Virus Content of Shope Papillomas of Cottontail Rabbits

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SUMMARY

The virus content of naturally occurring and experimentally induced papillomas of cottontail rabbits varies considerably. As a rule different individual papillomas on the same rabbit were found to have a similar virus content. The amount of virus usually increased as the tumors aged from 6 to 12 weeks. After 12 weeks there was no consistent pattern of change in virus content of papillomas, and dilution end points of $10^{-3}$ to $10^{-5}$ were commonly observed for at least 1 year. Tumors with low and intermediate levels of virus were induced by inoculation of 500 ID$_{50}$ and by 0.5 ID$_{60}$ of the same virus suspension. The white avascular portions of papillomas from the 2 cottontail rabbits tested were found to contain a substantially higher amount of virus than the dark portions of the same tumors. Papillomas tested several weeks before regression had a high virus content. Virus persisted in considerable amounts in these tumors during the entire period of regression.

INTRODUCTION

In Shope's first description of naturally occurring virus-induced papillomas of rabbit skin (11), it was reported that some of the tumors appeared to be free of infectious virus whereas others yielded substantial but variable amounts of virus. This was found to be true of both naturally occurring tumors and those induced experimentally in cottontails.

The present study was designed to examine some of the circumstances related to variations in the amount of virus present in Shope papillomas. Our chief interest was not in the presence or absence of infectious virus in the tumors but rather in the large differences in amounts of virus encountered in tumors on cottontail rabbits. The present study was not concerned with papillomas of domestic rabbits, which usually contain little if any infectious virus.

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MATERIALS AND METHODS

Rabbits

Cottontail rabbits (Sylvilagus floridanus) were obtained from Whidbey Island near Seattle or from Kansas. The domestic rabbits included New Zealand whites and San Juan rabbits (16).

Source of Virus

Unless specified otherwise the Washington B strain of Shope papilloma virus (1) of the 2nd and 3rd passages was used. This virus was derived from a single papilloma removed from a naturally infected cottontail rabbit trapped on Whidbey Island, 20 miles from Seattle, in 1962. It was maintained by passage in cottontail rabbits. Virus derived from Shope papillomas of wild cottontails trapped in Kansas was used to a limited extent in this study.

Processing Tumors for Assay of Virus

Amounts of virus in tumors were estimated by testing the infectivity of serial dilutions of tumor extract. Tumors were excised 1–2 mm above the base to permit regrowth and subsequent harvesting of same tumor. This allowed us to study the variations in virus content of a papilloma as it advanced in age. Tumors used for this purpose were usually 1–2 cm in diameter at the base. The tissue was either processed directly or kept in buffered 50% glycerin at 4°C. To prepare a crude virus suspension the tissue was washed in physiologic saline containing 1/100 Sorensen's phosphate buffer, pH 7.2, without Ca$^{++}$ or Mg$^{++}$ (PBS), weighed, minced with scissors, and ground in a mortar using Alundum. A sufficient amount of PBS was added to keep the tissue moist. The ground tissue was transferred to a centrifuge tube and the volume was adjusted with PBS to give the desired concentration, which was usually 10%. The suspension was centrifuged at 2000 rpm for 5 min and the supernatant fluid was dispensed in vials and stored at $-70^\circ$F.

Virus Inoculation

Cottontails were inoculated for the purpose of producing tumors which would be studied for virus content. Under ether
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TABLE 1
Typical Titration Results with Papillomas Representing the 3 Categories with Respect to Virus Content

<table>
<thead>
<tr>
<th>Papilloma category</th>
<th>Rabbit No.</th>
<th>Tumor No.</th>
<th>Dilution of tumor extract tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Age of tumor (weeks)</td>
<td>10^-1</td>
</tr>
<tr>
<td>Virus-rich</td>
<td>L 498</td>
<td>L-1</td>
<td>3/3</td>
</tr>
<tr>
<td>Intermediate</td>
<td>L 677</td>
<td>R-1</td>
<td>3/3</td>
</tr>
<tr>
<td>Virus-poor</td>
<td>L 506</td>
<td>L-2</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>L 659</td>
<td>R-1</td>
<td>1/2</td>
</tr>
</tbody>
</table>

\* R or L indicates the location of the tumor on the right or left side of dorsum. Each side had 4 tumors and they were numbered 1 to 4 from anterior to posterior position.
\* Number of sites positive/number of sites inoculated.

TABLE 2
Virus Content of Different Papillomas Harvested at the Same Time from Individual Cottontail Rabbits

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Tumor No.</th>
<th>Age of tumor (weeks)</th>
<th>Dilution of tumor extract tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbit No.</td>
<td>Age of tumor (weeks)</td>
<td>10^-2</td>
</tr>
<tr>
<td>L 674</td>
<td>R-1</td>
<td>6</td>
<td>3/3</td>
</tr>
<tr>
<td>L 682</td>
<td>R-1</td>
<td>6</td>
<td>3/3</td>
</tr>
<tr>
<td>L 663</td>
<td>R-2</td>
<td>6</td>
<td>3/3</td>
</tr>
<tr>
<td>L 680</td>
<td>R-1</td>
<td>6</td>
<td>0/3</td>
</tr>
</tbody>
</table>

\* Interval from virus inoculation to harvest of tumor.

RESULTS

For convenience we arbitrarily classified tumors as virus-rich, virus-poor, or intermediate according to whether the ID50 of the extract represented a dilution of 10^-4 or greater, 10^-4-10^-3 or less, or was between these 2 values. Table 1 illustrates typical titration results with the 3 types of tumors. In 1 experiment the virus content of 48 papillomas was determined. These tumors were harvested from 8 cottontails on 3 different occasions over a 17-week period. Twelve of these were virus-rich tumors, 13 were virus-poor, and the remaining 23 tumors were intermediate in virus content. In a 2nd experiment 33 papillomas from 20 cottontails were tested. The virus content was high in 6, low in 4, and intermediate in the remainder.

Concentration of Virus in the Several Tumors on 1 Rabbit

In most cases the several papillomas on 1 animal were found to contain similar amounts of virus at any given time. Typical data are shown in Table 2. The 2 tumors of cottontail L 674 were noninfective at the 10^-4 dilution, whereas the 2 papillomas of Rabbit L 674 diluted 10^-5 infected 5 out of the 6 test sites. The differences in virus content of tumors on 1 animal usually fell within a range of 10-fold but occasionally were as much as 100-fold.

Age of Tumor as Related to Virus Content

The virus content of a given papilloma may vary with time. There was usually an increase in titer, as the tumors aged from 6 to 12 weeks. In 1 experiment the virus content of 7 cottontail papillomas was determined at 6 weeks and again at 12 weeks. There was a 10- or 100-fold increase in the titer of 6 tumors. In the 7th papilloma the virus concentration remained unchanged. Twelve tumors removed from 6 cottontail rabbits used in the above experiment had the following virus content at 6 weeks: 5 were virus-poor papillomas, 1 was virus-rich, and 6 were intermediate in virus content. At 12 weeks 16 papillomas were harvested from the same 6 animals. Seven of these were rich in virus, 4 were virus-poor, and the remainder were intermediate in virus content. Table 3 shows the change in virus concentration which occurred in 3 cottontail papillomas as they advanced in age from 6 to 12 weeks.
Variation in Virus Concentration of Papillomas Induced on Same Specimen. All 6 rabbits were inoculated promptly after the virus was diluted. Tumors were harvested from all 6 rabbits on the same day.

Virus Content of Papillomas Induced with Inocula of Different Size and Before the Development of Malignancy. It was infective at a dilution of 10^-4 but not at 10^-5. Two papillomas were harvested from all 6 rabbits on the same day.

After 12 weeks there was no consistent pattern of change in the virus content of the papillomas, and end points of 10^-4 to 10^-5 were usual up to at least 1 year.

Virus Content of Papillomas Induced with Inocula of Different Size

In 1 experiment there was no apparent difference in virus content of papillomas induced by tissue extracts representing either 5% (50 ID50) or 0.5% (5 ID50) of the Washington B2 strain of virus. Table 4 shows that both virus-rich and virus-poor papillomas developed in rabbits that received the 5% inoculum as well as in those inoculated at the same time with a 10-fold greater dilution of the same virus suspension. All of the animals were inoculated on the same day by the same technique. They were kept under similar environmental conditions and received the same diet.

In a 2nd experiment the virus content of 6 cottontail papillomas was determined when they were 12 weeks old. Three of these tumors were induced by inoculation of 5 ID50, and the other 3 by inoculation of 500 ID50 of the Washington B2 strain of Shope papilloma virus. In each group there were tumors which had little virus and others which were intermediate in virus content. Two other cottontail papillomas had been induced by 0.5 ID50 of the same virus suspension as was used above. The virus content of the tumors at 12 weeks was low in one and intermediate in the other.

Virus Content of Papillomas Prior to and after Malignant Transformation

Each of 2 cottontail rabbits developed a carcinoma while carrying at another site a single benign papilloma. The papillomas and carcinomas were harvested simultaneously and tested for virus content. As shown in Table 5, no virus was recovered from the carcinomas. The papillomas, however, were infective at the 10^-3 but not at the 10^-4 dilution. The papilloma of Cottontail Rabbit 523 had been tested earlier, 13 months following virus inoculation and before the development of malignancy. It was infective at a dilution of 10^-4 but not at 10^-5. Two papillomas were harvested from a 3rd cottontail rabbit 6 weeks before carcinomaous change was first noticed. The tumors were 13 months old and both were found to be infective, one at the 10^-4 and the other at the 10^-5 dilution. It is of interest that the latter papilloma was largely white and avascular.

Virus Content of White Versus Dark Portions of Papillomas

Some papillomas have segments that are white and on microscopic examination appear to be a mass of keratin. In 2 instances the virus content was found to be higher in the white avascular portion than in the dark portion of the same papilloma. As shown in Table 6 the difference in virus content between white and dark parts was 100-fold in 1 case and more than 10-fold in the other.

Virus Content of Papillomas Prior to and during Regression

Spontaneous regression of papillomas occurs in cottontails with approximately the same frequency as in the case of domestic rabbits. The 5 papillomas shown in Table 7 were tested for infectivity at 6 weeks and all had a titer of 10^-4 or 10^-5. These 5 tumors underwent complete regression by the 12th week. Partial regression was found in a papilloma on 1 animal that had experienced complete regression of 7 other tumors. This single remaining papilloma was found to be infective at a dilution of 10^-5.
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**TABLE 7**

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Tumor No.</th>
<th>Dilution of tumor extract tested*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L 674</td>
<td>R-1</td>
<td>3/3</td>
</tr>
<tr>
<td>L 682</td>
<td>R-1</td>
<td>3/3</td>
</tr>
<tr>
<td>L 683</td>
<td>R-2</td>
<td>3/3</td>
</tr>
</tbody>
</table>

* All tumors were tested at 6 weeks after virus inoculation and all completely regressed by 12 weeks.

**TABLE 8**

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Dilution of tumor extract tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^-1</td>
</tr>
<tr>
<td>N 265</td>
<td>3/3</td>
</tr>
<tr>
<td>L 685*</td>
<td>4/4</td>
</tr>
<tr>
<td>N 204</td>
<td>3/3</td>
</tr>
<tr>
<td>O 208</td>
<td>0/3</td>
</tr>
<tr>
<td>L 684</td>
<td>0/2</td>
</tr>
</tbody>
</table>

* Washington B strain of virus was derived from this tumor.

All 8 papillomas on another cottontail rabbit had regressed. The scaly remnants collected at the site of 1 of the regressed tumors contained a substantial amount of virus; all of the 3 test sites inoculated with a 10^-4 dilution were positive. This specimen was not tested at a higher dilution. It is apparent, therefore, that papillomas which have had a high virus content undergo regression and that virus may persist in the tumor during the entire period of regression.

**Virus Content of Natural Papillomas**

Eight cottontail rabbits captured on Whidbey Island were found to have natural papillomas. The tumors were harvested at the time of arrival of the rabbits in the laboratory. Three were poor in virus content, 2 were virus-rich, and the other 3 tumors were intermediate in virus content. Table 8 shows that the variations in virus content of natural papillomas are similar to those observed in experimentally induced papillomas. One natural cottontail papilloma was tested on 3 successive occasions. On arrival in the laboratory it was found to be noninfective at a dilution of 10^-1. Four months later it was infective at 10^-1, the highest dilution tested. Seventeen months later the tumor was infective at the 10^-4 but not at the 10^-4 dilution. Obviously, variations occur in the virus content of natural papillomas as they age.

**DISCUSSION**

**Persistence of Virus in Regressing Papillomas**

Virus was shown to be present in a high concentration in papillomas tested a few weeks before regression, in a papilloma actively undergoing regression and in the scaly remnants of a tumor that had regressed. Similar findings on the persistence of virus in regressing rabbit papillomas have been reported by Kidd (4). It has been established in many tumor systems that regression is the result of an immune response directed against a tumor-specific antigen foreign to the host (8, 12). Rubin (10) has reported that interaction of immune cells with Rous sarcoma cells leads to death of tumor cells and loss of virus in chicken sarcoma. The mechanism responsible for regression of the Shope papilloma has not been clearly established. If an interaction similar to that described in the chicken sarcoma occurs in the Shope tumor, the target of the immune reaction might be limited to the basal and parabasal cells endowed with proliferative capacity. The dead keratinized cells and the highly differentiated granular cells which contain the mature virus particles might be an inaccessible target. Whatever the mechanism may be it is clear that virus may persist in considerable amounts in the papilloma during the regression period.

**Mechanisms Controlling the Virus Content of Cottontail Papillomas**

The factors that determine the quantity of virus in cottontail Shope papillomas are poorly understood. One concept that must be considered is that different variants of the papilloma virus induce virus-rich and virus-poor tumors. The fact that tumors of different animals varied greatly in virus content, even though they were induced with identical inocula, argues against this concept.

More than 25 years ago Kidd (5) attributed the lack of infectivity of the large, furred, and inflamed papillomas of cottontail rabbits to inactivation of the virus by extravasated antibody. There seems little doubt that neutralizing antibody in tissue harvested from such tumors must reduce the infectivity greatly. However, some small discrete cottontail papillomas yield little or no infectious virus. Moreover, the virus has been shown to persist in papillomas of cottontails even when they were infiltrated and covered by an extravasated serum coagulum (14). The present study has been concerned exclusively with small, discrete, well-ordered papillomas 5 to 18 mm in diameter. They were essentially devoid of inflammation. Neither grossly nor in microscopic section was hemorrhage noted as a common feature of tumors that were virus-rich or virus-poor.

A 3rd concept that must be considered is that a "helper" virus of the sort demonstrated in the Rous system (2) is required for synthesis of virus. Hartley and Rowe have isolated a 2nd virus, the rabbit-kidney vacuolating virus from some cottontail papillomas (3). However, it is unlikely that this virus acts as a helper for the synthesis of Shope virus since infectious papilloma virus has been recovered from tumors in which rabbit-kidney vacuolating virus could not be detected.

A 2nd argument against helper virus as the crucial factor in determining the virus concentration of the papillomas is the fact that tumors of low virus content and of intermediate virus content have been induced when the same virus suspension was inoculated in dilutions over a range of 1000-fold.

Another general concept worth considering is that some factor residing in the host animal is crucial in controlling the amount of virus present in the papilloma. This factor cannot be further
Virus Content of Shope Papillomas

defined from our present evidence. It might be genetically determined, it might be a hormonal or other physiologic state subject to natural fluctuation, or it might be related to an associated infectious agent. The finding that the multiple tumors of 1 animal contain similar amounts of virus strongly supports the concept that some systemic influence in the host animal is a major factor in determining the virus content of papillomas.

Rogers (9) has reported that injection of serine into infected rabbits increases substantially the virus content of papillomas. Mr. James Prince working in our laboratory was unable to find any consistent effect of serine treatment on the virus content of these tumors.

Absence of Virus from Carcinomas of Cottontails

The ability to recover virus from cottontail papillomas but not from co-existing carcinomas is in agreement with earlier reports (15). The demonstration of the Shope virus antigen in the carcinomas by the fluorescent antibody technic, and its persistence in cancers that have been serially transplanted for more than 20 years (6), is indicative of the presence of the virus in a "masked" state. The persistence of infectious virus in benign papillomas, present on cottontails that had developed malignancy in other papillomas, points to the possible participation of a local factor for masking of virus in the carcinoma. The most noticeable change in the transition of a benign papilloma to carcinoma is the loss of differentiation. The horny and the granular cell layers are poorly developed. These are the 2 layers in which the virus antigen and the mature Shope virus particles have been identified (7, 13). The absence of infective virus may simply reflect the paucity of those kinds of cells in which virus matures in papillomas.

ACKNOWLEDGMENTS

We wish to express our appreciation to J. J. Thomsen for his expert assistance. He carried primary responsibility for animal procurement and maintenance and carried out many of the inoculations, tumor harvests, and other technical procedures directly concerned with living animals.

REFERENCES

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