Histologic Features of Virus-rich and Virus-poor Shope Papillomas of Cottontail Rabbits

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SUMMARY

Certain histologic features of virus-rich cottontail papillomas were different from those of tumors poor in virus content. The virus-rich papillomas were characterized by a marked fragmentation of the horny layer and a well-developed granular layer which contained many keratohyalin bodies of unusually large size. A series of histochemical tests have confirmed that such large bodies are, in fact, keratohyalin granules. The virus-poor papillomas showed a rather homogenous horny layer and a granular layer that was ill developed or nonexistent in many areas. The horny layer was much thicker in virus-poor than in virus-rich papillomas, and in hematoxylin-and-eosin-stained sections, it was mostly pink in the former and blue in the latter.

Two other types of intracellular bodies were also identified in larger numbers in virus-rich papillomas. One was an eosinophilic cytoplasmic structure; the other was a basophilic intranuclear body. The eosinophilic inclusions were Feulgen-negative, but they sometimes contained a few Feulgen-positive granules. They could be identified in all layers of the papilloma and did not contain the Shope virus antigen, as judged by fluorescent antibody tests. These bodies might represent some form of abnormal cell keratinization. However, the possibility that they may be inclusion bodies of a second virus cannot be excluded.

The intranuclear basophilic bodies appear to represent virus-specific structures. These bodies were Feulgen-positive and occurred in the granular and horny layers. The fact that they contained a nucleoprotein resistant to DNase is consistent with the concept that such bodies might contain a mass of Shope papilloma virus particles. The demonstration by us and other workers that virus particles and virus antigen occur within nuclei in the granular and horny layers supports such a conclusion.

INTRODUCTION

The factors that determine the abundance of virus produced by cells infected with oncogenic viruses have been of interest for many years. One of the earliest well-known studies in this field was that by Bryan et al. (7), showing that tumors induced by a large inoculum of the Rous sarcoma virus as a rule contained a high concentration of new virus, whereas tumors induced by a small inoculum of that same virus commonly had little new virus. Subsequent work with the fowl tumor system showed that participation of two viruses in the synthesis of new virus accounts for Bryan's early observation (16).

The concentration of infectious virus in the cutaneous papillomas of cottontail rabbits described by Shope (25) varies over a wide range. Studies in our laboratory (13) have failed to reveal any difference related to the concentration of virus inoculated. In a search for biologic factors influencing the amounts of virus synthesized, we have investigated such variables as age of the tumor, strain of virus inoculated, and individual differences in the infected rabbits (13). Tumors harvested 6 weeks after virus inoculation were found to have, as a rule, lower concentration of virus than those harvested at 3 months or later. The several tumors on a given rabbit showed similar concentrations of virus, a fact pointing strongly to some systemic influence characteristic of the individual animal.

The present paper reports a further extension of our attempts to understand the mechanisms that control the amount of virus produced in individual papillomas (13) of cottontail rabbits by the Shope virus. In this study we explored the histologic and histochemical differences between tumors of high virus concentration and those of low virus concentration. The results as described in this paper demonstrate that differences in virus concentration do correlate with substantial differences in the usual histologic pattern. While this obviously reflects great variations in the physiologic processes of epidermal cell differentiation and maturation in tumors of high virus concentration as compared with those of low virus content, they do not provide an explanation in functional terms for the amounts of virus synthesized.

MATERIALS AND METHODS

Animals Used

The cottontail rabbits used were either from Kansas or from Whidbey Island near Seattle. Most of the domestic rabbits used were San Juan rabbits (27), but a few were New Zealand Whites.

Source of Virus and Virus Inoculation

The Washington B strain (13) of Shope papilloma virus of the second and third passages was used to induce tumors in both cottontails and domestic rabbits. The methods of virus inoculation and assay have been described in a previous paper (13).

Histochemical Tests

For most histochemical tests, 10% formalin in tap water and neutral buffered formalin were used as fixatives. The Feulgen
reaction (15) was used for the identification of DNA. The hema-
toxylin-permanganate method (14) was used to stain the ker-
atohyalin granules. Protein-bound sulphydryl (—SH) groups were
localized by means of the DDD (2,2'-dihydroxy-6,6'-dinaphthyl
disulfide) method of Barnett and Seligman (3). Acid phosphatase
activity was demonstrated by the method described by Barka
(2). Histochemical localization of RNA was performed by ex-
posing the sections to RNase (1 mg/ml distilled water) for 1 hr
at 37°C, followed by staining in toluidine blue. Duplicate sections
that had been incubated in distilled water served as controls.
Staining at 7 different pH values ranging from pH 2.5 up to pH
8.0 was accomplished with toluidine blue, methylene blue, and
aniline blue. The concentration of dye was \( 5 \times 10^{-4} \) M. The sec-
tions were left in the staining solutions at room temperature for
24 hr. Acridine orange staining and DNase treatment were carried
out as described by Williams (29).

Fluorescent Antibody Tests

\( \gamma \)-Globulin, fractionated from a pool of serum of a group of
cottontail rabbits whose tumors had regressed, was conjugated
with fluorescein isothiocyanate as described by Coons (11). The
tissue was fixed and processed according to the method reported
by Sainte-Marie (24).

RESULTS

We have arbitrarily classified the cottontail Shope papillomas
as virus-rich, virus-poor, or intermediate, according to whether
the ID0 was represented by a dilution of \( 10^{-5} \) or greater, by
\( 10^{-23} \) or less, or was between these two values (12, 13).

The virus concentrations of 6 representative cottontail rabbit
papillomas are shown in Table 1. Striking differences were noticed
on examining hematoxylin-and-eosin-stained sections of virus-
rich and virus-poor cottontail papillomas. These differences are
summarized in Table 2 and illustrated in Fig. 1-6.

Whereas the total thickness of the stratum corneum in virus-
rich papillomas greatly exceeded that of normal skin, it was not
as great as that on tumors with low virus content. There was a
great fragmentation of the horny layer in virus-rich tumors (Fig.
2). This is referred to as fibrillar fragmentation. In contrast, the
virus-poor papillomas presented a rather homogenous horny layer
with fewer fracture lines (Fig. 1). Whereas the horny layer
was predominantly blue (basophilic) in the virus-rich tumors, it
was mostly pink (eosinophilic) in the virus-poor papillomas. The
basophilia of the horny layer was usually most apparent in areas
overlying a well-formed granular layer that was rich in keratohy-
alin bodies.

The granular layer in a virus-rich papilloma was well developed
in practically all parts of the tumor and the keratohyalin gran
ules were numerous. Unusually large keratohyalin bodies meas
uring 10-14 \( \mu \)m in diameter have been identified (Fig. 4), and their
average number in a virus-rich papilloma was 10 per a single
microscopic field at a magnification of \( \times 430 \). In a virus-poor
papilloma, the granular layer was ill developed or nonexistent
in many areas. In other areas one could find a definite granular
area. The keratohyalin granules of the usual size were less nu
merous, and the large ones were sparse, averaging 5-10 in a
whole tumor section measuring 2 x 5 mm (Fig. 3).

<table>
<thead>
<tr>
<th>Category of tumor</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></td>
<td>( 10^{-1} )</td>
</tr>
<tr>
<td>Virus-rich</td>
<td>L 665</td>
<td>R-1</td>
<td>3/3*</td>
</tr>
<tr>
<td></td>
<td>L 682</td>
<td>R-2</td>
<td>3/3</td>
</tr>
<tr>
<td>Intermediate</td>
<td>L 660</td>
<td>R-2</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>L 662</td>
<td>R-4</td>
<td>3/3</td>
</tr>
<tr>
<td>Virus-poor</td>
<td>L 659</td>
<td>R-2</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>L 506</td>
<td>L-2</td>
<td>1/3</td>
</tr>
</tbody>
</table>

* Number of sites positive/number of sites inoculated.

| TABLE 2 |
|----------------|----------------|
| **Histologic Features of Virus-rich and Virus-poor Cottontail Papillomas as Seen in Routine Sections of Formalin-fixed Tissue Stained with Hematoxylin and Eosin** | |
| Stratum corneum | Considerably less thick |
| | Marked fragmentation |
| | Mostly basophilic (blue) |
| Stratum granulosum | Present in all areas |
| | Very well developed in practically all parts of tumor |
| Large keratohyalin bodies | Numerous |
| | 10/field at \( \times 430 \) |
| Cytoplasmic eosinophilic bodies | Numerous |
| | 1-2/field at \( \times 430 \) |
| Intranuclear basophilic bodies | Numerous |
| | 4/field at \( \times 430 \) |
| Virus-rich papillomas (ID0 > \( 10^{-5} \) | Much thicker |
| | Little fragmentation |
| | Predominantly eosinophilic (pink) |
| Virus-poor papillomas (ID0 < \( 10^{-5} \) | Nonexistent in some areas |
| | Poorly developed in many parts of tumor |
| | Few 5-10/whole tumor section (2 x 5 mm) |
| | Few 2-6/whole tumor section (2 x 5 mm) |
| | Few 3/whole tumor section (2 x 5 mm) |

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Two other kinds of cellular inclusions were also more numerous in virus-rich than in virus-poor tumors (Table 2). One (Fig. 6) was cytoplasmic and eosinophilic, and the other (Fig. 5) was basophilic and intranuclear.

It must be emphasized that most papillomas are of intermediate virus content. In them one finds a varying pattern from 1 area to another. Features suggestive of both high and low virus content are found to various degrees as one scans successive microscopic fields of a single section of a papilloma. Only by comparing tumors of high virus content with those of low virus content do the significant differences reveal themselves unequivocally. It should be stated that the finding in a cottontail papilloma of the histologic features described above would merely suggest that the tumor is either rich or poor in virus content. Bioassay is the method for measuring the virus concentration in a papilloma.

**Intracellular Bodies of Shope Papillomas**

The results of histochemical and fluorescent antibody tests for the 3 kinds of intracellular bodies of cottontail Shope papillomas are summarized in Tables 3, 4, and 5.

### TABLE 3

**Staining of the Intracellular Bodies of Virus-rich Shope Papillomas at Different pH Values**

<table>
<thead>
<tr>
<th>Character</th>
<th>Large keratohyalin bodies</th>
<th>Cytoplasmic eosinophilic bodies</th>
<th>Intranuclear basophilic bodies</th>
<th>Normal nuclei of papilloma</th>
<th>Horny layer of papilloma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of affinity to:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluidine blue at: pH 5.0</td>
<td>5.0*</td>
<td>5.0</td>
<td>2.5</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Methylene blue at: pH 5.0</td>
<td>5.0*</td>
<td>5.0</td>
<td>2.5</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Aniline blue at: pH 7.0</td>
<td>7.0*</td>
<td>7.0</td>
<td>6.0</td>
<td>5.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* They stained at pH 6-8 but not at lower pH values.  
* They stained at pH 2.5-6 but not at higher pH values.

### TABLE 4

**Characteristics of Large Keratohyalin Bodies of Cottontail Papillomas and of Keratohyalin Granules of Normal Skin**

<table>
<thead>
<tr>
<th>Character</th>
<th>Large keratohyalin bodies of cottontail papillomas*</th>
<th>Keratohyalin granules of normal skin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Stratum granulosum blue</td>
<td>Stratum granulosum blue</td>
</tr>
<tr>
<td>Hematoxylin and eosin</td>
<td>Blue</td>
<td>Blue</td>
</tr>
<tr>
<td>Hematoxylin permanganate</td>
<td>Blue</td>
<td>Blue</td>
</tr>
<tr>
<td>Feulgen reaction</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Treatment with ribonuclease</td>
<td>No digestion</td>
<td>No digestion</td>
</tr>
<tr>
<td>Sulphhydril groups</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Not detected</td>
<td>Present</td>
</tr>
<tr>
<td>Virus antigen</td>
<td>Not detected</td>
<td>Present</td>
</tr>
</tbody>
</table>

* Results of studies in our laboratory.  
* From published reports by others (19-21) based on studies of skin of various mammalian species. Normal rabbit epidermis is only 2-5 cells thick and therefore not differentiated sufficiently to provide a recognizable granular layer.

### TABLE 5

**Comparison of Keratohyalin Bodies with Eosinophilic Cytoplasmic Bodies of Cottontail Papillomas**

<table>
<thead>
<tr>
<th>Character</th>
<th>Keratohyalin bodies</th>
<th>Eosinophilic bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Granular layer</td>
<td>All layers</td>
</tr>
<tr>
<td>Hematoxylin and eosin</td>
<td>Blue</td>
<td>Pink*</td>
</tr>
<tr>
<td>Hematoxylin permanganate</td>
<td>Blue</td>
<td>Not stained</td>
</tr>
<tr>
<td>Loss of affinity to:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluidine blue at: pH 5.0</td>
<td>pH 5.0</td>
<td>pH 5.0</td>
</tr>
<tr>
<td>Methylene blue at: pH 5.0</td>
<td>pH 5.0</td>
<td>pH 5.0</td>
</tr>
<tr>
<td>Aniline blue at: pH 7.0</td>
<td>pH 7.0</td>
<td>pH 7.0</td>
</tr>
<tr>
<td>Feulgen reaction</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Ribonuclease treatment</td>
<td>No digestion</td>
<td>No digestion</td>
</tr>
<tr>
<td>Sulphhydril groups</td>
<td>Not detected</td>
<td>Present</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Not detected</td>
<td>Present</td>
</tr>
<tr>
<td>Viral antigen</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

* Basophilic, Feulgen-positive granules are present within some of the eosinophilic cytoplasmic bodies.

### Large Keratohyalin Bodies (Figs. 5, 6)

The results shown in Table 4 leave little doubt that the large cytoplasmic basophilic bodies are, in fact, unusually large keratohyalin masses. The only discrepancy was the failure to detect acid phosphatase activity in the papilloma granules. This enzyme has been reported to be present in normal skin keratohyalin (19). The Shope papilloma virus antigen could not be detected within the papilloma keratohyalin bodies and, as Noyes and Mellors have previously indicated (22), the antigen was found in nuclei in the granular and horny layers. The large keratohyalin bodies have been identified with the electron microscope, and no virus particles could be detected within them.

### Eosinophilic Cytoplasmic Bodies (Fig. 6)

Eosinophilic cytoplasmic bodies could be detected in all cell layers of the papilloma but mostly in the granular and horny layers. In the latter they existed as separate, well-defined eosinophilic masses. They were not so numerous as the keratohyalin granules. A single microscopic field of a virus-rich cottontail papilloma at a magnification of × 430 might reveal 1-2 eosinophilic cytoplasmic bodies and 30-40 large and medium-sized keratohyalin granules. Such eosinophilic bodies were rounded, oval, or dumbbell in shape and had a diameter of 5-15 μ. The large ones were usually found in the superficial part of the tumor. Table 5 summarizes the histochemical and fluorescent antibody results of the eosinophilic cytoplasmic bodies in comparison to the keratohyalin granules.

### Basophilic Intranuclear Bodies (Fig. 5)

Strongly basophilic rounded or oval intranuclear bodies were detected in the granular and horny layers of Shope papillomas of cottontail rabbits. They measured 8–14 μ in diameter and were frequently surrounded by a clear zone. They lost their affinity for methylene blue and toluidine blue at pH 2.5 and for aniline blue at pH 6.0 (Table 3). They were Feulgen-positive and resisted RNase treatment. They stained with acridine orange and had a
Distribution of Acid Phosphatase and Sulfhydryl Groups in Cottontail Shope Papillomas

In Shope papillomas, the stratum corneum was strongly reactive for acid phosphatase and for —SH groups. The other layers were mildly reactive for —SH groups and showed little or no reaction for acid phosphatase. The normal rabbit epidermis was very mildly reactive for —SH groups and showed little or no reaction for acid phosphatase. The normal rabbit skin was not attempted. Studies by others on skin of various other mammalian species have shown that the stratum corneum is mildly reactive for —SH groups and that the Malpighian layer shows a homogenous distribution of —SH groups (21). The acid phosphatase reaction was shown to be most intense in the granular layer of the skin, with gradual diminution towards the basal layer. The surface keratin was reported to show no reaction (28).

Findings in Domestic Rabbit Papillomas

Of 12 domestic rabbit papillomas tested, only 2 were infective: 1 at the 10⁻¹ and the other at the 10⁻² dilution. None of them showed the histologic features that characterized the virus-rich cottontail papillomas. They usually presented a combination of the features of intermediate- and low-titer cottontail papillomas. The large keratohyalin bodies were either absent or present in very small number. The cytoplasmic eosinophilic and the intranuclear basophilic bodies were also found, but they were very few.

DISCUSSION

Altered Differentiation and Keratinization

The fibrillar fragmentation of the horny layer in a virus-rich papilloma is evidence that the process of differentiation differs substantially from that of virus-poor papillomas which have a thick, relatively intact horny layer. Since the basophilia of the horny layer, another characteristic of virus-rich tumors, was, as a rule, most noticeable in areas overlying a well-developed granular layer, one might attribute the color of the horny layer to the relative abundance of the underlying keratohyalin bodies.

The results of the histochemical and fluorescent antibody tests support the conclusion that the large basophilic cytoplasmic bodies of Shope papillomas are keratohyalin granules. They differed from normal keratohyalin granules in 2 respects, their unusually large size and the negative results of tests for acid phosphatase. Acid phosphatases seem to play a role in the keratinization process (9), and abnormal keratinization in certain dermatologic conditions has been attributed to the absence of phosphatases and oxidases enzymes (19). In the Shope papillomas, there is an alteration of the normal process of keratinization as evidenced by the hyperkeratosis, parakeratosis, fragmentation of the horny layer, and the presence of a high concentration of —SH groups in the stratum corneum.

The abundance of large keratohyalin bodies in virus-rich cottontail papillomas could be explained either by an increased rate of synthesis or by a delayed incorporation of keratohyalin into the horny layer. It would be of interest to compare the rates of cell migration in virus-rich and in virus-poor papillomas. It is conceivable that in virus-rich tumors a retardation might occur in the rate at which granular cells are incorporated into the horny layer. This might lead to the accumulation and subsequent aggregation of keratohyalin granules giving rise to the large bizarre masses.

Cytoplasmic and Intranuclear Bodies in Shope Papillomas and in Human Warts

The nature of the eosinophilic cytoplasmic bodies which we found in Shope papillomas is not clearly understood. Our results indicate that they are not specific inclusions of the Shope virus. A variety of eosinophilic masses and cytoplasmic bodies have also been previously described in the Shope papillomas, but none was considered as a virus-specific inclusion (23, 25). The loss of affinity of the cytoplasmic eosinophilic bodies to toluidine blue and aniline blue at the same pH values as the horny layer suggests that they might represent some form of abnormal cellular keratinization.

The presence of Feulgen-positive granules within some raises the question of whether a virus, other than the Shope virus, might be the agent causing such inclusions. Hartley and Rowe (17) have isolated a second virus, the rabbit-kidney-vacuolating virus (RKV), from some cottontail rabbit papillomas. It would be of interest to compare the histopathology of Shope papillomas which contain the RKV with tumors that are free of RKV.

There is a great resemblance between rabbit papillomas and human warts. Electron microscope studies of the 2 tumors (18, 26) have shown that the morphologic sequence of development of virus is essentially the same. Three different types of intracellular bodies have been recognized in human warts: 1 cytoplasmic and 2 intranuclear (4, 5, 8). The available evidence indicates that the only virus-specific structure is the basophilic intranuclear body (1, 6, 29). Similar intranuclear inclusions have also been described in canine oral papillomas (10).

The basophilic Feulgen-positive intranuclear bodies of cottontail Shope papillomas resemble those inclusions described in human warts by Block and Godman (6). Electron microscope (26) and fluorescent antibody (22) tests have localized the Shope virus particles and virus antigen in nuclei of the granular layer. It seems, therefore, highly probable that the basophilic, Feulgen-positive intranuclear body is the only virus-specific structure of the 3 kinds of intracellular bodies that have been identified in Shope papillomas.

ACKNOWLEDGMENTS

The authors express their gratitude to Dr. N. K. Mottet for his helpful advice and assistance on the histopathologic and histochemical aspects of this study. We wish also to thank Dr. V. Chambers for carrying out the electron microscope examination and Mr. J. J. Thomaen who participated in the work with cottontail rabbits.
REFERENCES


Fig. 1. Virus-poor Tumor R-3 of Cottontail Rabbit L 659 showing a homogenous horny layer with little fragmentation. H, horny layer; C, connective tissue core. The space shown in this figure is probably due to retraction of the tissue. × 25.

Fig. 2. Virus-rich Papilloma R-4 of Cottontail L 665 illustrating the marked fragmentation of the horny layer. H, horny layer; C, connective tissue core. × 25.

Fig. 3. Virus-poor Tumor R-1 of Cottontail L 659 showing the presence of small and medium-sized keratohyalin bodies in the cells of the granular layer. Large keratohyalin granules are rarely seen in virus-poor papillomas. H, horny layer; G, granular cell layer; KH, keratohyalin granules. × 160.

Fig. 4. Virus-rich Papilloma R-4 of Cottontail L 665 showing many large keratohyalin bodies in cells of the granular layer. H, horny layer; G, granular cell layer; KH, keratohyalin granules. × 160.

Fig. 5. Virus-rich Tumor R-4 of Cottontail L 665 showing three intranuclear basophilic bodies in upper granular cells. IN, intranuclear basophilic bodies; KH, keratohyalin granules. × 400.

Fig. 6. Another area of the same tumor shown in Fig. 5 illustrating a cytoplasmic eosinophilic body in one of the cells of the granular layer. IC, intracytoplasmic eosinophilic body; KH, keratohyalin granules. × 400.
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