Studies on the Mechanism Responsible for the Spontaneous Regression of the Shope Rabbit Papilloma*

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SUMMARY

An attempt was made to demonstrate an immune reaction as the cause of the spontaneous regression of an autochthonous tumor, the Shope papilloma. It is well known that the incidence of regression is not correlated with antibody titer against the virus responsible for the neoplasm. Therefore, the regression mechanism may be directed specifically against papilloma cells. This hypothesis was tested by attempting to demonstrate anamnestic response in regressor animals upon secondary exposure to autologous papilloma tissue. Papilloma cells were obtained by infecting in short-term organ culture chips of skin, previously washed to free them of antiviral antibody. Papillomas developed from these explants when they were autografted to uninoculated, virus-immune, and papilloma-bearing rabbits. In animals in which papillomas had previously regressed either the papillomas failed to develop or the resultant tumors soon regressed. That the skin from regressor rabbits was fully capable of becoming papillomatous was demonstrated by grafting washed, virus-treated fragments into the cheek pouch of cortisonized hamsters. The results were consistent with the hypothesis that rabbits can develop immunity to autologous papilloma cells. Attempts to transfer this presumptive immune system are in progress.

The Shope rabbit papilloma is a virus-induced epidermal neoplasm indigenous to western cotton-tail rabbits and readily transmissible to domestic strains by cell-free preparations (18). In the domestic rabbit, the tumor may progress to carcinoma, remain benign, or regress completely. It was suggested as early as 1934 that regression may be effected by means of an immune mechanism (11). The regression is not correlated with antibody titer against the virus (9) and may be directed against another antigenic component of the papilloma cells. If this is so, regressor rabbits should manifest increased resistance on secondary exposure to papilloma cells.

How can regressor animals be provided with papilloma cells? Homografts of papilloma are not tolerated (11). The regressor can but rarely be reinfected by the classical method of rubbing Shope papilloma virus (SPV) into scarified skin because of the presence of circulating antiviral antibody (7, 8).

In the present experiments attempts were made to bypass the antiviral antibody present in the animal by using an in vitro technic. Skin explants were obtained from rabbits of four different immune states, infected by organ culture, and autografted to the original animal. The fate of these grafts supported previous suggestions that an immune phenomenon is responsible for the spontaneous regression of the Shope papilloma.

MATERIALS AND METHODS

Animals.—Giant Checker rabbits of mixed sexes weighing 1.5–2.5 kg. were housed in individual cages and fed water and Purina chow supplemented with fresh kale.

Apparatus.—Culture flasks were adaptations of Turner absorption bulbs† (Fig. 8). A rotator of original design was used to impart a gentle agitation to the culture flasks throughout the duration of the incubation period. This rotator was a 12″

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masonite disk mounted at 30° to the horizontal and rotated at 2 r.p.m. Eight aluminum clips spaced about the periphery held the flasks.

Media.—Eagle's medium (Difco) without added glutamine was supplemented with 30 per cent rabbit serum. This serum was drawn from normal young donors, pooled, and passed through a Seitz filter. There was no evidence of virus-neutralizing antibody in sera pools. Streptomycin and penicillin G were added to final concentrations of 40 µg/ml and 40 units/ml, respectively. The pH was adjusted to 7.4 with bicarbonate solution (Difco) and 95 per cent O2/5 per cent CO2. To one of a pair of flasks prepared for each animal's explants, 0.5 ml. SPV was added. The other was a control.

Virus.—Two kinds of virus preparations were used. The first, an extract of Kansas cottontail papillomas, was used only for inoculations by scarification. The second, used for immunization and culture, was purified by differential high-speed ultracentrifugation (3). Both inocula were of comparable infectivity.

Explantation technic.—A modification of the technic first developed by Medawar (10) for culturing small explants of rabbit skin in short-term organ culture was used. The abdominal fur was clipped with an electric razor, and the skin was washed with 70 per cent ethanol. The donor site was shaved with a razor blade held in a hemostat. Six cotton pledgets soaked in 70 per cent ethanol were then used to scrub the area. The site was blotted with sterile gauze pads and permitted to dry. Only skin areas in the nongrowing phase of the hair cycle were used. A scalpel was used to delinate a series of parallel crosshatchings 3—4 mm. apart. Another series was spaced at the same intervals at right angles to the first. The blade was drawn only partially through the dermis. Alternate squares were removed by grasping them at their centers with a small-toothed forceps and retracting upward. A sterile razor blade held in a hemostat was drawn across the base near the epidermal-dermal junction. A “pinch graft” resulted with rectangular boundaries 3—4 mm. and a maximum thickness of 0.5—1.0 mm. in the center. The explants were placed at once into a Petri dish with 5.0 ml. Hanks balanced salt solution, pH 7.4, at room temperature. When 40 were collected they were transferred to a sterile screw-cap tube and washed free of serum and clots by gentle inversion 10 times in 5 ml. of Hanks. This procedure was repeated with fresh Hanks for a total of 6 times. The fluid was then decanted, and twenty of the explants were placed in each of two flasks, together with 10 ml of media. The flasks were gassed with 1—2 liters of 100 per cent O2. They were then sealed and kept on the rotator at 36.0°C. for 3 days. Cultures were examined daily, but neither were they regassed nor were additions made to the media. The pH fell between 7.2 and 7.4 during the incubation.

Grafting technic.—At the end of the incubation period the explants were returned to the rabbit. The back was clipped with an electric razor, washed with 70 per cent ethanol, and permitted to dry. For orthotopic grafts, twelve 4-mm. circular excisions of full-thickness skin were removed down to the panniculus carnosus. These defects were arranged in two parallel rows, spaced about 4 cm. to either side of the midline. Six explants from each flask were grafted in each of the rows, virus-exposed explants on the right side of the animal, and control explants on the left. Neosporin2 ointment was applied to a 1-cm. gauze pad covered by an adhesive disk2 2-cm. in diameter. The disks were centered over the graft and firmly pressed to the surrounding skin.

For subcutaneous implants, twelve 1-cm. incisions were made ca. 6 cm. lateral to each of the orthotopic grafts. Through each of these incisions an explant was placed subcutaneously, at least 3 cm. lateral to the original incision. Again, virus-treated explants were on the right, controls on the left.

An over-all ventilated dressing composed of gauze pads overlaid by stainless steel wire mesh and edged by 9" adhesive tape prevented the animal from tampering with the grafts.

An area 2 cm. square on the outer aspect of each thigh was clipped with an electric razor and virus applied topically to freshly scarified sites and permitted to dry. Inspections were made at 14 days post-grafting and every 3 days thereafter until papillomas developed. Biopsies were taken of the largest subcutaneous cyst at 20—30 days, and of the orthotopic grafts when the situation warranted. All animals were observed for at least 2 months after grafting.

Experimental groups.—Four experimental groups were used: Group I consisted of animals never before exposed to SPV. Group II rabbits had been immunized with an intramuscular injection of 0.5 ml. of partially purified SPV, made directly into the thigh flexors via a small skin incision. The incisions were held open with forceps to prevent contact of SPV with epidermal cells. After withdrawal of the needle, the incision was washed with 70 per cent ethanol. Group III con-

1 Burroughs Wellcome and Company, Tuckahoe, New York.

2 "Sheer Spots"; Johnson and Johnson, New Brunswick, New Jersey.
sisted of animals bearing papillomas which had failed to regress. The papillomas were induced with virus in most animals, but some were induced with viral nucleic acid prepared by the cold-phenol method of Ito (8). Group IV animals at one time bore substantial papillomas which had subsequently regressed in the typical fashion. Any animals that died of intercurrent disease prior to the 14th day post-grafting were discarded from the studies.

RESULTS

Development of immunity to the Shope papilloma virus.—An attempt was made to determine the time at which an animal becomes resistant to reinoculation with SPV following an initial inoculation by scarification. For this purpose, each of six rabbits was given an inoculation of virus on days 1, 5, 6, 7, 12, and 15 of the experiment. All the animals developed papillomas at the first and second sites after average incubation periods of 12 and 22 days, respectively. At the third site, four of the six rabbits developed papillomas at 28 days. At the fourth site, only one rabbit developed papillomas at 35 days. Results at all the remaining sites were negative. The results suggest that the rabbits became resistant to reinoculation by scarification at about 6—7 days following initial inoculation.

Effect of SPV on migrating epidermis in vitro.—Medawar has described migration of rabbit epidermis about the edges and bottom of skin explants floating in a fluid medium (10). In the present studies it appeared that the rate of epidermal migration accelerated in the presence of SPV. To determine whether this was so, two to four explants were selected at random at the end of the 3-day incubation periods from each of the two flasks set up for an animal. The explants were fixed in 10 per cent formalin, sectioned through their maximum length, perpendicular to the hair follicle-bearing surface, and stained with hematoxylin and eosin. The extent of epidermal migration was measured with a calibrated ocular. The amount of epidermal migration was determined as the length of epidermis which extended from the last hair follicle at the edge of the section to the border of the migrating epithelium. From four to ten measurements were made on the specimens from each flask. Chart 1 shows the mean epidermal migrations in virus-treated and control explants. It is seen from this chart that in every instance the virus-treated epithelium migrated at a rate greater than that of the control.

Explants sectioned as described above were specifically studied for foci of proliferation and increased mitotic activity. Other than the effect on epidermal migration, no significant differences were noted between infected and noninfected explants (Figs. 1—8).

Assay of media for infective virus.—At the conclusion of the incubation period, fluids of 38 cultures were inoculated into the skin of normal rabbits. Of these samples, 24 (63 per cent) yielded papillomas, with an average incubation period of 35 days. From these results, it is clear that SPV persisted in most of the cultures. There was no evidence of virus replication. The prolonged incubation periods suggested a loss of active virus.

The Results of Inoculations in Specific Groups

Group I: normal rabbits.—Because the animals in this group had had no previous experience with the SPV, one would expect a high incidence of positive inoculations. That this was actually the case can be seen in Table 1. Autografts were positive in at least 50 per cent of the grafts in any given animal. Failure of some autografts to result in papillomas despite survival of these autografts as normal healthy skin, demonstrated by biopsy, can best be explained by failure of the infected cells to survive the trauma of the manipulations involved in autografting. Presumably, it was the membranous sheet of migrating epithelium which was infected (Fig. 3), since this sheet was the only part of the explant which demonstrated a consistent change when exposed to SPV. In addition, autografts macroscopically showed the first evidence of papilloma at this region (Fig. 5). Sections of early subcutaneous autografts showed the development of papilloma tissue in the migrating epithelium which formed the roof of such “external” cysts (Fig. 9).

Control virus inoculations by scarification were
uniformly positive. The relatively short incubation periods (14–25 days) reflected the absence of antiviral antibody.

The regression incidence in this group was 3/11, which is the expected incidence of spontaneous regressions (10–40 per cent).

**Group II: virus-immune rabbits.**—The animals in this group were subjected to explantation and autografting at varying intervals from the time of primary immunization. Papillomas never developed at the site of immunization. A considerable variation in antiviral immunity resulted as before to the presence of antiviral antibody. Increasing the number of washes to six in the remainder of the animals resulted in a high incidence of papilloma. The one animal that proved an exception (No. 9), died rather early in the experiment with a chronic gastroenteritis which progressively brought about a cachectic state. It is possible that the intercurrent disease was responsible for the results in this particular animal.

The prolonged incubation periods of control inoculations by scarification (28–35 days) and the high incidence of negatives reflect the high levels of antiviral antibody present in the rabbits. The animals (14–17) whose papillomas were induced by protein-free phenol extracts of wild cottontail rabbit papillomas demonstrated a variation in ef-
fectiveness of secondary inoculations which was not expected because of the supposed absence of SPV antigen in the original inoculum. Despite the absence of exogenous sensitization, the four rabbits demonstrated apparently different levels of antiviral immunity. Unfortunately, these were not quantitated by serum titrations before secondary inoculation. These results suggest that the animals were sensitized by viral antigen produced in their papillomas. The different levels of antiviral immunity are probably dependent upon the quantity of antigen liberated and the degree of the immune response in the individual rabbit.

It was observed in this group, as well as all the others, that there was a striking uniformity of the incubation periods of papillomas resulting from grafts infected in culture. The incubation periods varied from 14 to 21 days, with an average of about 16 days. These brief incubation periods of autografts, despite the prolonged incubation or even negative results of inoculation by scarification, strengthen the assumption that infection actually occurred in vivo, and not after autografting to the host.

No regressions occurred in this group. Since these rabbits had failed to regress their primary papillomas, it was not unexpected that the secondary papillomas also persisted.

**Group IV: regressor rabbits.**—The results of secondary inoculation in 11 regressor rabbits are shown in Table 4. Animals in this group were almost uniformly resistant to secondary inoculation, despite prolonged inoculation intervals and six washes prior to explantation. The one rabbit which developed macroscopic papillomas by the culture technic (Fig. 7) regressed the secondary papillomas in a period which was 15 days shorter than the original regression observed in this animal.

**TABLE 4**

<table>
<thead>
<tr>
<th>INOCULATION INTERVAL† (DAYS)</th>
<th>Skin infected in culture</th>
<th>Virus on scarified sites</th>
<th>Rabbit no.</th>
<th>Subcutaneous autografts/animal</th>
<th>Orthotopic autografts/animal</th>
<th>Incidence of papilloma*</th>
</tr>
</thead>
<tbody>
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<td>1–10</td>
<td>0/0</td>
<td>0/0</td>
<td>11</td>
<td>0/0</td>
<td>0/0</td>
<td>0/2</td>
</tr>
<tr>
<td>11</td>
<td>0/0</td>
<td>0/0</td>
<td>153</td>
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</tbody>
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* No. sites with papillomas/no. sites inoculated.
† Interval between primary and secondary inoculations.
‡ Regressed.

Biopsies were made of the largest subcutaneous cysts resulting from infected grafts in all regressor animals at approximately 20 days post-grafting. Many of these cysts showed hyperplastic epithelium which was suggestive but certainly not diagnostic of papilloma tissue (Fig. 10). Round-cell infiltrations and scant mitotic activity were noted almost uniformly in these cysts.

**INDUCTION OF PAPILLOMAS IN THE SKIN OF REGRESSOR RABBITS**

The object of this experiment was to demonstrate that the failure of secondary papillomas to occur in the autografts of regressor rabbits was not due to any acquired intrinsic refractoriness on the part of the epidermal cells themselves.

To this end, explants of skin 1 mm. square were excised from nine regressors, washed with Hanks balanced salt solution 3 times, and incubated 30 minutes at 36.0°C. in Hanks to which SPV had been added. The explants from each regressor were then grafted to the cheek pouches of two to four cortisonized adult Syrian hamsters, according to the method of Cohen (4). Animals were sacri-
ficed and grafts sectioned at 4–6 weeks after transplantation.

Although some of these heterografts were rejected, despite cortisonization, papillomas could be demonstrated developing from the epidermis of every regressor rabbit in at least one animal of each group of hamsters (Fig. 4).

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DISCUSSION

The mechanism responsible for the spontaneous regression of the Shope rabbit papilloma has been sought for many years. Earlier investigators suggested that the features of the regression were similar to those of homotransplanted tumors undergoing rejection (11). Regression is systemic in that all papillomas on the animal regress. It is specific in that it can be greatly increased by vaccination with homologous as well as autologous papilloma tissue (6), but not with various other tissue vaccines.

The level of antiviral antibody is not related to the incidence of regression (6, 9). In the present study, pre-immunization to SPV prior to attempts to infect the epidermis also failed to influence the regressions. Others (9) have described antiviral resistance which was proportional to the mass of papilloma in an individual rabbit. The finding reported here that antiviral resistance was present in rabbits that had papillomas induced with nucleic acid supports the suggestion that the degree of antiviral immunity can be significantly increased by release of SPV antigen from endogenous papilloma.

The acceleration of migrating epidermis in the presence of SPV described here confirms previous observations of Coman (5), who noted increased growth activity in vitro of rabbit epithelial cells of skin origin in the presence of papilloma virus. Acceleration of the rate of migration of wound epithelium in vivo in the presence of SPV has also been observed by Breedis (9).

The technic of organ culture used in these studies was developed primarily to provide autologous papilloma cells for grafting to rabbits that had previously been given inoculations of SPV and consequently developed varying degrees of resistance to reinoculation by conventional means. The effectiveness of this technic was shown to be

**COMPARATIVE ANTIVIRAL TITERS**

**CHART 2.—Results of direct comparison of the virus-neutralizing antibody levels in regressor and papilloma-bearing rabbits.**

**Comparison of Antiviral Antibody Titer in Papilloma-Bearing and Regressor Rabbits**

Despite the fact that it has been previously determined that antiviral antibody levels are not greater in regressors than nonregressors (9), it was of interest to compare these levels directly because of the limitation of the culture technic by antiviral antibody.

For this reason, serum from the individual rabbits was appropriately diluted and incubated 30 minutes at 36.0° C. with a standard virus preparation. The active virus present in the end mixture was assayed by inoculation into normal rabbits. The incubation period of the Shope papilloma is known to be related in an inverse manner to the concentration of active virus in a given inoculum (1). The incubation periods of comparative neutralization tests of sera of papilloma-bearing and regressor rabbits are demonstrated in Chart 2. From these curves, it can be seen that the sera of regressor rabbits did not possess more virus-neutralizing antibody than those of papilloma-bearers.

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Fig. 7.—Maximum development of papillomas which arose in regressor Rabbit 6.

Fig. 8.—Culture flask used in these experiments. Arrows indicate path of gas flow.

Fig. 9.—Subcutaneous cyst that developed in Rabbit 2, Group II, 12 days after autografting. Inflammatory cells are absent. Papillomatous epithelium is confined to the roof of the cyst. The original graft can be seen to the left of the cyst. X26.

Fig. 10.—Subcutaneous cyst that developed in regressor Rabbit 2. Note the hyperplastic epithelium with papillomatous pattern in keratin core of cyst. Subepidermal round cell infiltrations are present. X62.
limited in part by high levels of antiviral antibody. It is unlikely that the resistance of regressor animals to secondary inoculation can be explained by high levels of antiviral antibody interfering with the culture technic, since the levels in regressors were not greater than those of papilloma-bearers in which the culture technic was successful.

Others (9) have described failure to reinflect regressors by scarification. Only one animal of five regressors in their experiment could be reinfeeted. The secondary tumors regressed as did the similar positive inoculation by culture in the present study. Occasional positive secondary inoculations by scarification in papilloma-bearing rabbits have also been reported (7, 9).

Greene (7) has shown that failure to reinfect the skin of rabbits bearing papillomas is not due to a specific resistance of the epidermal cells themselves. The present report has extended this finding to the epidermis of regressor rabbits.

The results of these studies could possibly be confirmed by an in vitro technic. Direct inoculation of papilloma-bearing and regressor rabbits with infectious viral nucleic acid is theoretically possible because it has been shown (8) that this inoculum is unaffected by antiviral antibody. Resistance of regressor rabbits to such inoculations, and susceptibility of papilloma-bearers, would confirm the results reported here. Preliminary studies in this laboratory have shown that papilloma-bearing rabbits can be as readily infected as normal animals with this material. Attempts to transfer the regressor mechanism passively have so far been unsuccessful.

It is concluded that animals in which the Shope rabbit papilloma has spontaneously regressed are resistant to subsequent residence of this tumor, and that animals that have failed to regress do not demonstrate this resistance. The resistance is not attributable to levels of antiviral antibody and is not a property of the epidermal cells. The phenomenon of spontaneous regression has the characteristics of an actively acquired immune response, because it is systemic, specific, and subject to recall.

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