large growths when bled, yielded serum of only slight antiviral power.

TABLE II

Neutralization Tests with Serum of a Rabbit with Enlarging Cancers

(Second Tumor Generation—Rabbit F 5-82)

Time of test	Tumor size	Growths from mixtures of serum and virus in rabbits A to L					
		21st day			45th day		
		A	B	C	A	B	C
	mm.						
Prior to implantation		+++	+++	++	++++	++±	++++
Tyrode control		+++	+++	++±	++++	++±	++++
		21st day			42nd day		
		D	E	F	D	E	F
Implanted 118 days	50	+−	+	±	+	++	+
Implanted 139 days	50 (T)	+	+	±	++	+++	+
Tyrode control		++±	+++	+++	++++	++++	++++
		G	H	I	G	H	I
Implanted 154 days	30						
Reimplanted 14 days	4	0	±	±	+	±	0
	6*						
	6						
	10						
Tyrode control		++±	+±	++±	++++	+++	+++
		J	K	L	J	K	L
Implanted 202 days	61						
Reimplanted 62 days	15						
	27	0	±	0	±	±	0
	50						
Tyrode control		+++	+++	++++	++++	++++	++++

(T) = the tumor of rabbit F 5-82 was used for transplantation on the 139th day.

Virus procured from the papillomas of W. R. 54 was used in the test prior to implantation. In the second test virus W. R. 56 + 1-56 was used, and in the third and fourth tests virus W. R. 1-10.

* Retrogressed later.

In the 5th Tumor Gen. A "takes" and progressive growth were obtained in ten agouti animals. Blood from all of these was compared with that from three of the nine nega-

TABLE III

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers

(Third Tumor Generation)

Source of serum	Rabbit No.	Tumor size	Growths from mixtures of serum and virus W. R. 1-10 in rabbits M, N, O					
			21st day			42nd day		
			M	N	O	M	N	O
		mm.						
Rabbits with cancers (implanted 63 days)	6-78	13 28 28 30 35	±	0	0	±	0	0
	6-82	15 (T) 16 25 28	0	+	0	±	+	0
	6-69	11 (T) 12 12 18 20	±	+	0	±	±	±
	6-91	18	+++	+++±	+++	++++	++++	++++
	6-92	6	0	±	0	±	±	+
Implanted but negative	5-36		++±	+++±	++	++++	++++	++++
	5-48		++±	++	++±	++++	++++	++++
	6-81		++±	++±	+++	++++	++++	++++
	6-85		++	+++	++	++++	++++	++++
	6-87		+++	+++	++±	++++	++++	++++
Normal rabbits of same stock	7-70		+++	+++	++±	++++	++++	+++±
	7-71		++±	+++	++±	++++	++++	++++
	7-72		++±	+++	+++	++++	++++	++++
	7-73		++	++++	+++	++++	++++	++++
	7-74		+++	+++	+++	++++	++++	++++
With virus-induced papillomas (inoculated 84 days)	F 1	8 large growths on each (3–4 cm. across)	±	±	0	±	±	0
	F 2		0	0	0	0	0	0
	F 6		0	0	0	±	±	±
Inoculated 190 days	6-13	4 large growths	0	0	0	0	0	0
Inoculated 48 days	7-07	16 small growths	0	±	0	0	±	0
Tyrode control			+++	+++	++++	++++	++++	++++

(T) = the tumors of rabbits 6-82 and 6-69 were used for transplantation on the 65th day.

TABLE IV

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers

(Third and Fourth Tumor Generations)

Source of serum	Rabbit No.	Tumor size	Growths from mixtures of serum and virus W. R. 1-28 in rabbits A, B, C					
			18th day			43rd day		
			A	B	C	A	B	C
		mm.						
Rabbits with cancers of 3rd Gen. (implanted 165 days)	6-91*	30	0	0	0	±	±	0
Rabbits with cancers of 4th Gen. (implanted 99 days)	7-72	35 (T) 30 13 6 2	0	0	0	±	0	0
	7-70	7**	0	0	+	+	+	+
	7-89	36 (T)	±	0	0	±	++	+
	7-90[a]	23 (T)	+	++	±	++	+++	++±
	7-73	14*	+±	+++	++++	+++	++++	++++
Rabbits implanted for 4th Gen. but negative	7-71		++++	+++±	++++	++++	++++	++++
	7-74		++++	+++	++++	++++	++++	++++
	7-83		++++	+++	+++	++++	++++	++++
	7-87		++++	+++	++++	++++	++++	++++
Normal rabbits of same stock	5-52		++++	+++	++++	++++	++++	++++
	5-37		++++	+++	++++	++++	++++	++++
	5-35		+++±	+++	++++	++++	++++	++++
Rabbits carrying 4 virus-induced papillomas 3 to 8 cm. across (inoculated 94 days)	5-85		0	0	0	±	±	0
	5-46		±	0	±	+	++	+
	5-50		±	0	+	+±	++±	++±
	5-48		+±	0	+++	++±	+++	++++
Tyrode control			++++	+++	++++	++++	++++	++++

[a] Agouti rabbit.

(T) = the tumors of rabbit 7-90 were transplanted on the 61st day (series A), those of 7-72 and 7-89 on the 69th day (series B).

* Failed to manifest antibody in previous test (Table III) when the tumor was smaller.

** Disappeared before 87th day.

tive agouti rabbits of the same implanted group, as also with specimens from three normal individuals, and from five agouti rabbits carrying several papillomas each (Table V). Of the three rabbits inoculated with the test mixtures, one animal, C, was killed on the

27th day to obtain a picture illustrating how decisive the findings were (Fig. 19). The blood of all of the cancer rabbits had definite neutralizing power, though somewhat less marked in two instances than that of the rabbits with papillomas. All except one of the latter had carried its growths for many months whereas the cancers had been present only 60 days.

TABLE V

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers

(Fifth Tumor Generation Series A,—Agouti Animals)

Source of serum	Rabbit No.	Duration of growths	Diameter of tumors	Growths from mixtures of serum and virus W. R. 1-56 in rabbits A, B, C							
				17th day			26th day			36th day	
				A	B	C	A	B	C*	A	B
		days	*cm.*								
Rabbits with cancers	8-46	60	4 - 4 - 2.5 - 4 - 3 - 4	0	0	0	0	0	0	±	±
	8-54	"	3 - 3.5 - 2 - 3.5 - 3	0	0	0	0	0	0	0	±
	8-50	"	5 - 4 - 4 - 1.5 - 4 - 4	0	0	0	±	±	0	±	±
	8-49	"	5 - 2 - 2 - 3 - 2.5	0	0	0	±	±	0	±	±
	8-55	"	2.5 - 2.5 - 2.5 - 1.1 - 1.5 - 1.4	0	0	0	±	0	±	±	0
	8-60	"	1.2 - 1 - 1.8 - 1 - 1.6 (T)	0	0	0	±	0	±	±	±
	8-62	"	4 - 5 - 4 - 4 - 3 - 5 (T)	0	0	0	±	+	0	0	+
	8-61	"	3 - 3.5 - 3.5 - 3 - 4	0	0	0	±	+	±	±	+
	8-59	"	3.5 - 5 - 3 - 5 - 4 - 6	0	0	0	+	+±	+±	+	++
	8-45	"	3 - 4 - 3 - 4 - 4	0	0	0	++	+	+±	++±	+
Rabbits implanted with same material but negative	8-44			++	+++	+++	+++	++++	++++	++++	++++
	8-51			++	+++	++	+++	++++	++++	++++	++++
	8-56			++	+++	++	+++±	++++	+++±	++++	++++
Normal animals of same breed	9-07			+	+++	+++	+++	++++	++++	++++	++++
	9-08			+±	+++	+++	+++	++++	++++	++++	++++
	9-09			++	+++	+++	+++±	++++	++++	++++	++++
Rabbits with virus-induced papillomas	1-39	51	9 growths averaging 3 cm.	0	0	0	+	±	±	+	±
	6-12	315	6 - 6 - 6 - 8	0	0	0	±	0	0	±	0
	6-16	"	3.5 - 3.5 - 3.5 - 3.5	0	0	0	±	0	0	±	0
	6-10	"	6 - 6 - 6 - 6	0	0	0	+	±	±	+	+
	4-88	396	2 - 2 - 2 - 3	0	0	0	±	0	0	±	±
Tyrode control				++	+±	++±	+++	+++±	+++±	+++±	+++±

(T) = the tumors of rabbits 8-60 and 8-62 were used for transplantation on the 23rd day.

* Killed on 27th day for photographic purposes (Fig. 19).

Every rabbit thus far in which the cancer grew large had yielded serum with marked capacity to neutralize the virus. In the absence of any sign of change in this respect, the decision was made to do no more neutralizations until after the tumor had undergone several further transfers. Complement fixation tests were performed, however, with the sera of the cancer rabbits of the 5th and 6th Gen. B (Dutch belted animals). For comparison specimens were taken from two individuals of the 5th Gen. and three of the 6th Gen. which had not developed growths. At the same time eleven Dutch belted rabbits carrying virus-induced papillomas were tested. Some had served previously

TABLE VI

Complement Fixation Tests with Serum of Rabbits Bearing Transplanted Cancers or Virus-Induced Papillomas

(Fifth and Sixth Generations, Series B,—Dutch Belted Rabbits)

Source of serum	Rabbit No.	Time since implantation or inoculation	Size of growths	Complement fixation titer of serum				
				1:4	1:8	1:16	1:32	1:64
		days	*cm.*					
Rabbits with cancers (5th Gen.)	8-65	95	10 — 8	++++	++++	++++	++++	++++
	8-66	"	3.5 — 12 — 10	++++	++++	++++	++++	++++
	8-72	"	6 — 6 — 8 — 6 — 6 — 10	+++±	++++	++++	++++	++++
	8-79	"	8 — 4 — 10 — 7 — 3 — 4.5	++++	++++	++++	++++	++++
	8-74	74*	5 — 9 — 5 — 7 — 5 — 6 (T)	++++	++++	++++	++++	++++
	8-78	76*	5 — 4 — 6 — 5 — 4.5 — 4	+++±	++±	0	0	0
Implanted but negative	8-63	95		0	0	0	0	0
	8-64	"		0	0	0	0	0
Rabbits implanted with cancer (6th Gen.)	9-17	62	3.5 — 2.5 — 5 — 3 — 4	++++	++++	++++	+	0
	9-19	41*	2.5 — 2.5 — 5 — 2.5 — 2.3 — 3	++++	++++	++++	+++±	0
	9-20	62	3.5 — 5 — 2.5 — 8 — 5 — 5	++++	++++	++++	++++	++++
	9-22	"	4 — 4.5 — 8 — 3.5 — 5.5 — 3	++++	++++	++++	++++	++++
	9-24	"	4.5 — 4.5 — 7 — 4 — 3	++++	++++	++	0	0
	9-21	"	3 — 5	++++	++++	++++	++++	+++±
	9-26	"	2.5 — 3.5 — 3.5 — 4.5 — 3.5 — 3.5	+++±	++++	++++	++++	++++
Implanted but negative	9-18	"		0	0	0	0	0
	9-23	"		0	0	0	0	0
	9-25	"		0	0	0	0	0
Rabbits with virus-induced papillomas	5-45	154	5 — 7 — 6 — 5	++++	++++	++++	+++±	0
	5-46	"	6 — 7 — 6 — 7	+++	0	0	0	0
	5-51	"	6 — 7 — 6 — 5	++++	++++	+	0	0
	5-80	"	6 — 7 — 8 — 6	++++	++++	++++	+	0
	5-85	"	4 — 7 — 6 — 5	++++	++++	++++	+++±	0
	5-86	"	8 — 8 — 8 — 8	0	0	0	0	0
	5-88	"	8 — 8 — 8 — 7	+++±	++++	++++	+++	±
	6-75	121	6 — 8 — 8 — 7	++++	++++	++++	0	0
	6-81	"	6 — 6 — 8 — 6	++++	++++	+	0	0
	6-83	"	7 — 8 — 6 — 8	++++	++++	+	0	0
	6-89	"	12 — 10 — 12 — 10	++++	++++	++±	0	0

(T) = the tumors of 8-74 were used for transplantation on the 44th day.

++++ = complete fixation of complement (no hemolysis).

0 = no fixation of complement (no hemolysis).

2 units of complement in all tubes.

None of the sera was anticomplementary in control tests, nor was the antigen.

Antigen, W. R. 1-30 E, Berkefeld filtrate, 1:120.

* Moribund when bled on this day.

as normal controls while others had been implanted with the cancer as part of the groups of the 1st, 2nd, or 3rd Gen., but had failed to develop growths. The blood of a few had been tested previously and had proved devoid of the neutralizing property (*vide* rabbits 5-46, 5-86, Table I, and rabbit 6-81, Table III); and later inoculation with the virus had produced vigorous papillomas in them all.

The serum of every cancerous animal was found to fix complement strongly, whereas the results were negative with the specimens from the implanted controls (Table VI). Fixation was obtained with the serum of all except one of the papilloma rabbits, but it was wholly lacking in the case of this one, while in another it was slight, and the titer

TABLE VII

Neutralization Tests with Serum of Rabbits Bearing Transplanted Cancers

(Seventh, Eighth, Ninth, and Tenth Tumor Generations)

Source of serum	Rabbit No.	Time since implantation with cancer or inoculation with virus	Number of growths carried	Diameter of growths	Growths from mixtures of serum and virus W. R. 1-10 (Test rabbits A, B, C)					
					19th day			42nd day		
					A	B	C	A	B	C
		days		*cm.*						
Rabbits										
with 7th generation cancers (series B)	10-16	59	Five	5.0 - 4.5 - 5.0 - 5.0 - 4.0	0	0	0	0	0	±
	10-17	"	Three	2.5 - 3.5 - 0.8	0	0	0	0	0	0
	10-18	"	Four	5.0 - 5.0 - 6.0 - 3.0	0	0	0	±	±	0
	10-19	"	Six	4.0 - 4.0 - 6.0 - 5.0 - 4.0 - 3.0	0	0	0	0	0	0
with 8th generation cancers	9-65	86	Six	3.8 - 7.0 - 9.0 - 8.0 - 6.0 - 6.0 (T)	0	0	0	0	±	±
	9-67	"	Two	3.5 - 5.0	0	0	0	±	0	0
with 9th generation cancers	10-27	59	Six	5.0 - 5.0 - 6.0 - 6.0 - 4.0 - 4.0	0	0	0	0	0	0
	10-28	"	Five	6.0 - 7.0 - 3.5 - 6.0 - 2.8	0	0	0	±	0	0
with 10th generation cancers	10-37	47	Three	2.0 - 1.5 - 1.5	0	+	0	±	+	0
	10-38	"	Six	3.5 - 4.0 - 4.0 - 2.8 - 3.5 - 3.5	0	0	0	0	±	±
	10-40	"	Six	3.0 - 3.5 - 3.5 - 3.5 - 3.0 - 4.0 (T)	0	0	0	±	0	0
with virus-induced papillomas	10-07	67	Five	3.5 (average)	0	0	0	0	+	±
	10-08	"	Six	4.0 "	0	0	0	0	0	0
	10-09	"	Five	3.5 "	+	+	0	++	+++	0
normal	10-61				++±	++±	±	++++	++++	±
	10-62				++	+++	+	++	++++	+
	10-63				+++	+++±	±	++++	++++	±
Tyrode control					+++	++	0	++++	++++	±

(T) = the tumors of rabbit 9-65 were used for transplantation on the 27th day; those of rabbit 10-40 on the 91st day.

for the group as a whole was less than in the case of the cancer rabbits although these had had their growths for a shorter time.[2]

[2] To avoid confusion it should be pointed out that the plus signs of Table VI record the effectiveness of the blood antibody, as indicated by its capacity to fix complement in mixture with the virus, whereas in the neutralization tables these symbolize its ineffectiveness,—representing as they do there the fact that the mixtures of blood and virus gave rise to growths in greater or less number.

The neutralization tests were resumed after the cancers in the animals of the 10th Gen. had grown big. One of the group had been killed for transplantation purposes, leaving three with tumors. These were tested together with three normal agouti rabbits, three that carried papillomas, and all of the still surviving cancerous animals of the 7th Gen. B (Dutch belted rabbits) and of the 8th Gen. A and 9th Gen. A (agouti animals like those of the 10th Gen.). Table VII gives the results. It shows that the sera of the cancerous individuals,—each of which had good-sized tumors,—neutralized the virus markedly in every instance. The serum of one of the papilloma rabbits was relatively ineffective by comparison. One of the animals inoculated with the test mixtures (rabbit C) proved highly exceptional in possessing some natural resistance to the virus; it developed but few growths in response to inocula that caused many on rabbits A and B.

In brief the blood of every animal in which the cancer reached a considerable size had a definite and usually a marked power to neutralize the papilloma virus. Blood from normal animals and from those in which the tumor failed to grow was, on the other hand, devoid of effect under the conditions of our tests. When only small tumors had appeared the virus-neutralizing antibody was sometimes absent, but it developed later in the blood of those animals in which such tumors enlarged (Tables II and IV). The antibody titer attained seemed on the whole to be slightly higher than that reached in animals which had carried large virus-induced papillomas for a much longer time. There was no falling off in this titer as the propagation of the tumor went on. It became as high in the animals of the 10th Tumor Gen., tested a year and 4 months after the initial transfer of the tumor, as in the first new host in which it grew.

DISCUSSION

The tumor here considered is a squamous cell carcinoma, both in morphology and in all the phenomena of its enlargement and metastasis. Furthermore the cellular reaction occurring when it retrogresses is like that taking place about other transplantable neoplasms under similar circumstances. In previous papers the fact has been stressed that the cancers deriving from virus-induced rabbit papillomas tend in general to alter from one form to another in the direction of greater anaplasia (7, 4), and that in proportion as they do this the papillomatous traits usually disappear. They were gone from our cancer before it was first transferred, but it had not then wholly lost the ability to differentiate, and only of late and inconstantly has it become completely anaplastic.

The cancer forms large, solitary cysts containing quantities of glairy fluid under tension, in addition to necrotic debris. Another transplanted cancer deriving from a virus

papilloma did the same (6), and cysts with glairy contents have been encountered in two out of twenty cottontail cancers of similar origin (4),—from all of which one may infer this feature to be characteristic of certain tumors of rabbits. Only three epidermoid carcinomas have been successfully transplanted as yet in these animals, the present cancer, the one just referred to, and the Brown-Pearce carcinoma. This last seems to have arisen from a hair follicle cell or cells (15), and histologically it differs distinctively from our two tumors. It is rarely cystic, never contains glairy fluid, and the papilloma virus fails to persist after introduction into it (6).

The identical character of the two tumors we have transferred leads one to ask whether all cancers arising from the virus papillomas of domestic rabbits will not manifest similar traits on propagation. In favor of this possibility is the "pure line" origin of the growths from epidermal cells altered in a distinctive way by the papilloma virus (7, 16), as further their tendency to undergo changes later in a single direction, with anaplastic squamous cell carcinomatosis as the end product. But the cancers exhibit a considerable diversity within the limits outlined (7, 10, 4). A number of them have been successfully transplanted to the leg muscles of the animals in which they arose, with result in large growths in some cases (7, 10), and none of these growths thus far has contained cysts full of glairy fluid. The desmoplastic influence of the cancer now under propagation, while a striking feature of its growth, is no greater than that of a considerable proportion of human epidermoid cancers or of many of the cancers that we have elicited by tarring the skin of cottontail rabbits.

The ability shown by the tumor to flourish in hosts of a different breed after it had been propagated for a time, is no new finding with growths rendered increasingly effective by selective transplantation; but that it did better in alien animals than in blood relations of the original host is a highly exceptional phenomenon, though not without precedent. A spindle cell sarcoma of the fowl, rifted with blood sinuses and with a tendency to metastasize to the muscles, has been found to attain far greater success on transplantation to birds of the Plymouth Rock variety than in brown leghorns like the original host (17). The sarcoma proved due to a virus, and the possibility cannot be ruled out that it enlarged by an unobserved, secondary infection of neighboring elements as well as by intrinsic cell proliferation, in which case its remarkable success in Plymouth Rocks might have been consequent in part on a greater susceptibility of these birds to the virus. No such explanation will hold for the present rabbit carcinoma which can only have enlarged in the leg muscles by multiplication of the epithelial elements introduced there. Nor will the explanation hold for a tumor of Japanese mice which failed to grow in ordinary white mice but succeeded in hybrids (18).

As the transplanted cancer enlarged, an antibody capable of neutralizing the papilloma virus *in vitro*, and of fixing complement in mixture with the virus, regularly appeared in the blood of the new hosts, just as happens in animals with enlarging virus papillomas (5). This antibody was not present in normal rabbits or those in which the cancer failed to grow. It reached as high titer in the animals of the tenth successive group to which the tumor was transferred (by the implantation of small bits of large neo-

plastic masses[3]) as in the rabbit of the 1st Tumor Gen., tested 15 months previously. Since the neutralizing antibody is strictly specific for the virus (6) and is identical with the complement-fixing antibody (14), the conclusion seems inescapable that the virus or an agent nearly related to it was contained in the cancer and increased in amount as the latter proliferated in host after host.

The antibody is known to have no effect upon the causative virus contained in growing papillomas, these taking their course irrespective of its titer, and the virus increasing in association with them as they do so (5). The same facts evidently hold true for the transplantable cancer and the agent it contains. In animal after animal the tumor enlarged progressively although nourished by blood containing antiviral antibody in high titer; and on every transplantation it carried with it, undiminished in antigenicity, the agent calling forth this antibody,[4]—as blood tests of the new hosts have shown.

Rabbits carrying virus-induced papillomas differ widely in their response to the presence of the growth. Immunity to the virus, as measured in terms of the neutralizing power of the blood, becomes marked in most of them within a couple of months, but in some individuals it appears but slowly and may long remain ineffectual. Occasionally a rabbit develops papillomas on reinoculation although it has carried large growths for many weeks (2),—clear evidence that it has acquired no resistance worth the name. One of the animals of Table VI (rabbit 5-86), with four big papillomas 154 days old, had no immunity demonstrable by the complement fixation test. Instances of similar kind might have been expected amongst the animals carrying the transplantable cancer, but they were not encountered: every one of 44 individuals in which the cancer had reached a large size eventually yielded serum with marked neutralizing power, and animals with small tumors and blood devoid of this power acquired it as their tumors grew.

In a previous paper (5) we have discussed some presumptive causes for the great individual differences in the immunity elicited by virus papillomas. Living cells are

[3] The active virus cannot be diluted far without loss of pathogenicity: it gives but few growths at dilutions of more than 1:10,000,—in terms of weight of papillomatous tissue extracted,—and never yields any when diluted to 1:1,000,000, even though rubbed into a very large scarified area (5).

[4] The blood of rabbit 10-40 of the 10th Tumor Gen. was found to possess marked neutralizing power on the 47th day (Table VII) and its tumors were not utilized for further transfer until the 91st day. The resulting growths in the animals of the 11th Gen. elicited antiviral antibody in the usual high titer, as proven by complement fixation tests.

known to protect viruses in general from circulating antibodies (19),—a fact which explains the continued growth of papillomas in hosts with antibody circulating in high titer. It seems reasonable to suppose that virus associated with proliferating cells may sometimes not be liberated in quantity into the organism. However this may be, it is certain that cutaneous papillomas keratinize outwards, and that in proportion as this happens the virus associated with the differentiating cells is removed from the body. It is known too that the response of an animal to the presence of a virus is largely conditioned by how much of the latter gains access to the organism. In animals carrying papillomas this amount is often far less than that which elicits a maximum immunity response, as has been shown by the success of recent experiments to increase the neutralization titer of their blood by the intraperitoneal injection of papilloma tissue procured from other rabbits (20). The titer can be greatly stepped up in this way.[5]

The conditions are significantly different in the case of the transplantable cancer. The cells of this growth are constantly dying in large numbers amidst a reactive connective tissue. The papilloma virus is markedly resistant to autolytic processes, as we have found in experiments directed to the point. It follows that the opportunities for the organism to become immunized against the virus (or an agent related to it) should be especially favorable when they carry cancers that contain it. This may be one reason why the antiviral antibody so consistently appears in the blood of cancer rabbits and reaches a high titer.

What is the papilloma virus doing in the cancer, if anything? The question has the greater interest because a distinctive substance eliciting immunity responses on the part of the host, and having chemical and physical attributes closely resembling those of the papilloma virus, has recently been found in the Brown-Pearce rabbit carcinoma (21). True, this substance will not engender growths when extracted from the carcinoma. But extracts of our transplantable cancer produced none, and the papilloma virus itself can seldom be recovered in pathogenic state from the vigorous papillomas which it directly produces in domestic rabbits and of which it is known to be the actuating cause.

In an accompanying paper we have reviewed the literature on the extraneous viruses which have been shown to flourish in tumors (22). Not a few of them persist and increase in the neoplastic tissue after the host animal has developed a resistance sufficient to prevent them from establishing themselves elsewhere in its body on reinoculation. But these viruses are in general mere "riders," producing no significant alterations in the tumor. The papilloma virus constitutes a significant exception to the rule. Not only is it capable of infecting benign and malignant growths due to some other cause, namely, the tar tumors of rabbits, but it endows some of them with the ability to grow when implanted elsewhere in the

[5] It may be remarked in passing that the papillomas of the hyperimmunized animals continued to enlarge.

host, stimulates many to more rapid proliferation, alters the morphology of not a few, and makes some become cancers forthwith instead of remaining benign and ultimately vanishing (11, 22). The growths which it influences are epidermal papillomas and squamous cell carcinomas, that is to say, tumors deriving from cells of the kind that the virus habitually acts upon, and nearly like those for which it is directly or secondarily responsible. In view of its ability to stimulate and alter these growths to which it bears no causal relationship, there would seem to be a strong likelihood that it influences the squamous cell carcinomas arising from the papillomas it has itself engendered. Yet it might exert both a stimulating and an alterative effect upon them and still not be their actuating principle. In several previous papers we have brought evidence that the change of virus papillomas to cancers is the outcome of virus variation (23, 4).

Variant alterations of viruses are usually attended by alterations in their antigenic character, one result being that animals immunized by means of the variant may respond with a greater or less immunity to the parent virus than when this is employed. The serum of the cancer rabbits of the present work neutralized the papilloma virus as well, or even better than that of animals carrying papillomas; and the immunity thus expressed developed with greater regularity. But the conditions already discussed as determining the amount of antigen set free in the body may account for this finding.

The rabbit carcinoma is now growing rapidly in eight out of twelve agouti animals of the 14th Tumor Gen. For convenience sake it will be called "Carcinoma V2", with "V1" to designate the previous cancer of similar origin that was lost after a first transfer. The ease with which it can be maintained and the rapidity of its proliferation make it a useful experimental material, aside from its interest as a cancer of undetermined cause containing in masked or altered form the neoplastic virus concerned in its origin.

SUMMARY

A squamous cell carcinoma derived from a virus-induced rabbit papilloma has been propagated in fourteen successive groups of animals. It grows rapidly now in most individuals to which it is transplanted, killing early and metastasizing frequently. The original cancer was the outcome of alterations in epidermal cells already rendered neoplastic by the virus, and the latter, or an agent nearly related to it, has persisted and increased in the malignant tissue, as a study of the blood of the first ten groups of

cancerous animals has shown. An antibody capable of specifically neutralizing the virus *in vitro* appeared in the blood of every new host in which the tumor enlarged progressively, and reached a titer comparable with that obtaining in animals which had long carried large papillomas. The antibody was absent from normal rabbits and those in which the cancer failed to grow.

The implications of these facts are considered.

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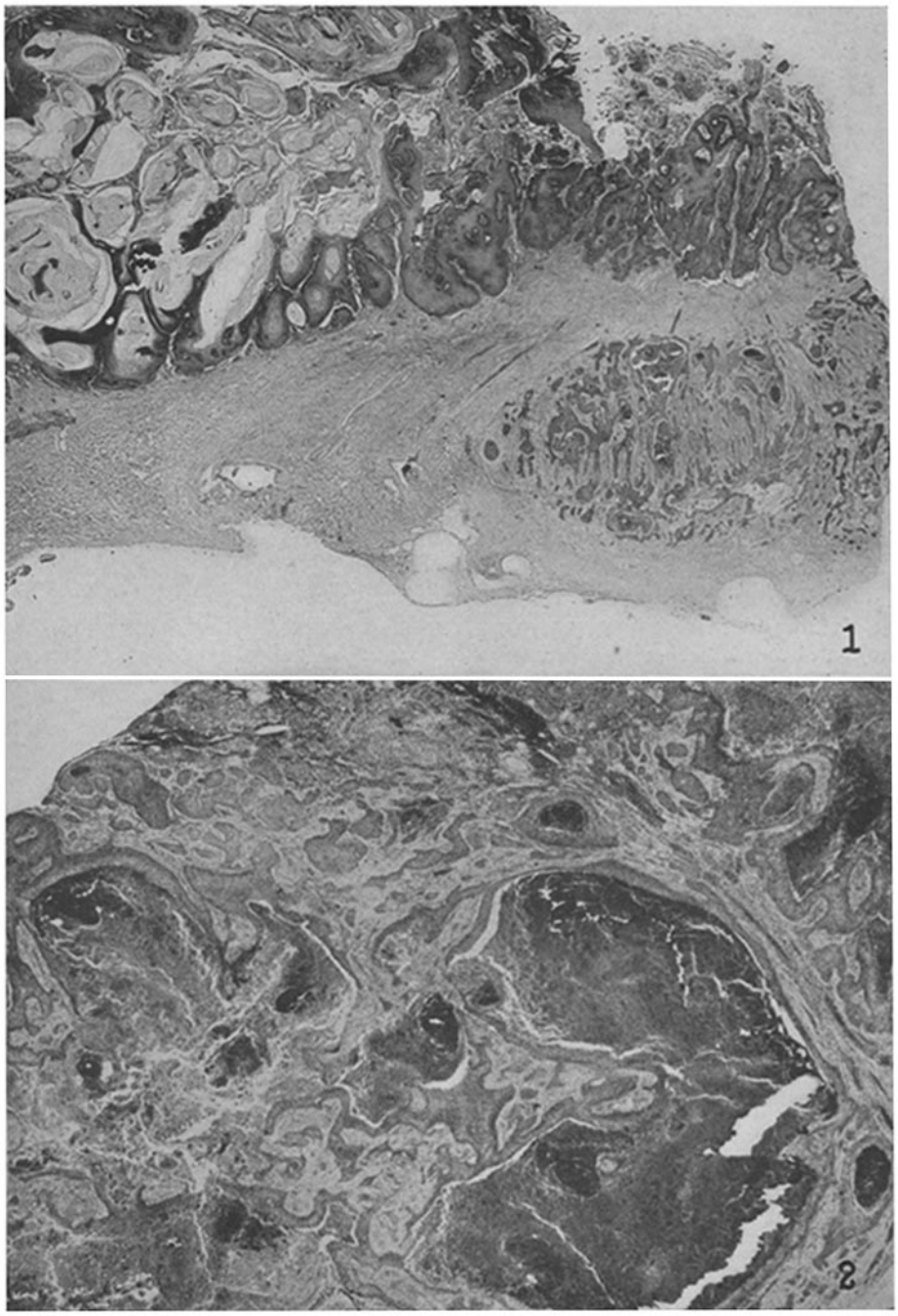
EXPLANATION OF PLATES

PLATE 42

FIG. 1. Radial section of one of the tumor masses on the side of the rabbit originally inoculated with papilloma virus,—to show the squamous cell carcinoma from which the metastases of Fig. 2 presumably derived. At the extreme left of the photograph normal skin with hair follicles can be seen, with keratinizing virus papilloma of the ordinary sort next it. Then comes malignant papilloma breaking up at two spots along the base into squamous cell carcinoma. At the extreme right (the center of the tumor mass) this latter has extended deep into the subcutaneous tissue. It is markedly desmoplastic, and small cysts have formed as result of early necrosis of the malignant cells. × 6½.

FIG. 2. Part of the metastatic growth in a regional lymph node, which was transferred to the leg muscles of the original host. The carcinoma shows the same features as the primary cancer. × 20.



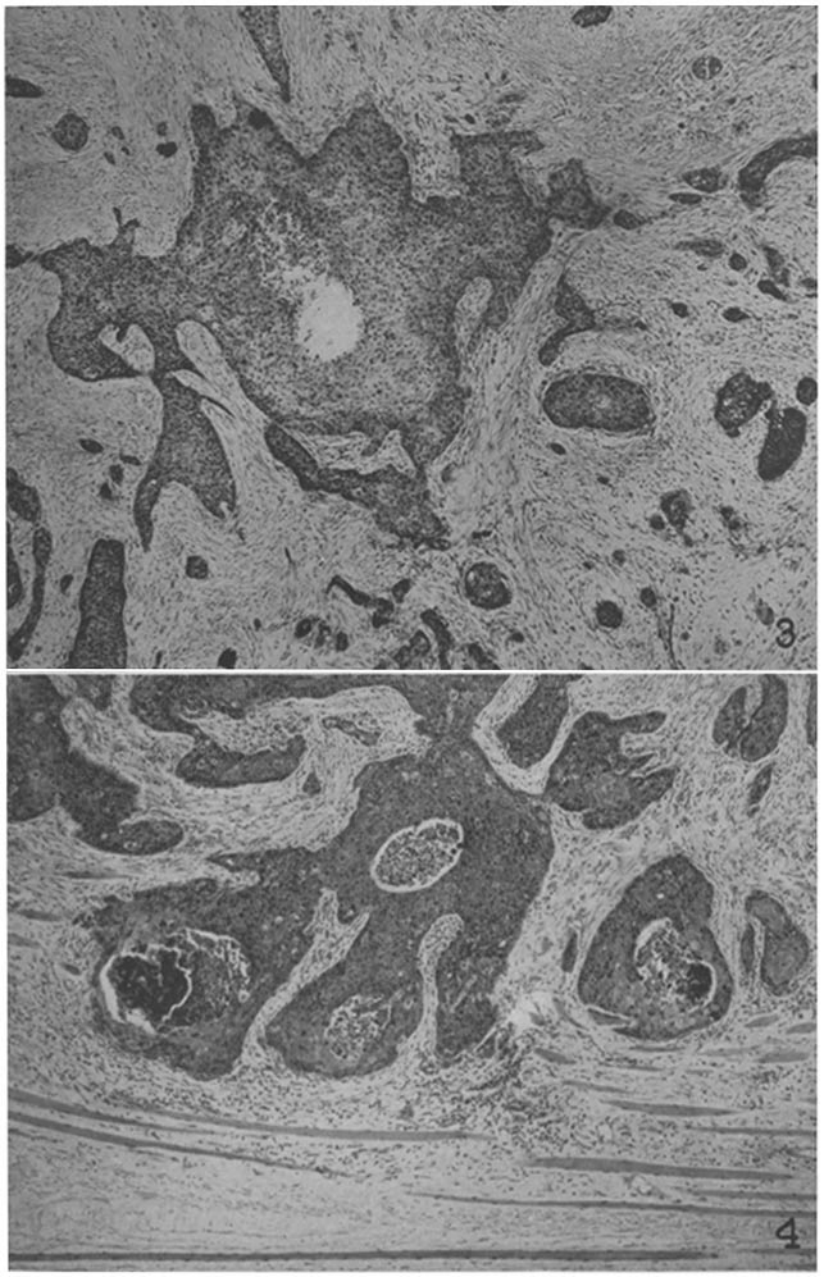
Photographed by Joseph B. Haulenbeek

(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

PLATE 43

FIG. 3. Part of one of the autotransplants in the leg muscles, which furnished the tissue that was implanted in other individuals. It shows an early stage in cyst formation. × 55.

FIG. 4. Margin of a growth in the leg muscles of a rabbit of the 6th Tumor Gen. The cancer has retained its initial character and again cysts are forming. Beyond the region of reactive connective tissue proliferation edema has forced the muscle fibers apart. × 57.



Photographed by Joseph B. Haulenbeek

(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

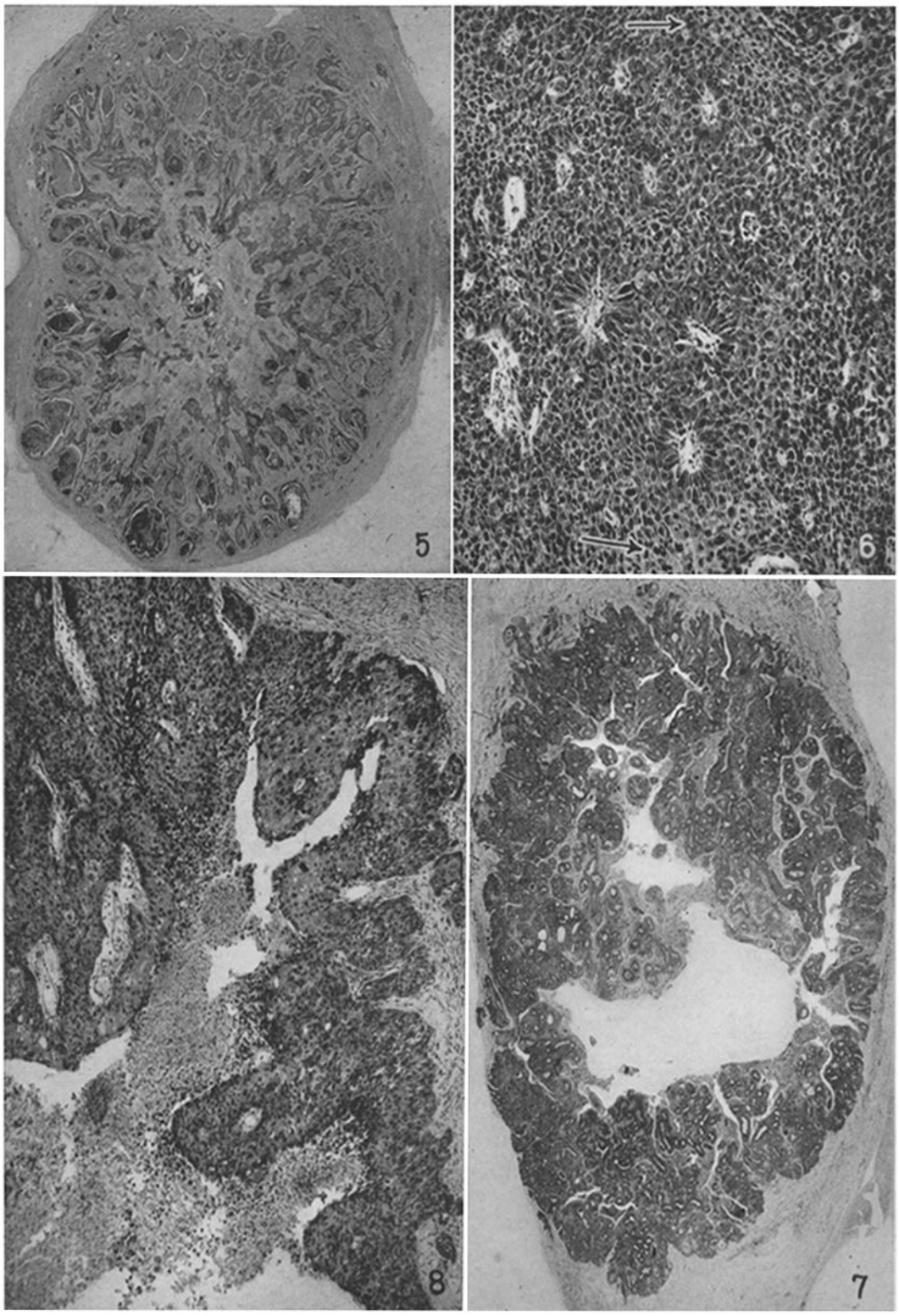
PLATE 44

FIG. 5. Cross-section of an intramuscular growth 2 cm. in diameter from an animal of the 5th Tumor Gen. A,—to show the coarse pattern of the cancer, beginning cyst formation, and encapsulation. There is more reactive connective tissue than usual, and hence the growth is still solid. Compare with Fig. 7. × 4.

FIG. 6. Part of a broad expanse of cancerous cells from a rabbit of the 6th Tumor Gen. B. The malignant elements immediately next the capillaries threading the tissue are crowded and radial, while further off they have begun to die (arrows). The edge of a small cyst containing debris can be seen beyond the beginning necrosis to which the lower arrow points. × 118.

FIG. 7. Cancerous nodule from an animal of the 5th Tumor Gen. A,—to illustrate the formation of papillae by ischemic necrosis. There is much less stroma than usual and the breakdown of the cancer is correspondingly extensive. As result of it a central cyst has formed, with numerous mural papillae. These have come into being as result of the necrosis of the cancer cells furthest from the blood stream. Their cores consist of blood vessels and they are covered with a thick layer of living cancerous elements. Toward the center of the cyst the papillae are dying. × 5½.

FIG. 8. A later stage in cyst formation. The papillae covered with cancerous cells have been reduced to mere blunt protrusions as result of interior pressure and continuing necrosis. Specimen procured at the second operation on rabbit F 5-82 of the 2nd Tumor Gen. (see also Fig. 17). × 52.



Photographed by Joseph B. Haulenbeek

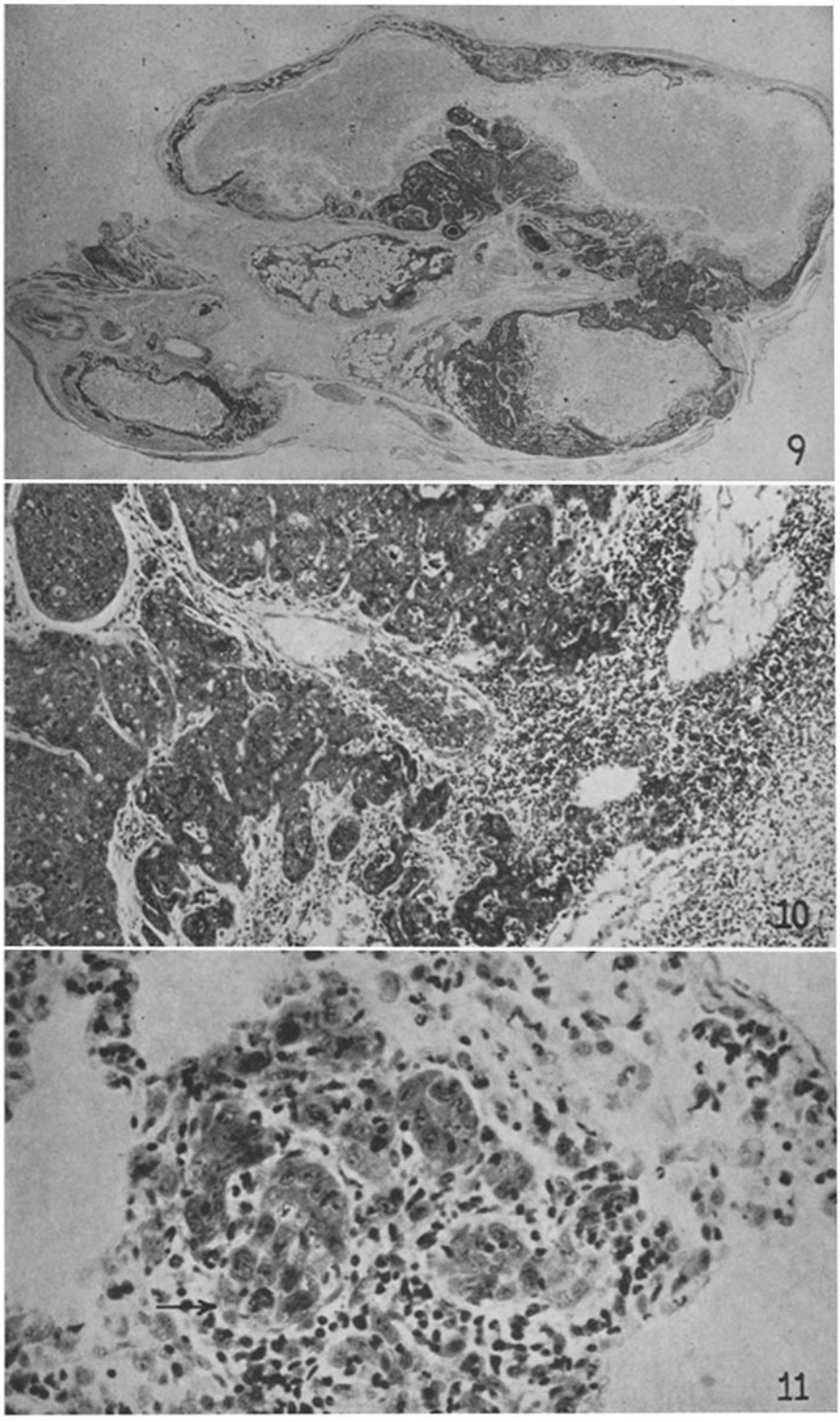
(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

PLATE 45

FIG. 9. Metastases in the iliac glands of a rabbit of the 6th Gen. B. Two glands have been entirely replaced by cancer and the process has been nearly completed in a third. The center of each growth has broken down, with result in a cyst with cancerous lining. × 6½.

FIG. 10. Part of the specimen of Fig. 9 at higher magnification,—to show direct invasion of the glandular tissue and the absence of connective tissue reaction. × 100.

FIG. 11. Pulmonary metastasis in an animal of the 8th Tumor Gen. B. There were secondaries in the axillary and iliac glands as well. The arrow points to a cell in mitotic division. × 112.



Photographed by Joseph B. Haulenbeek

(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

PLATE 46

FIGS. 12 to 16. Gross changes as the tumor enlarges (see text).

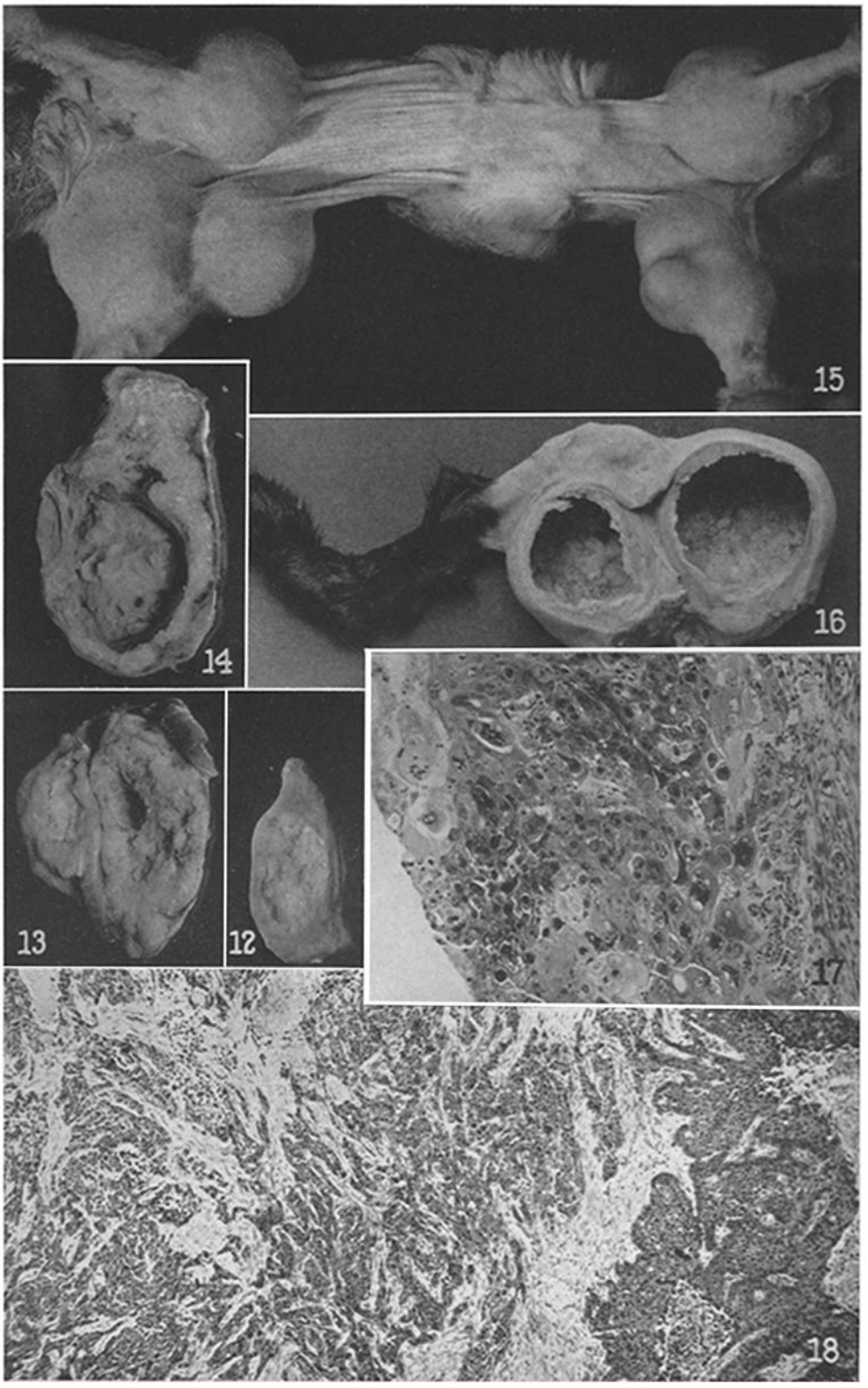
The rabbit of Fig. 15 was of the 5th Tumor Gen. A, and died of the cancer 89 days after implantation. The mass in the muscles of the right foreleg consisted of several cystic nodules, one of them about to herniate into the subcutaneous tissue. At each of the other situations there was a single, large, cystic growth. That one in the left posterior thigh was enormous.

Fig. 16,—from an animal of the 3rd Gen. killed when moribund on the 162nd day,—shows cystic tumors in the anterior and posterior thigh, emptied of their contents.

Figs. 12, 13, and 14 are natural size; Figs. 15 and 16, $\times$ ¼ and $\times$ ⅓, respectively.

FIG. 17. To show the alterations in the morphology of the cancer of rabbit F 5-82, consequent upon bacterial infection. For the previous character of the growth see Fig. 8. $\times$ 55.

FIG. 18. The extreme anaplasia found now and again in tumors of the later generations. Cancer of the ordinary type is also present, but there is no gradation from one form to the other. $\times$ 91.



Photographed by Joseph B. Haulenbeek

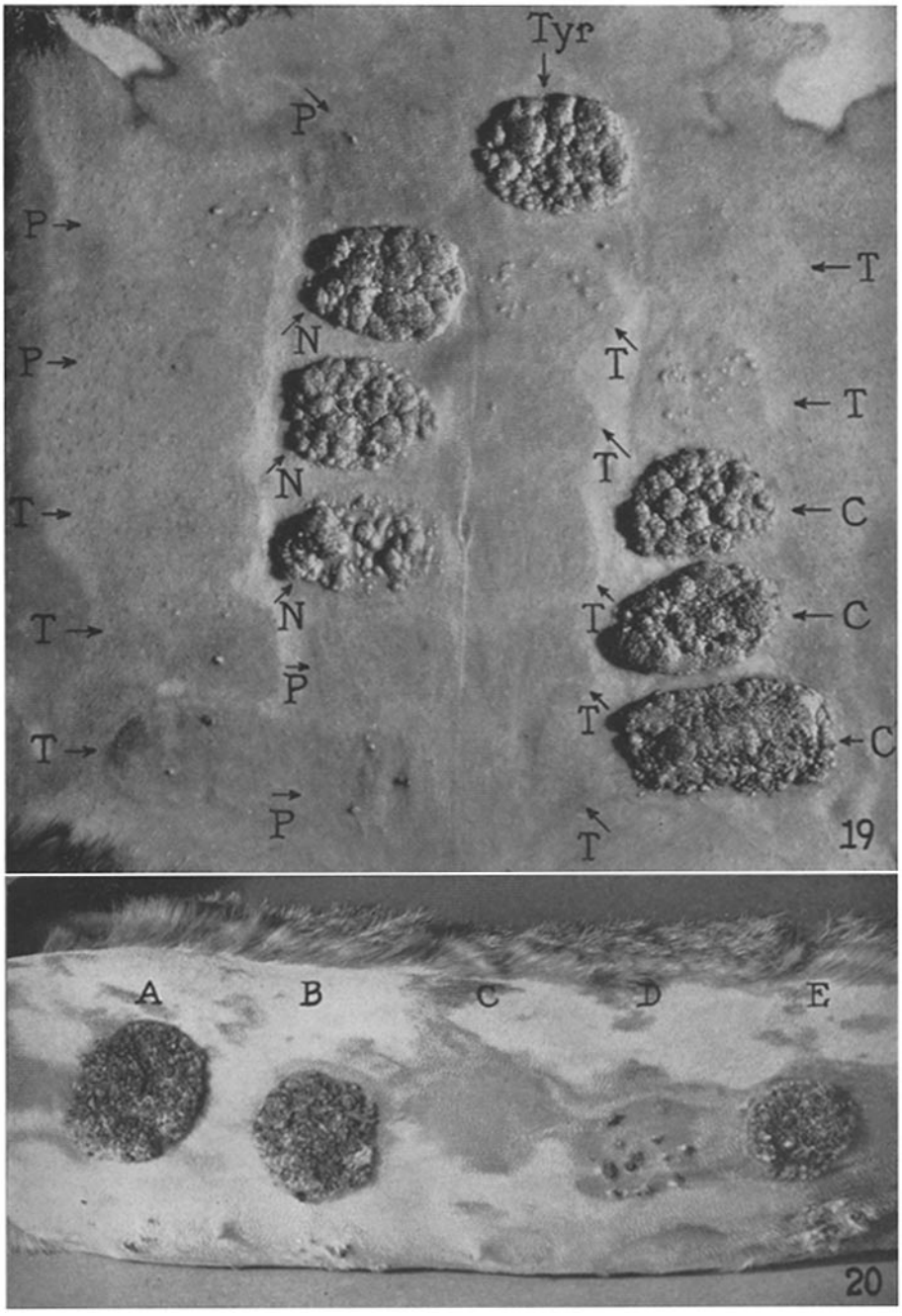
(Kidd and Rous: Transplantable rabbit carcinoma containing virus)

PLATE 47

FIGS. 19 and 20. The decisive character of the neutralization tests.

Fig. 19 shows the growths on the skin of test rabbit C of Table V (*q.v.*), which was killed for demonstration purposes on the 27th day after inoculation with 22 test mixtures. The skin has been dissected off and spread flat, and the strips of fur separating the inoculated areas have been shaved away. Where no growths cover the latter slight differences in their hue enable their extent to be discerned. At Tyr is an area completely covered by a confluent, papillomatous mass resulting from the inoculation of a virus-Tyrode mixture; and similar masses cover three areas (C) where mixtures of virus with the serum of 3 normal rabbits had been rubbed into the scarified skin. Five areas (P) were inoculated with mixtures containing sera from as many animals carrying large papillomas. Two of these sera prevented the virus from producing any growths at the inoculation sites, while in the other instances it caused very few. Five of the nine mixtures with the sera of cancerous animals (T) yielded no growths, two others caused only one or two, but in two instances a few more appeared than in the case of mixtures with the sera from papillomatous animals. For a record of the findings see Table V. × ½.

Fig. 20 shows five areas on the side of test rabbit A of Table I, 30 days after inoculation. Areas A and B had received mixtures of virus with serum specimens from 2 animals (5-46, 5-50) in which the cancer failed to grow after implantation. These areas are now covered with confluent papillomatosis. So too is area E which was inoculated with a mixture containing serum from a rabbit (5-52) in which a single nodule 4 mm. across appeared. At D a mixture was inoculated which contained serum from an individual (5-44) with three 4 mm. nodules in its leg muscles. A few growths have resulted. But there are none at all at site C where the mixture containing serum from rabbit 5-42, which had big tumors, was introduced. × ⅓.



Photographed by Joseph B. Haulenbeek

(Kidd and Rous: Transplantable rabbit carcinoma containing virus)