Correlation of Immunofluorescence and Infectivity in the Developing Bovine Cutaneous Papilloma*

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SUMMARY

Bovine papilloma antigen detected by fluorescent antibody was correlated with the appearance of infectious virus and with epithelial hyperplasia and hyperkeratinization in the developing bovine cutaneous papilloma. Neither antigen nor infectious virus was found in the earliest stages of growth characterized by connective tissue proliferation and by little or no epithelial change. The cellular localization of papilloma virus antigen in nuclear material of the superficial epithelium was the same when first observed as it was in the well developed wart. Regression of the warts was accompanied by a decrease in specific antigen. Marked variations in susceptibility of individual calves to bovine papilloma virus were observed, as well as differences in the histologic development of warts produced by virus administered intradermally and by scarification.

Specific antigen has been demonstrated in the transmissible bovine cutaneous papilloma by the fluorescent antibody method (14). This antigen was localized in the keratohyaline and keratinized regions of the hyperplastic epithelium, but not in the germinal basal cells—a distribution similar to that found in the Shope rabbit papilloma (10) and the human cutaneous wart (9, 15). Since these observations had been made on bovine warts in advanced stages of growth, it seemed of interest to attempt to follow the development of antigen in early stages of experimentally produced warts and to determine whether a correlation existed between infectivity and antigen detectable by fluorescent antibody.

MATERIALS AND METHODS

Antigen and antisera.—The preparation of bovine papilloma virus, a member of the papovavirus group (8), is described in detail elsewhere (14). The papilloma virus preparations were inoculated into calves, 1–2 months old, for the experimental production of skin warts and were also used for the production of immune bovine papilloma serum. The warts (isolate 277) used for most of the preparations were obtained from a cow with the natural disease. In an attempt to avoid possible difficulties in immunofluorescence from intraspecies antibodies, the same animal was hyperimmunized with the autologous wart antigen. Two intramuscular injections, 10 days apart, of 10 ml. of antigen emulsified with an equal volume of adjuvant (9 ml. Bayol F and 1 ml. Arlacel A) were given.

Serum collected 14 days following the last injection was used to prepare conjugates with fluorescein isothiocyanate. These autologous conjugates did not prove significantly superior to those prepared from sera from two immunized calves.

PREPARATION OF LABELED GLOBULINS

The separation of γ-globulin from immune sera was carried out on columns of DEAE-cellulose essentially according to the method of Levy and Sober (6). To eliminate the prolonged dialyses of the immune serum prior to DEAE-cellulose chromatography and of the final conjugated mixture for separation of free dye from conjugated γ-globulin, more rapid methods employing columns of Sephadex were devised by one of us (L.K.S.) and Dr. O. Smithies, and since they proved useful are described in detail below.

Column preparation.—For preparation of the different columns, both Sephadex G-25 and DEAE-cellulose (Selectacel-200) were first washed in a large volume of distilled water, allowed to settle, and the fine material was decanted. This process was repeated until all the material settled rapidly. All columns used were 18 cm. high and 3 cm. in diameter and with either Sephadex or DEAE were large enough to process easily 20–30 ml. of the various protein solutions applied. The column materials were added in the form of a slurry, allowed to settle but not to go dry. The DEAE column was packed gently with a rubber stopper fitted on a glass rod. All columns were washed with distilled water and then with appropriate

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buffers (see below). Before use, a disc of filter paper was placed on top of the material in the column to help insure a sharp 'front' when the protein solutions were applied.

**First Sephadex stage.**—Twenty ml. of immune papilloma serum, to which had been added 4 ml. of pH 5.9 buffer, 0.5 M in phosphate (Na), were carefully applied to a column of Sephadex G-25, which had been washed first with 100 ml. of 0.5 M phosphate buffer, pH 6.3, and then with 150–200 ml. of 0.015 M phosphate buffer, pH 6.3. This last buffer was used to elute the column, and 5-ml. fractions were collected. The fractions were tested for protein by applying a small drop of each to a strip of filter paper, drying, and immersing for 1–2 minutes in a solution of amido-black 10B dye (approximately 0.5 gm./100 ml. of decolorizing solution). The strip was blotted and decolorized by being soaked in several changes of a decolorizing solution of 50 volumes methanol, 50 volumes distilled water, and 10 volumes glacial acetic acid. Fractions which stained heavily for protein were pooled (and centrifuged, if necessary) for use in the later stages of the procedure. Usually the volume obtained was 2–2.5 times the volume of serum initially applied.

**DEAE-cellulose stage.**—The method of Levy and Sober (6) with minor modifications was used for the chromatographic separation of γ-globulin from the sera. The DEAE-cellulose column was first washed with 250 ml. of 0.5 M phosphate buffer, pH 6.3, and then with sufficient 0.015 M phosphate buffer (approximately 500 ml.) to maintain pH 6.3. Twenty to 30 ml. of pooled serum from the first Sephadex stage was applied to the column and eluted with 0.015 M phosphate buffer, pH 6.3. Thirty-five 5-ml. fractions were collected and tested for protein with Amido-black 10B dye as described in the first Sephadex stage. Electrophoretic analyses on starch gels showed that pure preparations of γ-globulin were almost always obtained from columns that had been carefully prepared.

**Conjugation stage.**—In general, the method of Marshall et al. (7) using fluorescein isothiocyanate was followed. Pooled γ-globulin was used directly in the following conjugation mixture: 18 ml. of the γ-globulin pool, 2.0 ml. of 0.5 M carbonate-bicarbonate buffer, pH 9.3, to bring the reaction mixture to pH 9.0, and 0.16 gm. NaCl. This mixture, chilled and maintained at 5°C, was stirred during the addition of 5 mg. of fluorescein isothiocyanate4 and for 12–16 hours thereafter.

**Second Sephadex stage.**—Unconjugated fluorescein isothiocyanate was removed on a Sephadex column previously washed with an isotonic eluting buffer (0.01 M phosphate, 0.15 M NaCl, pH 7.2). The free dye remained concentrated at the top of the column. The collected conjugated γ-globulin was stored in small aliquots at -65°C. Any precipitate present after thawing was removed by filtration through a Millipore filter in a Swinny type adapter.

Production of Bovine Warts: The papillomas to be examined were produced by injecting at multiple sites, on each of several calves, infectious bovine papilloma virus by intradermal (0.1 ml.) and by scarification (approximately 0.05 ml.) inoculation. Fourteen calves were given inoculations in four groups. Of these, five developed warts, and nine were completely resistant. In two groups, each consisting of two calves, all four animals were resistant. In a third group one of four calves, and in a fourth group four of six developed warts following inoculation. Biopsies were taken for study from four of the five calves in which papillomas had been initiated. The first biopsy was taken 4 weeks after inoculation, a time at which growth was usually first evident, and then from the same animal at 6, 8, 11, and 17 weeks. It was not always possible to obtain biopsies at every designated time period, for in some animals regression of the warts occurred before the end of the experimental period.

**Infectivity Tests of Biopsy Materials:** Portions of the successive biopsies, which had been stored at -65°C, were ground in a mortar and made into an approximately 10 per cent suspension in saline. Each suspension was then inoculated intradermally and by scarification of the skin of three test calves. No attempt was made to titrate the virus but only to determine whether the biopsy materials would initiate warts in the test calves. A control virus preparation of known infectivity was inoculated at the same time on all test calves. Not all biopsy materials were successfully tested for infectivity, since a number of test calves proved completely resistant, as demonstrated by the failure of the control virus preparation to induce papillomas. Sufficient biopsy material was usually not available to repeat the tests.

Staining Procedures: One part of the bovine papillomas was used for frozen sections. These were fixed in acetone and stained with immune conjugates. Following fluorescent observations these same sections were rinsed in buffered saline and then stained with hematoxylin and eosin.

The fluorescent staining procedure was carried out according to the method of Coons (5). The anti-bovine papilloma conjugates were sufficiently potent to be used at a dilution of at least 1:5 or 1:10, so that absorption with tissue powders was unnecessary to eliminate nonspecific background staining which sometimes occurred with more concentrated conjugates even when prepared from electrophoretically pure γ-globulin. Occasionally the specificity of staining was checked by twice absorbing immune conjugates with bovine liver or spleen powders. 'Blocking' controls with unlabeled normal and immune sera were also done. Fluorescence observations were made with a Corning U. V. exciter filter, No. 5480, and a Wratten K-2 excluding filter with an Osram, type HBO-200 mercury arc lamp.

**RESULTS**

Papillomas for study developed at both sites of inoculation in the four calves, showing that either route was equally effective in initiating growth. The intradermally produced warts, however, showed certain histologic differences and, in these animals, tended to regress more rapidly than those warts produced by scarification. Because of regression, it was possible to study a complete series of successive biopsies of intradermally produced warts over the 17-week experimental period in only one animal. The number of biopsies taken at various times and the results of examination of these with fluorescent antibody are...
shown in Table 1, along with the results of infectivity tests performed on some of these materials. The prominent histologic features of the developing cutaneous bovine papilloma, the appearance and localization of antigen detectable by fluorescent antibody, and the occurrence of infectious virus are presented according to time of biopsy.

**Four weeks.**—At both intradermal and scarified sites the first appearance of growth consisted largely of dense connective tissue proliferating in the dermis. In seven biopsies, only one from a scarified site showed a slight hyperplasia of the epithelium, though the cornified layer was not thickened (Fig. 1). Specific papilloma antigen was not detected by fluorescent antibody in any of these early biopsies. Tissues from three biopsies, including the one with the slight epithelial change, were not infectious for susceptible test calves.

**Six weeks.**—The papillomas were histologically similar to those of the 4-week stage, though the proliferation of connective tissue appeared to be greater. Two of the three papillomas produced by scarification showed some epithelial change; in one, this was only a slight hyperplasia, observed in only one area of the wart (Fig. 2); but in the other (which grossly was slightly roughened and horny) there was marked hyperplasia and thickening of the cornified layer. In some, but not all, of the sections of the latter papilloma there were a few specifically stained fluorescent nuclei and nuclear fragments in the keratinized region of the epithelium. Tissue from this papilloma was infectious for one of three susceptible test calves, suggesting the presence of a small quantity of infectious virus. Immune fluorescence was not observed in four other papillomas. Two of these were tested, but they did not initiate warts on calves.

**Eight weeks.**—Epithelial hyperplasia and hyperkeratinization were distinct in the three papillomas studied (Figs. 3 and 5). Fluorescent nuclear material was confined to scattered areas in the superficial epithelium in specifically stained sections (Figs. 4, and 6). The fluorescence was correlated with the presence of small amounts of infectious virus, since the three biopsy tissues initiated warts on some but not all of the test calves.

**Eleven weeks.**—The difference between the warts produced by virus administered intradermally and by scarification became most apparent at this time. The prominent features at the two scarified sites were a further increase in the thickness in the epithelium (Fig. 7) and an increase in specific fluorescence which was still confined to the superficial epithelium (Fig. 8). Connective tissue still comprised the major portion of the intradermally produced wart, and specific fluorescence in the epithelium was not appreciably increased. The three biopsy materials, however, were infectious in a 10⁻¹ dilution, but, since they were not titrated, it was not established that the greater amount of specific antigen seen in the two warts produced by scarification was associated with a larger quantity of infectious virus.

**Seventeen weeks.**—The findings were essentially the same as at 11 weeks, (Figs. 9, and 10), except that the warts produced by intradermal inoculation were regressing and the amount of specific fluorescence was correspondingly decreased. The three biopsy tissues were still infectious for calves.

**Twenty weeks.**—At this time regression of warts produced by intradermal inoculation was complete and was also occurring at the scarified sites. No biopsies were taken.

### DISCUSSION

These investigations establish that papilloma virus capable of producing warts in calves is not found in the early stages of growth but only after considerable epithelial hyperplasia has occurred and that infectivity is associated with the appearance of specific nuclear antigen detectable by fluorescent antibody. The nuclear material is therefore probably complete virus. Further, it was found that the cellular localization of virus detected by fluorescent antibody did not vary and was the same at the time of its first appearance in early stages of growth as it was in the well developed wart (14). At no stage of growth was immunofluorescent material found in the proliferating basal cells or connective tissue. To account for the stimulation of connective tissue and epithelial proliferation seen in the early stages, in the absence of complete virus detectable by fluorescent antibody or by infectivity tests, it must be assumed either that the quantity of infectious virus providing the stimulus for proliferation is too small to be detectable by the two methods employed or that the stimulus is something other than complete virus. In any case, the pattern for production of complete virus must persist, at least in some cells, throughout these early stages, since in later stages identifiable virus appears in older cells of the proliferating epithelium.

Throughout this study the biopsy tissues and the control
virus preparations exhibiting the most immune fluores-
cence initiated warts on test calves in shorter periods of
time than were required for those materials containing
smaller amounts of specifically stained papilloma antigen.
Since several successive biopsy tissues were tested on
the same calves, the differences in the length of incubation
periods were independent of variations in susceptibility
between individual calves and, hence, may provide a crude
measure of comparative amounts of virus in different
inocula. For example, tissue from the 11-week, intra-
dermally produced wart, which showed less immune
fluorescence than did the corresponding wart produced by
scarification, initiated warts in three of three test calves,
but in these the incubation period was 2–4 weeks longer
than with tissue from the wart produced by scarification.
Decreases in latent periods related to increased dosage
have also been reported for the bovine papilloma virus in
the skin of the horse (13), for the Shope rabbit papilloma
(3), and the Rous chicken sarcoma (2) viruses.

Differences in the response of calves to papilloma virus
administered by different routes were noticed during this
study; similar findings have been reported by others.
(1, 4). In the intradermally produced warts a stimulation
of connective tissue, observed in the first biopsy, usually
remained a prominent feature in all subsequent biopsies,
whereas in warts produced by scarification hyperplasia
occurred in the epithelium at an earlier time and was
accompanied by specific antigen production in the super-
ficial epithelial cells.

Marked variations in susceptibility of young calves com-
plex studies of experimental bovine papillomatosis, and
the experiments described above, therefore, are not as-
sumed to be representative in terms of the exact time of
appearance of the various changes, but only as regards the
general features of development. In these studies only
five of fourteen calves developed warts following the
injection of infectious virus. Whether this resistance is
dependent on specific antibody acquired through previous
exposure or, in very young calves, by passive colostral
transfer is not known.

The ultimate size which a papilloma attains and the time
when regression of a wart commences are the two develop-
mental features exhibiting the greatest variability in
bovine cutaneous papillomatosis. In the experiments
described, the maximum diameter of all the experimentally
produced warts was never greater than 2–3 inches, and
regression had occurred in some warts as early as 2 months
and, in all, by 5 months after infection of the virus. It is
well known that, in some animals, naturally occurring or
experimentally produced warts may become very large and
persist for a much longer time (11). These variations may
be a manifestation either of a state of partial resistance of
the animal at the time of infection or of the development
of resistance during infection. They may also be in-
fluenced by such other factors as dosage, route of inocula-
tion, differences in virulence of virus strains, and the
 genetic background of the host.

Although dosage and route of inoculation have some
obvious effects on the development of warts, it is not at all
clear whether these are not, in fact, affected by the im-
 munological status of the animal. In the absence of a
satisfactory test for neutralizing antibody, it is difficult to
assess the role of specific antibody in the developmental
variations of the bovine wart. Antibody capable of
neutralizing infectious virus preparations is poorly devel-
oped by the bovine (12, 13). That considerable amounts
of an antibody may be present without noticeably sup-
pressing development was suggested by the observation
that serum taken from a cow with large, actively growing
warts contained sufficient antibody to prepare a usable,
specific fluorescein conjugate. After hyperimmunization
of this animal with infectious virus more potent conjugates
were possible, but even at this time regression of all the
warts had not commenced.4

The factors underlying resistance in bovine cutaneous
papillomatosis, therefore, remain obscure, as do the de-
velopmental events that may be suppressed or modified by
these factors. These aspects of bovine papillomatosis
warrant investigation for their possible bearing on suscepti-
bility and resistance of other species to tumor viruses.

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Fig. 1.—A section of a 4-week-old bovine papilloma (scarified inoculation) showing characteristic fibroplasia and minimal epithelial changes. No fluorescence was observed in this or other sections of the wart. H. & E., Mag. X100.

Fig. 2.—A section of a 6-week-old bovine papilloma (scarified inoculation) showing fibroplasia and a region of moderate epithelial hyperplasia with slight hyperkeratinization adjacent to normal appearing epithelium. Immunofluorescence was not observed. H. & E., Mag. X100.

Fig. 3.—A section of an 8-week-old wart (intradermal inoculation) showing epithelial hyperplasia and moderate thickening of the cornified layer. The field is the same as the fluorescent field shown in Fig. 4. H. & E., Mag. X100.

Fig. 4.—The field shown in Figure 3 but stained with fluorescein-labeled antiserum. A few fluorescent nuclei and nuclear fragments confined to the cornified layer are seen. Only a few areas in the wart contained fluorescent material. Mag. X100.

Fig. 5.—A section of an 8-week-old bovine wart (scarified inoculation) stained with hematoxylin and eosin following the fluorescent observations shown in Figure 6. Note the indipping of the cornified layer. Mag. X100.

Fig. 6.—A fluorescence photomicrograph of the field of bovine papilloma illustrated in Figure 5. Fluorescent material, limited to the cornified layer, was seen in only a few areas of the wart. Mag. X100.
FIG. 7.—A section of an 11-week-old bovine wart (scarified inoculation) stained with hematoxylin and eosin following immunofluorescent staining shown in Figure 8. Hyperplasia of the epithelium accompanied by extensive hyperkeratinization are seen throughout the wart. Mag. X100.

FIG. 8.—A fluorescence photomicrograph of the field in Figure 7, showing the distribution of fluorescent material in the keratoxyline and keratinized layers of the epithelium. Fluorescence was distinct throughout the wart in these layers. Mag. X100.

FIG. 9.—A section of a 17-week-old bovine papilloma (scarified inoculation) stained with hematoxylin and eosin following the fluorescent observations shown in Figure 10. Hyperkeratinization is prominent. Mag. X100.

FIG. 10.—The same field as shown in Figure 9 but stained with fluorescein-labeled antiserum. Fluorescent nuclei and nuclear fragments are present in the keratinized region. This characteristic distribution of fluorescence was prominent in all sections of the wart. Mag. X100.
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