Antigen of Bovine Cutaneous Papilloma Detected by Fluorescent Antibodies*

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SUMMARY

Specific antigen was detected in the bovine cutaneous papilloma by fluorescent-labeled antiserum prepared against an infectious extract of bovine papilloma. Antigen was present in the nuclei of cells in the keratohyaline and keratinized layers of the hyperplastic epithelium but not in the germinal basal cells. These findings are similar to those reported for the Shope rabbit papilloma and the human cutaneous papilloma.

The fluorescent antibody method of Coons (3) has been useful in determining the intracellular localization of several animal tumor viruses. With the Rous sarcoma (10) and the polyoma (4) viruses in cell cultures, the sites of viral propagation have been established, and information regarding the developmental events leading to production of mature virus has been obtained with this method. Also, recent studies of the Shope rabbit papilloma and Rous chicken sarcoma in vivo (6) have demonstrated the characteristic distribution of specific viral antigens in the tumor cells of the natural host.

In the following studies, the fluorescent antibody method was employed in a search for specific viral antigens in the bovine papilloma.

MATERIALS AND METHODS

**Antigen.**—Extracts of bovine papillomas containing infectious virus, as established by their ability to produce papillomas on the skin of test calves, were used as antigens. The extracts were prepared from a 15–20 per cent suspension of ground bovine warts in 0.15 M NaCl. This suspension was first centrifuged at 480 × g for 20 minutes in the cold to remove the coarse material, and then at 18,400 × g for 30 minutes. The sediment was discarded, and the supernatant fluid, which had an infectivity titer of $0.1 \times 10^{-5}$ to $10^{-6}$ intradermally on the skin of calves, comprised the antigen. It was stored in small aliquots at $-65^\circ$ C until used either for the immunization of calves or in complement-fixation tests.

**Antibody.**—Antisera were prepared by injection of the bovine wart extracts into calves. Two calves, 12 months old and recovered from experimentally induced papillomatosis, were immunized by three intramuscular injections, 10 days apart, of 10 ml of wart antigen emulsified with an equal volume of adjuvant (9 ml Bayol F and 1 ml Arlacel A). Sera collected 14 days following the last injection were used to prepare fluorescein conjugates. These sera had antibody titers of 1:128 determined in a complement-fixation test employing bovine papilloma antigen. Whether or not the complement-fixing antibody is the one responsible for specific fluorescence, it appeared to be useful in selecting sera suitable for labeling with the dye: conjugates prepared from two normal sera lacking complement-fixing antibodies produced no fluorescence in sections of bovine papillomas, whereas conjugates made from post-immunization sera containing this antibody caused specific fluorescence in similar sections of the papillomas.

**Preparation of labeled globulin.**—Gamma-globulin was precipitated from the sera with 50 per cent saturated ammonium sulfate. After dialysis against 0.01 M phosphate-buffered saline, pH 7.2, the γ-globulin fractions were freeze-dried, then stored in a desiccator jar at 4°C. Conjugation of γ-globulin with fluorescein isothiocyanate (Sylvana) was carried out according to a method previously described (5). The final reaction mixture
contained 200 mg. of freeze-dried γ-globulin, 10 mg. of the dye, 18 ml. of buffered saline (0.01 M phosphate, 0.15 M NaCl, pH 7.2), and 2 ml. of 0.5 M carbonate-bicarbonate buffer, pH 9.0. The conjugate was dialyzed at 4°C for several days against phosphate-buffered saline, pH 7.2, to remove unconjugated dye and then stored in small aliquots at −65°C. Immediately before use, conjugates were twice absorbed with acetone-extracted bovine liver powder (100 mg. for each ml. of conjugate) (9). The liver powder was prepared from a pool of equal portions of liver taken from eight normal adult cows, with a view toward removing any intra-species tissue antibody that may have developed in the sera during immunization with the bovine wart preparations.

Staining procedure.—Bovine papillomas were produced by the intradermal injection of bovine wart material into calves. Ninety to 120 days after infection, the cauliflower-like growths, approximately 2–6 inches in diameter, were removed, and representative pieces were stored at −65°C. Frozen sections of the bovine papillomas were cut, air-dried, and used either the same day or within a few days, in which instance they were kept in a desiccator in the refrigerator. Immediately before being stained the sections were fixed for 10 minutes in acetone and then dried for an hour in a 37°C incubator. Fluorescein conjugates were applied for 30 minutes at 37°C. After being rinsed, the sections were mounted in buffered glycerol consisting of 3 parts of 0.01 M phosphate buffer, pH 8.0, and 7 parts of glycerol.

Frozen sections of several warts were stained directly with fluorescein-labeled conjugates prepared from each of the two post-immunization sera and from two normal bovine sera. The “blocking” control to determine inhibition of specific fluorescence was carried out by applying either the unconjugated antiserum or normal serum conjugate. Additional controls consisted of frozen sections of normal bovine skin stained with either the normal serum or immune serum conjugates. In each experiment, unstained sections were examined for autofluorescence. Subsequent to fluorescent observations, papilloma sections were rinsed in buffered saline and stained with hematoxylin and eosin.

Fluorescent microscope.—The ultraviolet light source was an Osram, type HBO-200 mercury arc bulb in a Reichert housing and used with a Spencer monocular microscope. A Corning U.V. exciter filter, No. 5480, and a Wratten K-2 excluding filter were used.

RESULTS

The morphologic structure of bovine papilloma, described elsewhere in detail (1), is illustrated in Figure 1. The hyperplastic epithelium consists of proliferating basal cells, an intermediate prickle-cell layer, the keratohyaline region, and the superficial cornified layer. Specific fluorescence was observed in nuclei of cells in the outer layers of the papilloma (Figs. 2, 4). When juxtaposed with photographs of the same fields (Figs. 1, 3) taken after the sections had been counter-stained with hematoxylin and eosin, the antigens of bovine papilloma virus were seen to be confined largely to nuclei in the keratohyaline and keratinized layers; occasionally, a fluorescent nucleus occurred in the deeper prickle cells, but was never seen in the proliferating basal cells or in the cells of the underlying connective tissue. No specific fluorescence was noted in the cytoplasm of any cell. The green nuclear fluorescence was observed in papilloma sections stained with conjugated antisera but not with conjugated normal sera and was completely inhibited by a prior application of unlabeled immune serum but not by unlabeled normal serum. The epithelium of normal bovine skin showed no fluorescence after being stained with either immune or normal serum conjugates absorbed with

Fig. 1.—A section of bovine papilloma stained with hematoxylin and eosin subsequent to staining with fluorescein-conjugated antiserum. The field is the same as the fluorescent field shown in Figure 2 and illustrates the morphologic structure of the papilloma. The hyperplastic epithelium is comprised of proliferating basal cells (A), keratohyaline region (B), and keratinized layer (C). Note correspondence of arrows to those in Figure 2. Mag. ×100.

Fig. 2.—The same field in the section of bovine papilloma shown in Figure 1 but stained with fluorescein-labeled antiserum. The discrete areas of bright fluorescence are confined to the keratohyaline and keratinized layers of the epithelium. The fluorescent nuclei indicated by arrows occur in the keratinizing region of the prickle-cell layer. Mag. ×100.

Fig. 3.—A section of bovine papilloma stained with hematoxylin and eosin following the fluorescent observations shown in Figure 4. Note the characteristic epithelial layers in the papilloma. Mag. ×100.

Fig. 4.—A fluorescence photomicrograph of the field of bovine papilloma illustrated in Figure 3. Fluorescing antigen is evident against the background autofluorescence of the cornified layer. Mag. ×100.

Fig. 5.—Fluorescence photomicrograph of a single nucleus, greatly enlarged, with the characteristic “mottled” appearance resulting from the irregular distribution of bovine papilloma antigen. The nucleus was in a cell in the keratohyaline region of the papilloma. Mag. ×3000.

Fig. 6.—Fluorescence photomicrograph illustrating the association of bovine papilloma antigens with nuclei and nuclear fragments in the cornified layer of the papilloma. Mag. ×450.
bovine liver powder.

In specifically stained sections of papilloma some vividly fluorescing nuclei were seen in the large, vacuolated cells in the outer region of the prick-cell layer (Fig. 2). Characteristically, the intensity of the fluorescence varied within the same nucleus, giving it a mottled appearance (Fig. 5). Antigens of bovine papilloma virus were often associated with the flattened, elongated nuclei and nuclear fragments in the cornified layer (Fig. 6). Autofluorescence in bovine papilloma sections was minimal and limited to the superficial, keratinized epithelial layer where it appears (Figs. 2, 4) as a uniform, grayish background fluorescence.

DISCUSSION

The characteristic distribution of bovine papilloma virus in the outer layers of the tumor is essentially similar to the findings in rabbit papilloma (7). Studies of the human wart virus with the electron microscope indicate that this virus, too, is present in the superficial, epithelial layers (8, 11). The similarity of these findings suggests a common pattern of viral development in the cutaneous papillomas of the three species. Noyes and Mellors (1957) postulated from their fluorescent antibody studies of rabbit papilloma that virus, which should be present in the germinal, basal cells to stimulate the characteristic epithelial hyperplasia of the wart, was there, but in some nonantigenic, incomplete form (possibly as viral nucleic acid) and, therefore, not detectable by fluorescent antibody. This hypothesis would seem to apply as well to the bovine papilloma.

The bovine papilloma, however, differs somewhat from the rabbit papilloma in that the connective tissue element is usually greater in the bovine wart. Further, under some conditions, a bovine tumor may be produced in which connective tissue comprises the larger part of the growth (1). This fibromatous type of growth is also characteristic of the tumor produced in the horse by bovine papilloma virus (9). The intracellular localization of bovine papilloma virus in tumors consisting predominantly of connective tissue is not yet known. In the present study no antigen of bovine papilloma virus was seen in the connective tissue cells of the bovine papillomas examined.

REFERENCES

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