

## An Immunopathologic Study of the Bovine Prepuce

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**Abstract.** The prepuces of 83 bulls with macroscopically normal reproductive tracts were obtained at slaughter and microbiological, immunological, and histologic studies were done and the findings were correlated. Some bulls had been vaccinated on several occasions against *Campylobacter fetus*.

Mean concentrations of intrapreputial immunoglobulins (Ig) in 27 bulls were IgG<sub>1</sub> - 1.8 ± 5.2; IgA - 0.16 ± 0.15; and IgM - 0.24 ± 0.24 mg/ml. High concentrations of IgG<sub>2</sub> in some bulls precluded precise estimation but mean concentration was in excess of 11.0 mg/ml (range 0 to 20+ mg/ml). Differences between these concentrations were significant (P < 0.005).

Mean prevalences of class specific, immunoperoxidase-labeled plasma cells in the preputial dermis of 35 bulls were IgG - 39.0 ± 9.3; IgA - 16.6 ± 6.6; and IgM - 2.2 ± 1.8 labeled cells/100 nuclei (P < 0.001). The prevalence of IgG labeled cells in the preputial dermis was, however, negatively correlated with the concentration of intrapreputial IgG (IgG<sub>1</sub> + IgG<sub>2</sub>) (r = -0.4; P < 0.05).

Except for an apparently lower intrapreputial Ig concentration in *Trichomonas foetus*-infected bulls than in negative ones, there were no significant correlations between intrapreputial immunoglobulin concentration, histologic findings, and age, infection, or vaccination status of the bulls.

Plasma cells frequently have been observed in apparently normal male and female genitalia [18, 28, 41] and are believed to increase as a result of repeated infectious challenge resulting in chronic inflammation [37, 47]. In the bull, an increase in plasma cells in the preputial and penile epithelium appears to be associated with age and is considered to result from repeated infectious challenge [6, 33]. A similar observation has been made in the uterus [41].

Many different organisms may localize in the prepuce of the bull [3, 20, 25, 29, 38]. Some infections generate a local immune response [2, 17] and differences in ratios of antibody classes in the prepuce as compared to other organs and serum [11, 15] suggest that there must be a local mechanism for the production of protective local immunity. Furthermore, vaccination against campylobacteriosis in bulls [7] and heifers [14] is an effective method of both curing and controlling the disease.

It is postulated that the antibodies in the preputial cavity probably are produced

by plasma cells in the dermis of the prepuce and penis, and gain access to the preputial cavity by diffusing across the nonsecretory epithelial lining thus clearing infection from animals, and protecting vaccinated animals from infection. This study was designed to demonstrate any link between the presence of plasma cells in the prepuce, intrapreputial immunoglobulins and vaccination, or infection status of bulls.

## Materials and Methods

### Experimental animals

Eighty-three *Bos indicus* crossbred bulls, 12 to 84 months of age, originating from seven herds throughout northern Queensland were used in this study. Fifteen of the bulls, 40 months of age, came from a closed herd and had been vaccinated one year, two months, and one month prior to slaughter with a formalin-killed suspension of *Campylobacter fetus* Type 1 and Subtype 1 plus mineral oil adjuvant (Vibrovax-Bivalent, Commonwealth Serum Laboratories, Melbourne, Australia). Genitalia from all bulls were examined grossly and microscopically, and bulls with lesions were discarded.

Entire reproductive tracts were collected at the time of slaughter in the abattoirs, and they were identified individually. The bulls were aged at slaughter by dentition [32]. The penis of each bull was cut dorsal to the sigmoid flexure and was removed from the ventral abdominal wall with the prepuce and contiguous skin. It was placed inside a sealed plastic bag, packed with ice, and was maintained at 4°C until processing within five hours.

### Sampling procedures

At the laboratory, preputial hairs were clipped from around the orifice and the penis was extended fully from the prepuce by grasping the glans with "rat-tooth" forceps. Bacterial swabs were taken immediately from the penis and prepuce in the area of the fornix.

Scrapings of the preputial epithelium obtained by using a scalpel blade on the median raphe area of the everted penis and preputial sac were transferred immediately to 0.85% saline at room temperature. After approximately 20 minutes, one ml of the saline containing the scrapings was withdrawn from 3 mm below the surface and transferred to *Campylobacter* enrichment medium at 4°C [12]. The remainder was transferred to modified Plastring's medium [16] and maintained at room temperature for up to five hours.

The penis was returned to within the prepuce and 2 to 3 ml of phosphate buffered saline containing sodium azide 0.01% as a bacteriostat was infused into the preputial cavity. After massaging for 15 to 30 seconds, the preputial washings were aspirated into a sterile syringe, transferred to a sterile vial, immediately snap frozen to -70°C and stored at -55°C. Samples that were visibly contaminated with feces or blood were discarded.

Triplicate samples of preputial and penile epithelium approximately 1-cm square were taken from close to the fornix (avoiding the previously scraped median raphe area) and placed respectively in Carnoy's fluid [44], absolute alcohol at 4°C [39], and phosphate buffered saline at 4°C [9]. The left ampulla and epididymis from each bull were sectioned and blocks were collected into Carnoy's fluid.

### Microbiology

Specimens in Plastring's medium or *Campylobacter* medium were processed and examined for *Trichomonas foetus* and *C. fetus* subsp. *fetus* using procedures previously described [16, 21]. Bacterial swabs were cultured for aerobes and anaerobes. Identification of the isolates to generic and species level was based on biochemical tests.

### Assessment of intrapreputial immunoglobulins

Preputial washings from the 27 bulls processed as above were thawed in a waterbath at 37°C, centrifuged in a refrigerated centrifuge at 1500 rpm for ten minutes at 4 to 8°C, refrozen to -55°C, and maintained at that temperature until examined.

The centrifuged washings were thawed at 37°C, and filtered through 0.2- $\mu$ m pore-sized disposable filters (Acrodisc<sup>R</sup>-Gelman Clemco, Sydney, Australia). The samples were screened for the presence of IgG<sub>1</sub>, IgG<sub>2</sub>, IgA, and IgM by immunodiffusion using monospecific anti-bovine immunoglobulin antisera (Miles Yeda, Rehovot, Israel) [36]. Concentrations of IgG<sub>1</sub>, IgG<sub>2</sub>, IgA, and IgM were measured using the single radial immunodiffusion technique with monospecific antisera [22, 31]. For IgG<sub>2</sub>, values in excess of 20 mg/ml were recorded as 20 mg/ml of preputial washing.

### Histology

Blocks of preputial and penile epithelium, ampulla and epididymis fixed in Carnoy's fluid, were trimmed to 3 mm, placed in 70% ethyl alcohol, dehydrated through ascending grades of alcohol, cleared in xylene, impregnated with paraffin wax (Paraplast<sup>R</sup>-Sherwood Medical, St Louis, Mo., U.S.A.), and embedded. Sections were cut to 6  $\mu$ m and stained with hematoxylin and eosin (HE); some also were stained with alcian blue and methyl green-pyronin. HE-stained sections were examined for the presence of neutrophils and intraepithelial lymphocytes ("lymphoepithelium"), mast cells, globule leukocytes, epidermal plasma cells, and lymphoid follicles, and for the quantitation of dermal plasma cells (scored 0 to 4, where approximate actual numbers of plasma cells were 0 = 0 cells per high power field, 1 = 10, 2 = 30, 3 = 70, 4 = 100). Alcian blue and methyl green-pyronin stained sections were used to confirm the presence of mast cells and epidermal plasma cells [8].

### Immunohistology

Blocks of preputial and penile epithelium from 35 bulls were fixed in cold ethanol [39] or washed in phosphate buffered saline at 4°C for 48 hours [9], then trimmed to 3 mm, dehydrated in alcohol at 4°C, and cleared in xylene at 4°C. The tissues were impregnated with paraffin wax, processed as above, embedded, and kept at 4°C until immunostaining.

Because of several inherent advantages over immunofluorescent methods [43], the immunoperoxidase method of staining was used by the indirect (sandwich) technique [34].

Sections were cut at 6  $\mu$ m and mounted on glass microscope slides using glycerin:albumin as an adhesive, and dried for 10 to 18 hours in a 60°C incubator. Sections then were cooled to room temperature before dewaxing in two baths of xylene and passing through three baths of alcohol to water. After two minutes in tap water, and five minutes in distilled water [4], the slides were dried and flooded with serum of an unrelated species (sheep) for 20 minutes [10]. After washing three times in phosphate buffered saline and drying, specific rabbit anti-bovine IgA, IgG, or IgM (Miles Research Products, Elkhart, Indiana, U.S.A.) were applied to the sections for three hours at room temperature and 15 to 18 hours at 4°C at dilutions predetermined by lattice titration against peroxidase-linked goat anti-rabbit antiserum (Miles Yeda, Rehovot, Israel). After washing in phosphate buffered saline, draining and wiping, the peroxidase-linked goat anti-rabbit serum was applied for 1½ hours at room temperature, again at the dilutions predetermined by the lattice titration.

The slides were washed again in phosphate buffered saline, drained, wiped, and then they were stained with freshly mixed and filtered diaminobenzidine/hydrogen peroxide for one to ten minutes. After washing twice with distilled water, 0.2% toluidine blue in 60% ethyl alcohol was applied as a counterstain [45], and the sections were passed through three baths of alcohol,

two baths of xylene, and mounted in a neutral synthetic medium (DePex<sup>R</sup>—Hopkin and Williams, Chadwell Heath, U.K.) under a glass coverslip for microscopic examination.

Cells strongly stained by peroxidase polymerized diaminobenzidine were considered to contain a specific immunoglobulin. Only immunoglobulin-containing cells in the dermal papillae, between the rete pegs, were counted, and cell numbers were expressed as a percentage of the total number of cell nuclei counted in the same fields.

Specificity tests undertaken by the commercial suppliers of the antisera were accepted. Specificity controls of peroxidase labeling procedures were undertaken by replacing rabbit anti-bovine IgG/IgA/IgM antiserum with phosphate buffered saline; replacing goat anti-rabbit peroxidase labeled antiserum with phosphate buffered saline; replacing rabbit anti-bovine IgG/IgA/IgM antiserum and goat anti-rabbit peroxidase labeled antiserum with phosphate buffered saline; applying unlabeled goat anti-rabbit antiserum (Miles Yeda, Rehovot, Israel) before applying peroxidase labeled goat anti-rabbit antiserum; and applying pig anti-bovine IgG antiserum (Biogenes, Pinner, U.K.) before applying rabbit anti-bovine IgG antiserum [24].

Lack of staining of plasma cells with diaminobenzidine following application of each of these procedures indicated that the plasma cells stained by the immunoperoxidase method had contained and/or been coated with the specific immunoglobulin.

### Statistical methods

Data were ranked for all analyses involving IgG<sub>2</sub> concentrations. Friedman's method for randomized blocks [42] permitted analysis of variance of Ig concentrations. Using the Statistical Package for the Social Sciences computer program [35], Spearman's rank order correlation, and the Kruskal-Wallis one-way analysis of variance tested for relationships between IgG<sub>2</sub> or total immunoglobulin levels and other parameters, while the Student's *t*-test and analysis of variance (multiple classification) tested for relationships between presence of neutrophils in the epithelium and total plasma cell count with campylobacteriosis, trichomoniasis, other bacterial infections and age.

## Results

### Microbiological studies

Of the 83 bulls examined, *T. foetus* and *C. fetus* were isolated from 14 and four prepuces, respectively. Other recognized bacterial pathogens were isolated from five of the prepuces. These included isolation of *Corynebacterium pyogenes* from four bulls and *Pseudomonas aeruginosa* from one bull. The remaining samples yielded no isolates (five bulls), or commensals only (73 bulls). These commensals included streptococci (isolated from 64 prepuces), micrococccaceae (58), *Bacillus* sp. (47), corynebacteria other than *C. pyogenes* (42), coliforms (38), *Bacteroides* sp. (21), *Vibrio* sp. (11), clostridia (10), *Acinetobacter* sp. (5), *Streptomyces/Nocardia* sp., and pseudomonads other than *P. aeruginosa* (each from four bulls), as well as single isolations of a *Pasteurella* sp., and *Achromobacter* sp. Three of the four bulls with *C. fetus* infection also were infected with *C. pyogenes* and one also was infected with *T. foetus*.

### Intrapreputial immunoglobulins

The predominant immunoglobulin present in 27 bulls was IgG<sub>2</sub> which was at least five times more prevalent than any other immunoglobulin measured. Concentrations

**Table I.** Mean concentration of immunoglobulins in preputial washings and prevalence of peroxidase-labeled cells in the preputial dermis of slaughtered bulls

	Number of bulls	IgG <sub>1</sub>	IgG <sub>2</sub>	IgA	IgM	Significance
Ig concentration (mg/ml)	27	1.77 ± 5.21 <sup>a</sup>	> 11.07 <sup>b</sup> ± 9.30	0.16 ± 0.15	0.24 ± 0.24	p < 0.005
Ig-containing cells (Labeled cells per 100 nuclei)	35		39.00 <sup>c</sup> ± 9.30	16.64 ± 6.56	2.23 ± 1.80	p < 0.001

<sup>a</sup> Mean ± standard deviation.

<sup>b</sup> In 11 animals intrapreputial IgG<sub>2</sub> concentrations in excess of 20 mg/ml were recorded as 20 mg/ml.

<sup>c</sup> IgG<sub>1</sub> and IgG<sub>2</sub> containing cells were both stained as IgG.

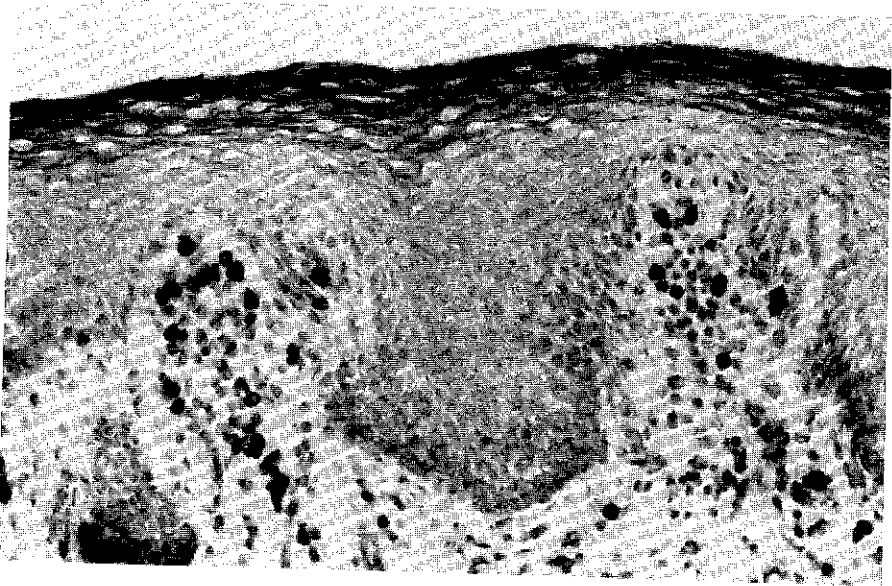


Fig. 1: Section of preputial epithelium with darkly staining IgA-containing cells in the dermis, especially in the dermal papillae; non-specific labeling of superficial epidermis. Immunoperoxidase-toluidine blue.

of immunoglobulin in different washings, and especially IgG<sub>2</sub>, were, however, quite variable (table I).

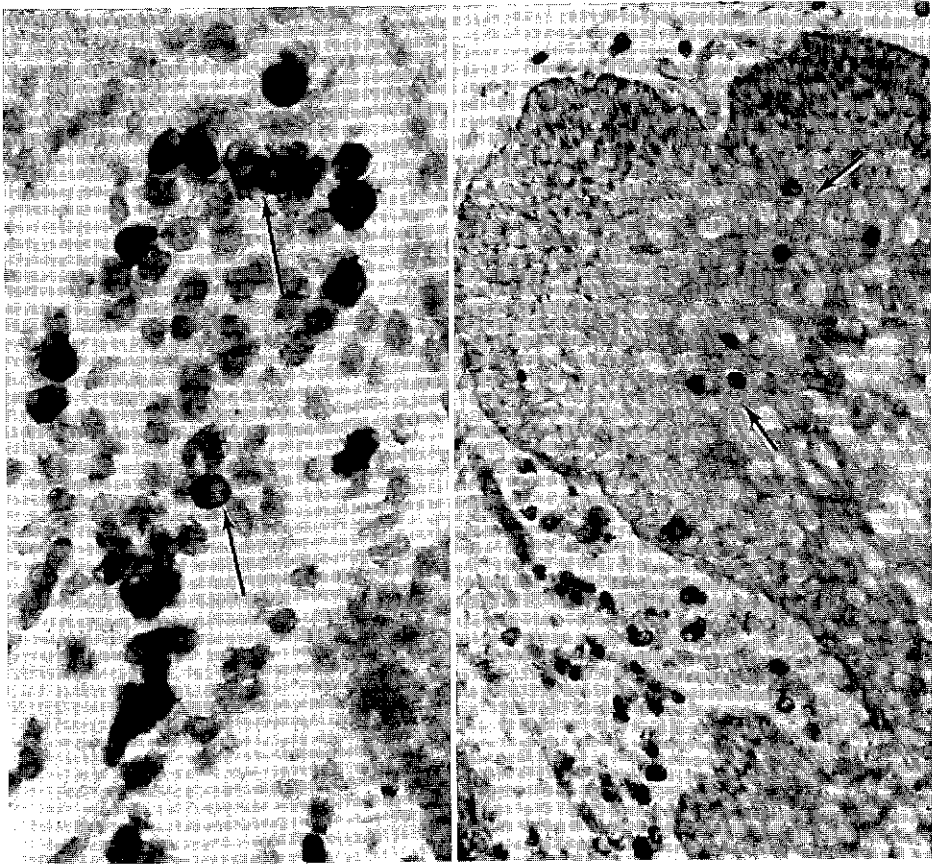
Total immunoglobulin levels were not correlated with age of bulls, presence of *C. fetus*, or total plasma cell estimates in routinely stained histological sections. It appeared, however, to be related to infection status in regard to *T. foetus*: being greatest in bulls from noninfected herds, less in noninfected bulls from infected herds, and least in bulls infected with *T. foetus* ( $p < 0.05$ ). The concentration of intrapreputial IgG (IgG<sub>1</sub> plus IgG<sub>2</sub>) was, however, negatively correlated with IgG-labeled cells in preputial dermis ( $r = -0.4$ ;  $p < 0.05$ ).

#### Immunohistochemical studies

In the preputial dermis, IgG-containing plasma cells were the most numerous. There were approximately half as many IgA-containing cells, and few IgM-containing cells (table I). The labeled plasma cells were concentrated primarily in the dermal papillae close to the epidermis (fig. 1, 2), although in prepuces that were heavily infiltrated with plasma cells they also were common in the deeper layers. Plasma cells containing IgA, IgG, and IgM also were noted in the epidermis of some bulls (fig. 3).

#### Histologic findings

In addition to plasma cells, other mononuclear cells including large and small lymphocytes, mast cells, neutrophils, large cells with an irregular dense nucleus and



**Fig. 2:** Higher magnification of fig. 1 with intracytoplasmic distribution of immunoglobulin in some cells (arrows). Immunoperoxidase-toluidine blue.

**Fig. 3:** Preputial epithelium with occasional labeled IgA-containing cells in epidermis (arrows) as well as dermis. Immunoperoxidase-toluidine blue.

large intracytoplasmic eosinophilic globules resembling globule leukocytes, and lymphoid follicles frequently were present in the preputial dermis. Mononuclear cells, neutrophils, and cells resembling globule leukocytes also were seen, although much less frequently, in the epidermis (fig. 4, 5). The cytoplasm of cells resembling intraepidermal plasma cells were stained positively by the alcian blue methyl green-pyronin method for ribonucleic acid. No neutrophilic infiltrates were related to the recognized pathogens isolated.

The relative frequency of these findings is summarized in table II. The intensity of each of these changes varied considerably between bulls. The mean dermal plasma cell "score" for all bulls was 2.7 (approximately equivalent to 55 cells per high power field), and although such features as dermal lymphoid follicles, marked lymphoid infiltration of epithelium overlying these follicles ("lymphoepithelium"—see fig. 4),

**Table II.** Occurrence of microscopic findings in the prepuces of 83 slaughtered bulls

	Bulls with finding (%)
Plasma cells—Epidermal	66
—Dermal	100
Dermal lymphoid follicles	73
Intraepithelial lymphocytes ("lymphoepithelium")	71
Mast cells	100
Globule leukocytes—Epidermal	5
—Dermal	96
Intraepidermal neutrophils	70

and intraepidermal neutrophils each were observed in the majority of prepuces (table II), they were prominent only in a few bulls. Likewise globule leukocytes, though present in most bulls, were seen usually as solitary cells scattered in or near the basal layer.

No correlations or trends were found in regard to preputial isolates with neutrophils, lymphoid follicles, or globule leukocytes. Although not significant, plasma cell numbers increased with age, *C. fetus*-infection, *T. foetus*-infection, and appeared to be greater following vaccination against *C. fetus* (table III). It also was noted that lymphoid follicles were approximately twice as prevalent in vaccinated bulls as in unvaccinated bulls; in addition, fewer preputial mast cells were seen in older bulls with mean prevalences of 4.3, 3.8, and 2.9 mast cells per high power field in young, mature, and old bulls, respectively.

### Discussion

This study revealed basic information about plasma cell populations, the immunoglobulin class specificity of plasma cells present, and the types of cells infiltrating the preputial integument of *Bos indicus* crossbred bulls. Useful information also was obtained concerning the immunoglobulin levels and microbial flora of the bovine prepuce.

Microbiological studies were designed to diagnose the presence of trichomoniasis and to establish the range of bacteria present so they could be related to immunopathological events to each bull. The frequent isolation of streptococci, micrococcaeae, and corynebacteria agrees with previous studies [38].

Apart from known pathogens, it is difficult to attach importance to the species of bacteria present in the bovine prepuce because of its potential for constant contamination. Perhaps some isolates, though nonpathogenic in the prepuce, should be considered potential pathogens. Recent work with rams has confirmed that the preputial flora ultimately may be important higher in the genital tract; when rams were stimulated hormonally, certain bacteria migrated up the genital tract causing seminal vesiculitis or epididymitis [26].



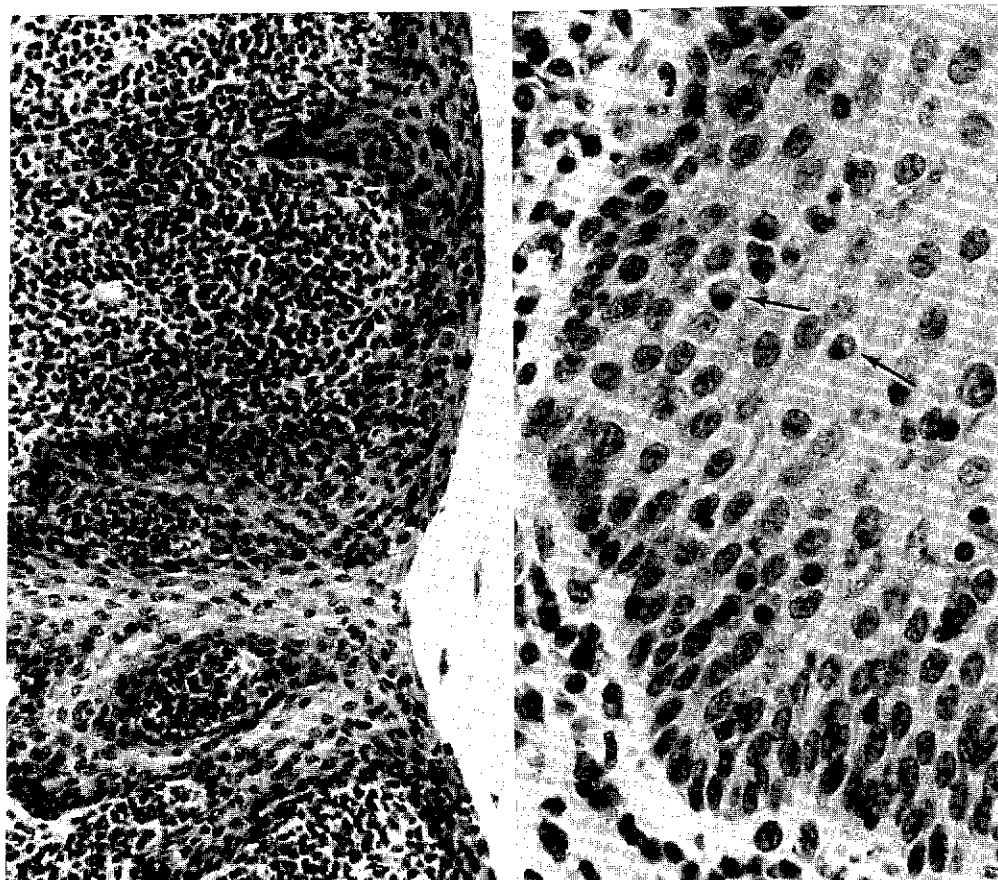


Fig. 4: Preputial epithelium with pronounced dermal accumulation of lymphoid and plasma cells and marked infiltration of lymphoid cells into overlying epidermis. HE.

Fig. 5: Preputial epidermis containing cells having morphology and staining characteristics of plasma cells with apparent Russell bodies in several cells (arrows). HE.

Both the mean total intrapreputial immunoglobulin concentration and variation in this concentration found in the present study, were considerably greater than those found previously; whereas we found total IgG<sub>1</sub> and IgG<sub>2</sub> levels of  $1.8 \pm 5.2$  and greater than 11.0 mg/ml, respectively, levels observed in older bulls in a previous study [5] were  $0.59 \pm 0.48$  (IgG<sub>1</sub>) and  $1.47 \pm 1.08$  mg/ml (IgG<sub>2</sub>), respectively. Although these differences may have been due partially to technique (variable recovery of infused saline; urine and/or ejaculate being deposited in the prepuce at slaughter), no obvious accumulations of urine or semen were observed in prepuces of these bulls or other bulls examined after slaughter [33]. Additionally, the high concentrations of IgG<sub>2</sub> we observed were unexpected, and it is conceivable that these resulted partially from post-mortem bacterial splitting of the immunoglobulin molecule between slaughter and freezing of preputial washings [48]—in spite of their being maintained at 4°C until freezing.

**Table III.** Preputial plasma cell prevalence relative to age, *C. fetus*, *T. foetus*, and vaccination status of bulls

Variable	Category	Bulls in each category (Number and %)	Plasma cells per high power field <sup>1,2</sup> (Mean number)
Age (years)	Young (1-3.5)	10 (12)	46
	Mature (3.5-7)	41 (51)	52
	Old (> 7)	30 (37)	64
<i>C. fetus</i>	Not infected	44 (54)	55
	Herd infected	33 (41)	58
	Bull infected	4 (5)	80
<i>T. foetus</i>	Not infected	33 (41)	56
	Herd infected	34 (42)	58
	Bull infected	14 (17)	61
Vaccination	Not vaccinated	66 (81)	56
	Vaccinated	15 (18)	64

<sup>1</sup> Least square constants.

<sup>2</sup> No significant differences between groups with regard to age, *C. fetus* or *T. foetus* infection, or vaccination status were found.

With due regard to these technical considerations, we believe that differences between this and the previous study [5, 6] were because of the greater diversity of bulls which we studied in regard to age, herd of origin, and infection status. Although it was not possible to establish a clear relationship between infection and intrapreputial immunoglobulin, the majority of bulls in this study were from herds infected with known preputial pathogens—*C. fetus* and *T. foetus*. Furthermore, previous studies have revealed that the majority of bulls in the herds are serologically positive to the virus of infectious bovine rhinotracheitis-infectious pustular vulvovaginitis [29].

It is difficult to explain why the total intrapreputial concentration was greater in noninfected bulls than in bulls from which *T. foetus* was isolated. In view of the diverse nature of the bulls studied, this finding might best be regarded with caution; comparable immunoglobulin studies in experimental *T. foetus* infection would resolve the question.

The concentration of labeled plasma cells in the dermal papillae close to the epidermis was anticipated from previous histological studies [6, 33] and immunofluorescent examination which revealed that IgG-containing cells predominated [30]. In the present study, IgG-containing plasma cells again were found to be the most common, as was expected because of the high levels of IgG that were found in the prepuce, both in this and previous studies [5, 6].

As IgG was the most prominent intrapreputial immunoglobulin, and IgG-containing cells the most prevalent, it seems contradictory that these features were negatively correlated; other than to suggest that many clones of subepithelial plasma cells simultaneously attain maturity (and therefore exhibit optimal labeling) and then

secrete their contained immunoglobulin (ultimately into the preputial lumen), no satisfactory explanation of this observed correlation can be offered.

In previous studies there was no attempt to specifically label immunoglobulin-containing cells. The present study therefore represents the first attempt to relate the presence of such cells to levels of intrapreputial immunoglobulin in the same bulls. It is interesting to note that the ratios of different immunoglobulin class cells present in this study and one other study using immunofluorescent labeling of plasma cells [30] were similar to the ratios of preputial antibody classes found previously [5]. The number of IgG-containing cells found here was approximately twice the number of IgA cells, which were in turn seven times more prevalent than IgM cells.

Though it seems logical that immunoglobulin secreted by the plasma cells under the epidermis would diffuse across the epidermis into the preputial cavity, the mechanism of immunoglobulin transfer in this location has not been elucidated. It is quite possible that under normal circumstances, some extracellular fluid might diffuse from the dermis into the preputial cavity via the intercellular spaces (depending on the nature of the intercellular junctions), taking with it immunoglobulin secreted by the plasma cells.

The high frequency of immunoglobulin-containing plasma cells actually located within the epidermis (fig. 3, 5) was a surprising finding, and it is interesting to speculate on their purpose. It seems most likely these cells would have entered the epidermis as motile immunoblasts [23], and subsequently differentiated into immunoglobulin producing cells in this location. Their presence may represent another mechanism whereby antibody gains access to the preputial lumen.

As with intrapreputial immunoglobulin levels, the trend for plasma cells to increase in the dermis in the presence of *C. fetus* infection was expected. However, the trend for plasma cell prevalence to increase with *T. foetus* infection was less convincing (table III). There does not appear to be a clear expression of local immunity to *T. foetus* in the male even though a recent report has shown that systemic response to vaccination may be effective in reducing the prevalence of *T. foetus* in young bulls [13]. *Trichomonas vaginalis* infection in humans elicits a local immune response in women but not in men, who continue to harbor the parasite [1].

The apparent increase in plasma cells and lymphoid follicles in the preputial dermis of bulls vaccinated against *C. fetus* may indicate that such parenteral vaccination induces the establishment of circulating lymphocytes which may then in some way recognize, and proliferate in response to *C. fetus* antigen in the prepuce. Although the vaccinated bulls were from a herd not infected with *C. fetus*, the related vibriolike organisms which frequently contaminate the prepuce could presumably initiate a "homing" response leading to dermal plasma cell infiltration and lymphoid follicle development.

The greatest concentration of epidermal inflammatory cells was found in the "lymphoepithelium" which was associated primarily with lymphoid follicles. As in tonsils, preputial "lymphoepithelium" seems to allow direct communication between

the lumen and the underlying lymphatic nodules and is probably an important factor in cell mediated immunity in infections such as infectious bovine rhinotracheitis-infectious pustular vulvovaginitis [19, 27]. The need for further studies to elucidate the role of cell mediated immunity at body surfaces has been stressed [46]. Although this study has provided some knowledge concerning immunopathology of the bovine prepuce, further correlative studies are needed using more uniform groups of bulls screened for all likely pathogens, and using artificially induced preputial infections and specifically labeled antigens [40] in a more precisely controlled environment.

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