Infectiousness of the Shope Papilloma Virus after Growth in Embryonic, Neoplastic, and Cancerous States of Domestic Rabbit Skin

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Transfer of the Shope virus from the cottontail to the domestic rabbit results in the formation of papillomas indistinguishable from those of the original host, but, in the majority of cases, such passage is associated with a loss of infectivity. The presence of virus in the domestic papillomas can be demonstrated by immunological technics, and loss of pathogenicity is referred to as masking. It has been suggested that masking of the virus is a function of the body fluids of the domestic rabbit, but studies undertaken in this laboratory indicate, rather, that the process is independent of body fluids and related to a variation in the constitution of the epithelial cells of the epidermis (2, 3, 4, 8). The investigation to be reported was undertaken in an attempt to determine whether the ability to mask the virus was limited to the adult state of domestic rabbit epidermis or shared by embryonic and neoplastic states.

MATERIALS AND METHODS

In brief, the experiments consisted of the in vitro infection with cottontail virus of embryonic rabbit skin, Shope papilloma tissue, and fragments of the V1 carcinoma; transfer of the infected material to rabbits and hamsters; and subsequent test of the transplants for the presence of infectious virus.

Embryonic rabbit skin was obtained from domestic rabbit embryos between the 16th and 31st days of gestation; the papilloma tissue was removed by biopsy from induced papillomas in domestic rabbits; and the V1 carcinoma represented tumor carried for stock purposes in rabbits' brains. In addition, fragments of the tissue grown in rabbits' brains were retransplanted to the subcutaneous spaces of hamsters, and, after a further month of growth, the transplants were removed and tested for infectivity.

The rabbits used for assay of infectious virus were kept under observation for 2 months, and throughout this period the scarified skin areas were examined at weekly intervals for the presence or absence of papillomas. In a number of cases in which papillomas occurred, serial passage by skin scarification was attempted.

RESULTS

Embryonic skin.—The homologous and heterologous transplantation of embryonic skin resulted in takes in the great majority of cases. (The presence or absence of papillomatous transformation was not determined, since the growths were not sufficiently large to permit both histological examination and assay. In a parallel series of control studies, the incidence of papillomas varied from 0 to 100 per cent, with an average of 40 per cent.) The growths resulting from individual experiments were pooled, ground to a paste, mixed with sterile saline, and tested for the presence of infectious virus. The results of this test are shown in Table 1.

In total, 48 embryos, obtained from different does at different periods of gestation, were used in the study, and the growths derived from twenty of these were found to contain infectious virus. Some variation in the incidence of recovery of infectious virus was associated with the type of transplantation procedure employed. Following transfer to the rabbit’s brain, 25 per cent (nine out of 36) of the embryos tested produced growths containing in-
per cent subcutaneous site and when the issue first
from the used related to be trans-
ments of tissue, representing little more than one-
 tenth of the bulk of tissue used in passing the in-
fected virus may have been present but in ins-
ufficient quantity to induce papillomas on test.
In other cases, exposure of the tissue to virus may
not have been adequate to insure infection. It is
highly suggestive in this respect that, in recent ex-
periments not directly concerned with the pres-
ent investigation, infectious virus was recovered
TABLE 1
INFECTED EMBRYONIC DOMESTIC RABBIT SKIN
RECESSION OF INFECTIOUS VIRUS

<table>
<thead>
<tr>
<th>ANIMALS WITH PAPILLOMAS</th>
<th>NUMBER ANIMALS EXPLANTED</th>
<th>RECOVERY OF INFECTIOUS VIRUS OR OTHER METHOD OF TRANSPANTATION</th>
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<tbody>
<tr>
<td>Rabbit brain to Hamster brain</td>
<td>1/14 0/7 0/4</td>
<td>0/4 1/3 1/2 2/4</td>
</tr>
<tr>
<td>Rabbit brain to Hamster skin</td>
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<td>0/2 0/1 0/3 1/4</td>
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<td>Rabbit brain to Hamster skin</td>
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<td>1/1 0/2 0/2</td>
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</table>
onic skin was incubated overnight in the presence of virus prior to transplantation.

In a small series of subsidiary experiments, the subcutaneous space of the DBA mouse was used as a nidus for the growth of transplanted tissues. Embryonic rabbit skin infected in vitro, as well as papillomas arising in such tissue after transplantation to the rabbit brain, was used for transfer. Takes occurred with high frequency, and the resulting growths were found to contain infectious virus in approximately the same incidence observed after transfer to the hamster.

The papillomas arising in adult domestic rabbit skin after treatment with the transplanted material differed in several respects from those usually obtained after similar treatment with glycerolated cottontail material. Control rabbits treated with the cottontail virus preparations used for in vitro infection of embryonic skin invariably showed lesions by the end of the 2d week, and the resulting papillomas were always multiple and confluent. In contrast, the papillomas arising in test rabbits treated with transplants of infected embryonic skin were rarely recognizable before the 6th week and were generally few in number and discrete. It seems probable that many of the test animals released from the experiment at the end of 2 months as "negative" might have eventually developed papillomas since, in several instances, such animals were held in the colony for other purposes and, on subsequent examination—as late as 6 months after inoculation—were found to bear small papillomas in the scarified skin area. Despite the variation in number and time of occurrence, the induced papillomas resembled in appearance the isolated, discrete growths sometimes found following inoculation with cottontail virus. They grew to large keratinized tumors, rarely underwent regression, and showed an identical histological structure.

An attempt was made to determine whether or not the infectivity of the virus for adult domestic rabbit skin was maintained beyond the first generation. Accordingly, papillomas from sixteen of the assay rabbits were removed and tested individually by rubbing a suspension of ground tissue on scarified areas in the skin of normal adult animals. It was found that the papillomas of nine of the rabbits contained infectious virus and induced identical growths in the second generation. No apparent differences in site or host of origin or in subsequent transplantation history distinguished the infectious from the noninfectious papillomas. The infectious group included two papillomas derived directly from the transplants in rabbit brains; three were grown in hamsters after induction in rabbit brains; one was of similar origin but was subsequently grown in the DBA mouse; and three were induced in skin transplanted to the hamster. The noninfectious group contained two papillomas of the rabbit brain–hamster sequence and none representative of the DBA-grown tissue, but was identical in other respects.

Six of the infectious papillomas have been carried by serial inoculation to the present time and are now growing in the eighth consecutive passage. The incidence of infection has shown some increase but has varied widely in different generations and only rarely has reached 100 per cent. The length of the induction period has not decreased with continued passage, and the general characteristics of the papillomas have not changed.

Filtrates or cellular suspensions of the serially induced papillomas are infectious for both rabbit and rat embryonic skin. The papillomas arising in transplants of such skin are infectious for adult domestic rabbit skin, but the rate of infection is not increased over that of the original preparation. Similarly, transfer of the papillomas to the rabbit brain or the hamster subcutaneous space is not associated with either an increase or decrease of infectivity. The papillomas are infectious for adult cottontail rabbits, but the growths resemble those of the domestic rabbit rather than those generally produced in cottontail skin with cottontail virus. Further, the infectivity of the virus for domestic rabbit skin is not altered by cottontail passage.

The retention of infectivity by the Shope virus in transplants of embryonic skin after papilloma formation is in distinct contrast to the situation in intact adult skin. All the preparations used for in vitro infection of embryonic skin were tested for behavior in adult skin in intact domestic rabbits. In all instances, papillomas developed in first-generation animals but failed to occur on second passage. In further control experiments, papillomas derived from the skin of domestic rabbits were transplanted to hamsters and rabbits and the resulting growths tested for infectivity. A large group of assay animals were employed, but no papillomas resulted.

The ability to retain the virus in an infectious state is apparently not a function of the stage of embryonic development. The embryos used in the present investigation were obtained between the 16th and the 31st days of gestation, and the ability was manifested in all age groups. The relationship with embryonic length is shown in Table 2. It will be noted that the number of embryos whose skin yielded infectious papillomas remained relatively constant irrespective of size or age.

In a smaller series of experiments, the infec-
Infectiousness of the virus grown in embryonic rat skin transplanted to the subcutaneous space of adult rats was tested. It had previously been found that papillomas occurred in approximately 50 per cent of such transplants and gross examination suggested a similar incidence in the present group (1). Assay of the transplants resulted in the occurrence of papillomas in the skin of domestic rabbits and in two instances the papillomas were carried by serial inoculation to the third generation.

Newborn skin.—To determine whether or not the ability to retain infectious virus was carried over into postnatal life, the skin from five different groups of newborn rabbits was tested by in vitro infection, transplantation, and subsequent assay of the induced papillomas. The results of this test are shown in Table 3.

<p>| TABLE 3 |
| RELATIONSHIP BETWEEN THE ABILITY TO RETAIN INFECTIOUS VIRUS AND THE SIZE OF THE EMBRYO |</p>
<table>
<thead>
<tr>
<th>Crown-rump length (cm.)</th>
<th>No. infectious</th>
<th>No. non-infectious</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0–3.0</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>3.1–4.0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>4.1–5.0</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5.1–6.0</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>6.1–7.0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>7.1–8.0</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>8.1–9.0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>9.0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>28</td>
</tr>
</tbody>
</table>

The skin of all the five newborn animals developed papillomas on transfer to the various sites, but, on assay, only those derived from a single individual contained infectious virus. The papillomas derived from the skin of this individual induced growths in the skin of adult domestic rabbits identical with those described above, and the growths proved to be infectious on second-generation passage.

Adult skin.—A control experiment was carried out utilizing adult domestic rabbit skin in place of embryonic skin, and the various procedures were duplicated. The results obtained on assay of the papillomas arising in the transplants are shown in Table 3. The skin from eight animals was tested, but in no instance were the induced papillomas found to be infectious.

To determine whether the failure of the papillomas to retain infectious virus in these cases was related primarily to the adultness of the skin used or to other factors peculiar to adult domestic skin, a second group of experiments was undertaken utilizing adult cottontail skin rather than adult domestic skin. Skin from four different adult cottontail rabbits was infected with the Shope virus and transplanted to rabbits and hamsters, and the resulting papillomas were assayed for infectious virus. Infectious virus was present in the growth derived from all four of the animals and persisted on serial passage in domestic rabbits. The papillomas induced in domestic rabbits were of the same type noted after inoculation with papilloma tissue derived from transplants of infected embryonic skin.

It is of interest that one of the papillomas induced in infected skin of cottontails transplanted subcutaneously to a hamster was carried for four serial transplantation passages in this species prior to assay. The tissue was in residence in the hamster, a species resistant to infection with the Shope virus, for 3½ months, yet retained infectious virus and produced papillomas when applied to the scarified skin of domestic rabbits.

Domestic papillomas.—Papillomas induced in the scarified skin of adult domestic rabbits after the application of glycerolated cottontail virus constituted the experimental material in this phase of the study. The papillomas from eight different animals were used, and the tissues of each were divided into portions for control experiments and portions for in vitro infection with virus fluid.

Control experiments with the untreated tissue consisted of immediate application to the scarified skin of other domestic rabbits and transplantation to the subcutaneous space of hamsters with subsequent removal and assay. As a further control, the papillomas of ten additional domestic rabbits were subjected to the same procedures. In no instance, whether with tissue obtained directly from the domestic rabbit or after a month's growth in the hamster, was evidence of the presence of infectious virus found on assay.

The hamster's subcutaneous space was the only transplantation site for infected papilloma tissue used in this experiment. The transplants grew in the majority of hamsters, and the tissue available for assay at the end of a month was sufficient in
amount to be representative of each individual case. The transplants of six of the treated papillomas were found to contain infectious virus on assay (Table 4), and two of these have been carried by serial passage of virus fluid to the sixth generation in adult domestic rabbits. The papillomas obtained are also infectious for embryonic rabbit and rat skin and for the skin of adult cottontail rabbits. Further, other papillomas in the domestic rabbit

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>ASSAY OF TRANSPLANTS OF INFECTED PAPILLOMA FOR THE PRESENCE OF INFECTIOUS VIRUS</th>
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</thead>
<tbody>
<tr>
<td>Source of tumor</td>
<td>Exposure to virus</td>
</tr>
<tr>
<td>DBA subcutaneous space</td>
<td>½ hour at room temperature</td>
</tr>
<tr>
<td>DBA subcutaneous space</td>
<td>Overnight in incubator</td>
</tr>
<tr>
<td>Domestic rabbit brain</td>
<td>½ hour at room temperature</td>
</tr>
<tr>
<td>Domestic rabbit muscle</td>
<td>Overnight in incubator</td>
</tr>
<tr>
<td>cottontail rabbit brain</td>
<td>Overnight in incubator</td>
</tr>
<tr>
<td>cottontail rabbit muscle</td>
<td>Overnight in incubator</td>
</tr>
</tbody>
</table>

found to be noninfectious by assay became infectious after treatment with virus fluid obtained from the growths under discussion.

All the growths found in domestic rabbits, either as a result of assay or of continued passage, were identical with those arising from inoculation with the other treated and transplanted tissues discussed above. Moreover, inoculation of the skin of cottontail rabbits resulted in papillomas of the same type rather than of those associated with infection with the cottontail virus.

*Cottontail papillomas.*—In view of the constancy of the type of papilloma found in this investigation, it seemed desirable to determine whether the cottontail papilloma itself, if subjected to the same transplantation procedure, would yield papillomas comparable to those obtained on direct inoculation of domestic rabbits or of a sort similar to those found in the present experiments. Accordingly, papilloma tissue was transplanted directly from the cottontail rabbit to the subcutaneous space of hamsters and DBA mice, and, a month later, the growths were removed for assay in domestic rabbits. Both hamster and DBA-grown tissue proved to be infectious, and at the present time the infection has been maintained by serial passage to the fifth generation of domestic rabbits. The papillomas induced are discrete, few in number, and, in all other respects, resemble those obtained with other transplanted tissues.

\( V_2 \) carcinoma.—Attempts have been made to infect the \( V_2 \) carcinoma, using donors and recipients of various species as well as different methods of exposure to the Shope virus. Sixteen individual experiments were carried out, but infectious virus was found in the resulting tumors in only four cases (Table 5). In one of these, tumor tissue obtained from the eleventh passage in the DBA mouse was kept overnight in the ice box in contact with virus fluid prior to transfer to the succeeding generation. The animals of this generation were killed 3 weeks after transfer, and their pooled tumors were assayed in domestic rabbits. In another case, tumor derived from an intramuscular transplant in the domestic rabbit was bathed in virus fluid for ½ hour at room temperature and transferred to the brain of a cottontail rabbit. The animal was killed and the tumor assayed at the end of 3 weeks. In the two remaining instances, tumor derived from the domestic rabbit's brain was incubated overnight in a mixture of tissue culture and

<table>
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<tr>
<th>TABLE 5</th>
<th>ASSAY OF TRANSPLANTS OF INFECTED ( V_2 ) CARCINOMA FOR INFECTIOUS VIRUS</th>
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<tbody>
<tr>
<td>Source of tumor</td>
<td>Exposure to virus</td>
</tr>
<tr>
<td>DBA subcutaneous space</td>
<td>½ hour at room temperature</td>
</tr>
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<td>cottontail rabbit muscle</td>
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The animals were killed and the tumors assayed 3 weeks after transplantation. Serial passage of the infectious virus has been carried to the third consecutive generation of domestic rabbits in the first two cases, and the characteristics of the papillomas obtained are identical with those described in the previous paragraphs.

DISCUSSION

An evaluation of the results of the present investigation requires some comment with reference to the methods employed. Assay of the experimental material was carried out in adult domestic rabbits, and an interpretation of the significance of the occurrence of papillomas is restricted by the fact that a "masked" form of the virus has been demonstrated immunologically in some materials incapable of inducing papillomas in this strain of animal. Accordingly, the possibility that the failure of some of the transplanted tissues to induce papillomas on assay resulted from masking of the virus by such tissues cannot be disregarded.

Immunological tests for the presence of masked virus were not performed in the present experiments, and the possibility remains, but there is other evidence to suggest that the failures in question resulted from the methods used rather than from modification of the virus. As previously noted, the mass of tissue available for assay was much less than that utilized in transferring the infection from the cottontail to the domestic rabbit, and it is probable in some cases that infectious virus was present but in too small quantity to induce papillomas. A point of pertinence in this respect is that the bulk of tissue obtained from the transplantation of adult rabbit papillomas was much greater than that of embryonic skin transplants, and infectious virus was recovered in 75 per cent of the experiments, in contrast to 34.4 per cent in the case of the embryonic tissues. Further, the amount of tissue present in embryonic skin transplants after both rabbit brain and hamster subcutaneous space transfer was several times that obtained after rabbit brain transfer alone, and the incidence of infectious virus recovery was 45 per cent, in contrast to 25 per cent. In other cases, the negative results of assay may stem from an original failure to infect the tissues in question and may relate to the methods of exposure employed. It is suggestive that, in recent experiments involving overnight exposure to the virus in tissue culture fluid, the recovery of infectious virus from transplants increased to 100 per cent.

In any case, the point of significance in the present investigation is that infectious virus could not be demonstrated in papillomas induced in adult domestic skin, whether in intact animals or in transplants, whereas the virus derived from infected transplants of embryonic, neoplastic, or carcinomatous skin remained infectious. The failure to demonstrate infectious virus in some instances may be interpreted as evidence of "masking" by the tissues of the transplant, despite suggestive data to the contrary. But, even in such case, the frequency of recovery established the existence of a pronounced difference in "masking" ability between the tissues in question and adult domestic rabbit skin.

The papillomas induced in domestic rabbits with material from the transplants were similar in character irrespective of the nature of the transplant but differed from those induced with material obtained directly from the cottontail rabbit with respect to incubation period and number. Inasmuch as the same differences characterized the papillomas elicited with transplants of infected cottontail skin, it is possible that they represented a quantitative variation relating to the smaller amount of tissue available in the transplants than in the rabbit itself. However, the occurrence of a lasting qualitative change in the virus incident to growth in the transplants was evidenced by the retention of infectivity following passage in the adult domestic rabbit.

Such passage almost always results in the loss of infectivity of virus derived directly from the cottontail rabbit, but this loss was not sustained when tissue transplants were used as intermediate hosts, and the virus proved to be capable of inducing papillomas in second and successive domestic rabbit generations as well as in the first. It would appear, therefore, that multiplication of the virus in the transplanted tissues was associated with both an absence of the "masking" effect characteristic of growth in adult domestic skin and a modification inhibiting the subsequent action of adult domestic rabbit skin in this respect.

Numerous attempts to effect second-generation infection in domestic rabbits with cottontail virus not subjected to previous passage in transplanted tissues resulted in failure, but other workers have reported success in this direction. Shope succeeded in inducing papillomas following second-generation passage in domestic rabbits in thirteen of 58 attempts and, in one instance, carried the infection for fourteen serial passages (7). Selbie and Robinson obtained a transmissible strain subsequent to simultaneous infection of a domestic rabbit with the papilloma virus and the sheep dermatitis virus and carried the infection for fifteen passages in this breed (5, 6). In both of the cited cases, the serially induced papillomas bore a closer resem-
blance to the growths obtained in first-generation
domestic rabbits than did those under question. Further, the infectiousness of Selbie's virus for
domestic rabbits was lost after passage in a cottontail,
while, in the present instance, the infectiousness
of the virus was not altered by such procedure. Despite their apparent differences, the vari-
ant viruses possess the significant common property
of resistance to the masking effect of adult do-
mestic skin and, as such, offer material for studies
of possible pertinence in a clarification of the oper-
ation of the so-called masking process.

The infectious virus obtained by Shope appar-
ently arose spontaneously in cottontail papillomas,
Selbie's was initiated in a domestic rabbit bearing
a second viral infection, and, in the present in-
stance, the occurrence of the variant was associat-
ed with growth in transplanted tissues. It appears
highly probable that the procedures employed in
the present investigation were operative in effect-
ing the variation, since, in all instances, the cottontail
virus used for in vitro infection of the trans-
planted tissue was tested by second passage to
domestic rabbits, and the absence of papillomas in
this generation contrasted sharply with the con-
tinued infectiousness of the virus grown in the
transplants.

The procedures employed involved growth of
the cottontail virus in a variety of tissues trans-
planted to different sites in an assortment of hosts,
but the variant virus occurred irrespective of such
divergent factors. The subcutaneous space and the
brain were effective as transplantation sites and
the hamster, domestic rabbit, and mouse as hosts,
while transplants of cottontail papillomas or in-
fected adult cottontail skin proved as efficient
media as the various nonadult states of domestic
skin. Papillomas were induced in the transplants
of embryonic domestic and adult cottontail skin,
but changes incident to growth of the virus were
not found in the other tissues, and no related vari-
ation in the occurrence of infectious virus was
noted. Transplants of the various nonadult tissues
appeared equally impotent in masking the virus,
while transplants of infected adult domestic skin
proved as effective in this respect as did the skin
of intact animals.

The fact that the variant virus was produced in
transplants of cottontail papillomas and infected
cottontail skin and occurred only rarely, if at all,
in the intact cottontail rabbit indicates that the
factors operative in inducing the variation were
concerned in some manner with the conditions
of transplantation. As noted above, these were of
nonspecific character and included such diversified
environments as to suggest that the essential fea-
ture common to all was merely removal from the
cottontail rabbit. The suggestion that some factor
in the cottontail rabbit may inhibit the develop-
ment or multiplication of the variant virus is in
line with Selbie's observation that transfer to this
breed resulted in the loss of his infectious strain.
The present failure to repeat this observation may
relate to dosage or to some extraneous factor, and
attempts will be continued. Other investigations
have also been instituted involving the assay of
tissues containing the variant virus after trans-
plantation to cottontail rabbits, but the difficulty
of obtaining such animals in sufficient quantity
has delayed completion of the study.

Unlike cottontail skin, adult domestic skin will
not support development of the variant virus fol-
lowing transplantation, and, accordingly, the fac-
tor concerned appears to be a function of the skin
itself rather than dependent on residence in the in-
 tact animal as in the case of cottontail skin. How-
ever, this factor is concerned with evolution and
not multiplication of the virus, since, once estab-
lished, infection can be passed serially. The factor
is a developmental acquisition, not present in em-
 bryonic life but usually expressed at birth and is
lost in the conversion from adult to neoplastic or
cancerous states.

SUMMARY

Growth of the Shope papilloma virus in embry-
onic, neoplastic, and cancerous states of domestic
rabbit skin was not accompanied by the loss
of pathogenicity so frequently associated with
growth in adult domestic skin. The ability to
"mask" the virus was a developmental acquisition,
not present in embryonic life, but usually ex-
pressed at birth. It characterized the adult state
of the skin, but was lost in the conversion to neo-
plastic and cancerous states.

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Infectiousness of the Shope Papilloma Virus after Growth in Embryonic, Neoplastic, and Cancerous States of Domestic Rabbit Skin

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