

THE REACTION OF OVINE NEUTROPHILS TO *HISTOPHILUS OVIS* IN RELATION TO GENITAL INFECTION OF RAMS

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ABSTRACT

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Histophilus ovis was shown to be phagocytized by neutrophils when the organisms enter the lumen of the reproductive tract of the ram. The phagocytosis and destruction of *H. ovis* by neutrophils was demonstrated *in vitro* by the viable count method and by electron microscopy. It was shown that immunoglobulins and complement had no influence on the phagocytosis and destruction of *H. ovis*. Phagocytosis and killing of *H. ovis* was accomplished equally well by neutrophils from immunized and non-immunized rams. Immunized rams showed a massive infiltration of neutrophils into the walls, epithelium and lumen of their ampullae when dead *H. ovis* were introduced into their lumen.

INTRODUCTION

Dodd & Hartley (1955) isolated a gram-negative, bipolar staining, pleomorphic organism from lesions of suppurative epididymitis affecting young rams in New Zealand. Roberts (1956) suggested that this organism should be named *Histophilus ovis*. It subsequently proved to be an important pathogen in rams, occurring as it does in several sheepbreeding countries. Claxton & Everett (1966), for instance, recovered an organism resembling *H. ovis* in Australia from the genitalia of a ram showing clinical epididymitis. Van Tonder (1979) showed that *H. ovis*, which he included in his group referred to as *Actinobacillus seminis*, is also an important pathogen of rams in South Africa.

Although *H. ovis* plays an important role in the economy of sheep production, the relevant documented information covers only its description and the lesions caused by it, but little is known about aspects that can lead to the prevention and control of the infection. An investigation into the nature of the resistance to *H. ovis* therefore seemed justified and hence, as a start, the study reported in this article.

Jansen's (1980 b) conclusions that bacteria enter the genital tract of a ram through the prepuce and then migrate via the urethra to the accessory glands and further along the vas deferens to the epididymis and testis, served as a basis for investigating the immune mechanism operative on the surface of the epithelium of the genital tract and in the seminal fluid.

Since the presence of abundant neutrophils in the semen of rams with a bacterial infection of the genital tract is a common phenomenon, we chose to study the interaction between ovine neutrophils and *H. ovis*.

MATERIALS AND METHODS

Experimental animals

Twelve-month-old Merino rams were tested for freedom from bacterial infection of their genitalia with semen obtained through electro-ejaculation in as sterile a manner as possible. Smears were prepared from the semen, stained by the modified Stamp's method (Van Tonder, 1979) and with Giemsa's stain and examined for the presence of inflammatory cells and bacteria. Cultures were prepared on 10% horse blood tryptose-agar*** and incubated for 48 h in 10% CO₂ at 37 °C. The rams found free from bacterial infection of their genitalia except for their preputial cavity were kept intensively on regularly cleaned concrete floors and used for further experimentation.

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Bacterial strain and culture methods used

H. ovis strain 0782* was used for all the experimental work. It was grown on surface culture on 10% horse blood tryptose-agar in a 10% CO₂ atmosphere at 37 °C for 48 h.

Dead bacterial cells were prepared by washing the growth from the surface of a solid culture medium in a Petri dish with 5 ml of Hank's balanced salt solution (HBSS) (Kruse & Patterson, 1973), adding an equal volume of 10% formalin buffered at pH 7.0 and leaving the suspension at room temperature overnight. The organisms were then cleared of the unbound formalin by centrifugation and washing thrice with sterile HBSS.

The dead organisms were finally suspended in HBSS and the density of the suspension adjusted to give 50% light transmittance at a wavelength of 560 nm, using a Corning Colorimeter 252. This density was accepted as the equivalent of 1 unit. Their death was checked by culturing.

Immunization of sheep with H. ovis antigen

H. ovis was grown on surface culture in Petri dishes for 48 h. The growth was then washed from the surface with HBSS and the suspension adjusted to an opacity of 1 unit. Formalin was added to a final concentration of 0.3% and incubation continued for 48 h at 37 °C. Culture of the suspension showed that all the organisms were dead at this stage. Potassium alum was then added to a final concentration of 1% and the pH adjusted to 5.6. This constituted the final antigen suspension of which the sheep received 3 subcutaneous injections of 2 ml each at intervals of 30 days. Injected sheep were termed immune. Immune serum was obtained 7 days after the last injection.

Blood granulocytes derived from immunized and non-immunized rams will be designated immune and non-immune cells respectively in this text.

Surgical procedures

Dead bacterial suspensions in HBSS were introduced into the tubule of the epididymis by the method described by Jansen (1980 a).

For the introduction of dead bacterial suspensions into the intra-abdominal section of the vas deferens proximal to the accessory glands, laparotomy was performed under general intravenous chloral hydrate anaesthesia. The injection was done in the direction of the ampulla to imitate the natural flow of the semen through the ampulla. A No. 26 Luer-Lok hypodermic needle attached to a 1 ml tuberculin syringe was used and a volume of 0.2 ml was injected.

* Obtained from Dr E. M. van Tonder, Veterinary Investigation Centre, Middelburg 5900

The rams were killed 24 h after the operation and ampulla specimens were collected for histologic examination. The specimens were fixed in 10% formalin buffered at pH 7.0, and the sections were stained with haematoxylin-eosin.

In vitro phagocytosis of H. ovis by blood granulocytes

The *in vitro* determination of the phagocytosis and intracellular killing of *H. ovis* was done essentially according to the method described by Van Furth, Van Zwet & Leijh (1978). Granulocytes were isolated from the peripheral blood of sheep according to the method of Carlson & Kaneko (1973). The cells were suspended in HBSS and the density of the suspension adjusted to 0.65×10^6 cells per ml, as determined by means of a haemocytometer. About 80% of these were neutrophils. The viability of the granulocytes in the final suspension was about 90%, as measured by the trypan blue exclusion technique.

Glass tubes, measuring 100×15 mm and fitted with rubber stoppers, both items being siliconized, were used to contain the reaction mixtures consisting of the following ingredients in the volumes indicated. The total volume in each tube was 4.5 ml and where a particular ingredient was left out, as in the control tube for that ingredient, its volume was replaced with an equal volume of HBSS.

- (a) 1.0 ml of bacterial suspension. The organisms were washed from the surface of a 48 h culture of *H. ovis* and suspended in HBSS to a density of 0.3 absorbance unit. This suspension was diluted 1:100 for use. The number of organisms per tube in each test was determined by the viable count in the relevant control tube which contained only the bacteria and the foetal calf serum in the total volume of 4.5 ml.
- (b) 0.5 ml of foetal calf serum inactivated for 30 min at 56 °C.
- (c) 1.0 ml of either immune or non-immune fresh sheep serum. Where the effect of complement was investigated, the sera were inactivated for 30 min at 56 °C. Control tubes were included in which either the immune or the non-immune serum was left out.
- (d) 1.5 ml of granulocyte suspension.
- (e) 0.5 ml of a 1:10 dilution of fresh guinea-pig serum, where the effect of complement was investigated. In the control tube and in tests where complement was not required, 0.5 ml of HBSS replaced the guinea-pig serum.

The tubes were stoppered and gently shaken for 1 h in a horizontal position in an incubator room at 37 °C.

After incubation, the mixtures were centrifuged at 450 g for 4 min to deposit the granulocytes and the supernatant fluid was removed with sterile precautions. The deposited cells were disrupted by the addition to the tube of 4.5 ml of sterile distilled water at 4 °C. After 30 sec a 1 ml sample of the contents of the tube was appropriately diluted with HBSS to count the live bacteria released by the disruption of the cells (Van Furth *et al.*, 1978).

The viable organisms in the supernatant fluid and those adhering to the granulocytes and released by their disruption were counted by preparing serial tenfold dilutions in HBSS, distributing 0.1 ml of each dilution onto each of 3 blood tryptose-agar surfaces in Petri dishes and counting the colonies formed after 48 h incubation. The average of the counts on the 3 Petri dishes was used for calculating the number of viable organisms in the particular dilution.

Whether immune serum *per se* can have any lethal action on *H. ovis* was investigated by the viable count method by replacing all the ingredients except the organisms and the immune serum by HBSS. The number of live organisms was counted immediately after mixing and again after shaking in an incubator for 1 h at 37 °C.

The possibility of a lethal effect of immune serum during longer exposure of the organism to it was investigated by punching wells in blood tryptose-agar plates, filling the wells with immune serum on 3 occasions, allowing the serum to diffuse into the medium, and then spreading a diluted culture of *H. ovis* evenly over the surface of the medium and incubating it for 48 h. During the incubation the serum in the wells was replenished on 2 further occasions. At the end of the period of incubation the culture plates were examined for any signs of inhibition of growth around the wells.

Detection of specific immunoglobulin in serum of vaccinated sheep

An enzyme-linked, immunosorbent assay method, as described by Voller, Bidwell & Bartlett (1976), with minor modifications as indicated below, was used for the detection of IgG antibodies against *H. ovis* in the serum of vaccinated sheep. A lipopolisaccharide, prepared according to the method of Westphal & Jann (1965), was used as antigen. The hot phenol-water extraction procedure was applied to an *H. ovis* cell wall preparation for the extraction of the lipopolisaccharide. This antigen, made up in a 0.05 M carbonate-bicarbonate buffer at pH 9.6 and a concentration of 5 µg/ml, was distributed into the wells of a polivinyl chloride microtitre plate and left overnight at 4 °C.

The globulins were precipitated from sheep serum with a saturated ammonium sulphate solution according to the method of Herbert, Pelham & Pittman (1973). The IgG was subsequently isolated from the mixture of globulins by fractionation on a Sephacryl S-300* column. The concentration of IgG was determined spectrophotometrically and made up to a 1% solution in a 0.8% NaCl solution. It was stored at -20 °C.

Rabbits were immunized by an intramuscular injection of 1.0 mg IgG in complete Freund's adjuvant. The injection was repeated after 30 days and the rabbits bled 10 days later. The IgG was recovered from the rabbit serum by the same methods used to isolate the IgG from the sheep serum. The rabbit IgG was then linked to alkaline phosphatase Sigma type VII with specific activity 300-1100 U by the glutaraldehyde method (Avrameas, 1969).

Para-nitrophenyl phosphate Sigma 104 at a concentration of 1 mg/ml was used as substrate for the test.

One per cent bovine serum albumin was added to the serum diluent to reduce the non-specific adsorption of serum constituents to the plate.

Foetal calf serum was used as negative control. The incubation lasted for 2 h at 31 °C.

The substrate reaction was stopped after 30 min by the addition of 50 µl 3 M NaOH and the absorbances measured in a Titertek Multiskan**.

All wash procedures were carried out in a Multi-wash** apparatus.

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Electron microscopic studies

To study visually the phagocytosis and destruction of *H. ovis*, duplicate tubes to those set up for studying the effect of various factors on phagocytosis of *H. ovis* by blood granulocytes by the viable count method, were prepared.

After the tubes had been incubated, with shaking, for 30 min, they were centrifuged at 450 g for 4 min. The supernatant liquid was decanted and the pellet of granulocytes fixed in 4% glutaraldehyde freshly diluted in buffer (Millonig, 1961) at pH 7.4. Staining was done with a saturated aqueous solution of uranyl acetate for 30 min (Watson, 1958), and then with a 0.2% solution of lead citrate (Reynolds, 1963) for 5 min.

Statistical analysis of data

Where the effect of different treatments on the same factor was investigated, for example, phagocytosis in the presence of inactivated immune serum, inactivated immune serum plus complement and untreated immune serum, the t-test was applied to the results obtained.

For the analysis of the results obtained by investigating the effect of different factors, for example, phagocytosis by immune or non-immune granulocytes, or phagocytosis in the presence of immune or non-immune serum, the test described by Tukey (1960) was applied. For this purpose, the final bacterial counts were converted into logarithmic equivalents and then compared by an analysis of variance.

RESULTS

The response of rams to injections of dead *H. ovis*

An example of the titre of IgG antibodies obtained by means of the enzyme-linked immunosorbent assay in the sera of a sheep injected with killed *H. ovis* organisms is set out in Table 1. Foetal calf serum (FCS) was used as negative control.

TABLE 1 Enzyme-linked immunosorbent assay of IgG antibodies in the serum of a ram. Adsorbances read at 405 nm

Dilution series	FCS	Test serum
1/10	0,220	0,682
1/20	0,188	0,574
1/40	0,169	0,456
1/80	0,176	0,400
1/160	0,183	0,318
1/320	0,172	0,254
1/640	0,183	0,223
1/1280	0,181	0,199
1/2560	0,183	0,187

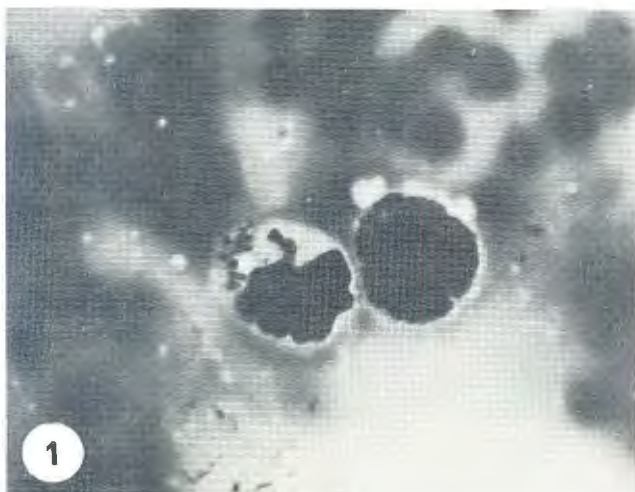


FIG. 1 *H. ovis* phagocytized by neutrophil: $\times 600$

These results leave no doubt that the serum of sheep injected with the whole cell antigen contained IgG antibodies against *H. ovis*. The serum containing IgG antibodies was designated immune serum although this was not interpreted as resistance to infection by the organism on the part of the donor of the serum.

No IgG antibodies could be detected by the enzyme-linked, immunosorbent assay in the semen of the rams showing a serum IgG antibody titre.

The fate of killed *H. ovis* in the epididymal tubule

Smears, prepared from the epididymis of a ram 24 h after the introduction of killed *H. ovis* via the vas deferens, gave ample evidence that the organisms were phagocytized by neutrophils (Fig. 1). Histological sections of the same tissue showed neutrophils migrating through the normal epithelium of the duct (Fig. 2). Neutrophils could be seen lying free in the lumen of the duct (Fig. 3). The mononuclear cells seen in the basal section of the epithelium were not more abundant than normal.

The phagocytosis of *H. ovis* by blood granulocytes in vitro

The results in Table 2 reflect the reduction in the number of organisms brought about by immune and non-immune granulocytes in the presence of immune serum. The control tubes in this test contained immune serum, but no granulocytes.

TABLE 2 Phagocytosis of *H. ovis* by immune and non-immune granulocytes in the presence of immune serum (viable bacterial count $\times 10^3$ per tube)

Test No.	Control	Immune leucocytes	Non-immune leucocytes
1	60,3	13,8	8,7
2	16,45	5,1	4,85
3	373,4	12,67	30,55
4	745,0	1,75	1,62
5	27,15	0,72	2,2

A statistical analysis of these data shows that immune granulocytes brought about a highly significant reduction in the number of bacteria ($P < 0,01$), as did non-immune granulocytes ($0,05 > P > 0,01$). There was no significant difference in the degree to which immune and non-immune granulocytes reduced the number of bacteria ($P > 0,05$).

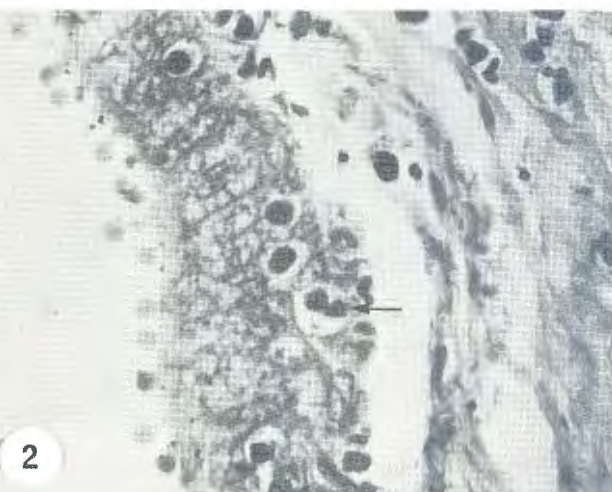


FIG. 2 Neutrophils migrating through epithelium of epididymal duct: $\times 450$

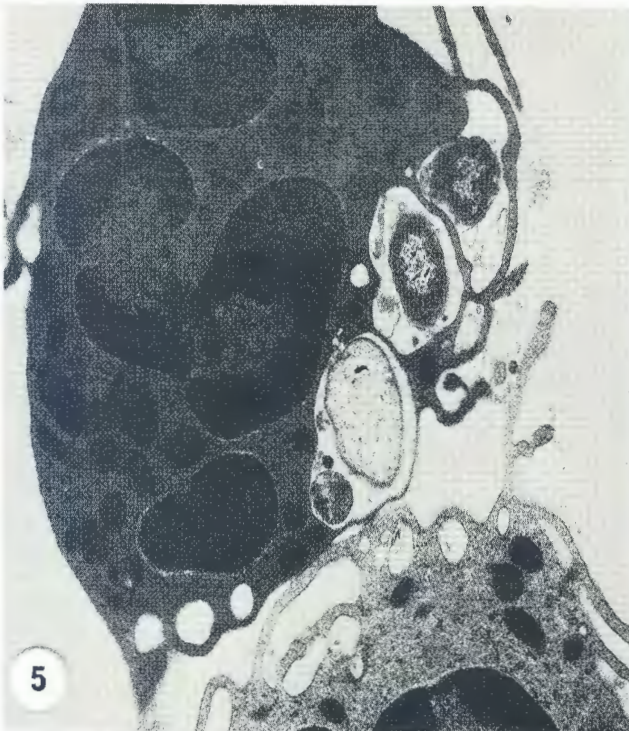
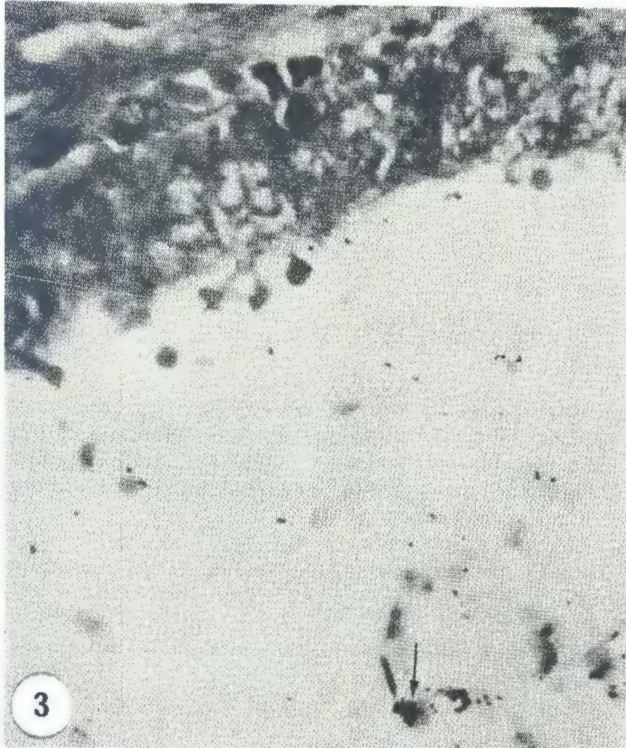


FIG. 3 Neutrophils lying free in lumen of duct: $\times 450$

FIG. 4 *H. ovis* engulfed by neutrophil: $\times 17\ 300$

FIG. 5 *H. ovis* engulfed by neutrophil: $\times 21\ 600$

FIG. 6 *H. ovis* showing signs of lysis inside neutrophil: $\times 33\ 200$

On the same basis the phagocytosis by immune and non-immune granulocytes in the presence of non-immune serum was investigated and the results recorded in Table 3. The control tubes in this test contained non-immune serum but no granulocytes.

TABLE 3 Phagocytosis of *H. ovis* by immune and non-immune granulocytes in the presence of non-immune serum (viable bacterial count $\times 10^3$ per tube)

Test No.	Control	Immune leucocytes	Non-immune leucocytes
1	74,5	1,7	6,4
2	84,1	7,8	2,4
3	161,1	7,2	11,5
4	538,5	11,5	12,2
5	273,5	45,9	77,7

A statistical analysis of these data shows that both immune and non-immune granulocytes brought about a highly significant reduction in the number of live *H. ovis* by phagocytosis ($P < 0,01$). There was no significant difference ($P > 0,05$) in the degree to which these 2 types of cells reduced the number of bacteria.

A statistical comparison of the results in Tables 2 and 3 shows that there was no significant difference ($P > 0,05$) between the effects of immune and non-immune serum.

The influence of complement on phagocytosis and the killing of *H. ovis* by blood granulocytes can be seen from the results recorded in Table 4. In the relevant experiment granulocytes from non-immune sheep were incubated with *H. ovis* in separate trials in the presence of immune serum, immune serum heated to 56 °C for 30 min, and inactivated immune serum plus 0,5 ml fresh guinea-pig serum. The control tubes contained no granulocytes.

TABLE 4 The influence of immune serum, inactivated immune serum and inactivated immune serum plus guinea-pig complement on the phagocytosis of *H. ovis* by granulocytes (viable bacterial count $\times 10^3$ per tube)

Key reagent	Test No.	Bacteria in control tube	Bacteria after phagocytosis
Immune serum	1	32,57	2,07
	2	68,50	17,00
	3	2 900,00	0,19
	4	431,67	3,07
	5	1 193,34	0,56
Inactivated immune serum	1	700,00	0,35
	2	40,00	1,58
	3	4 566,67	22,12
	4	1 273,34	3,31
Inactivated immune serum + complement	1	462,00	1,88
	2	703,50	94,12
	3	1 741,67	429,08
	4	866,67	66,28

A statistical analysis of these results shows that:

- (1) in the presence of immune serum a significant reduction in the number of viable bacteria is effected ($0,05 > P > 0,01$);
- (2) in the presence of inactivated immune serum a highly significant reduction is brought about ($P < 0,01$);
- (3) in the presence of inactivated immune serum plus complement a significant reduction is brought about ($0,05 > P > 0,01$); and
- (4) the degree to which the bacteria are destroyed in the presence of immune serum does not differ significantly from that to which the bacteria are destroyed in the presence of inactivated immune serum plus complement ($P > 0,05$).

It is clear, therefore, that complement does not play any decisive role in the bactericidal action of granulocytes on *H. ovis*.

The effect of immune serum on *H. ovis*

The direct effect of immune serum on the viability of *H. ovis* was investigated by exposure of the organisms to serum in tubes and counting the live organisms after 60 min incubation. The results are set out in Table 5.

TABLE 5 The effect of immune serum on the viability of *H. ovis* (viable bacterial count $\times 10^3$ per tube)

Exposure time (min)	Control tube	Immune serum
0	410	410
60	603	560

From these results it is clear that the immune serum had no significant bactericidal effect. This result was confirmed in the test where the organisms were allowed to grow on medium into which immune serum had been allowed to diffuse from wells punched in the medium. There was no inhibition of growth around the wells during the 48 h of incubation.

Phagocytosis of *H. ovis* in the presence of seminal fluid

To ascertain whether the phagocytosis and killing of *H. ovis* would proceed in the presence of seminal fluid, the following combinations were tested in the usual manner:

- (1) Organisms alone in HBSS.
- (2) Organisms plus 1 ml of sterile seminal fluid per tube.
- (3) Organisms plus seminal fluid plus granulocytes.

Duplicate tubes were prepared for electron microscopical examination.

A typical set of results is recorded in Table 6.

TABLE 6 The phagocytosis of *H. ovis* in the presence of seminal fluid

Medium	No. of organisms $\times 10^3$
HBSS	2 410
HBSS + seminal fluid	9 480
HBSS + seminal fluid + leucocytes	3 090

From these results it can be seen that the presence of seminal fluid in the medium stimulates the multiplication of the organisms but does not interfere with the phagocytic and bactericidal effect of the granulocytes.

This finding corresponds with the picture seen under the electron microscope (Fig. 4), where the organisms were freely phagocytized in the presence of semen.

Electron microscopical studies

Phagocytosis of *H. ovis* by granulocytes was evident in the preparations from all the different *in vitro* reaction mixtures.

In Fig. 4 & 5 the bacteria can be seen in the process of being engulfed by polymorphonuclear neutrophils. Some of the bacteria show signs of lysis, as can be more clearly seen from the higher magnification in Fig. 6.

No bacteria could be seen in any type of leucocyte other than neutrophils.

These findings signify that *H. ovis* is phagocytized by immune and non-immune neutrophils in the presence of immune and non-immune serum. This phagocytosis also proceeds in the absence of any serum and is not impaired by the presence of seminal fluid.

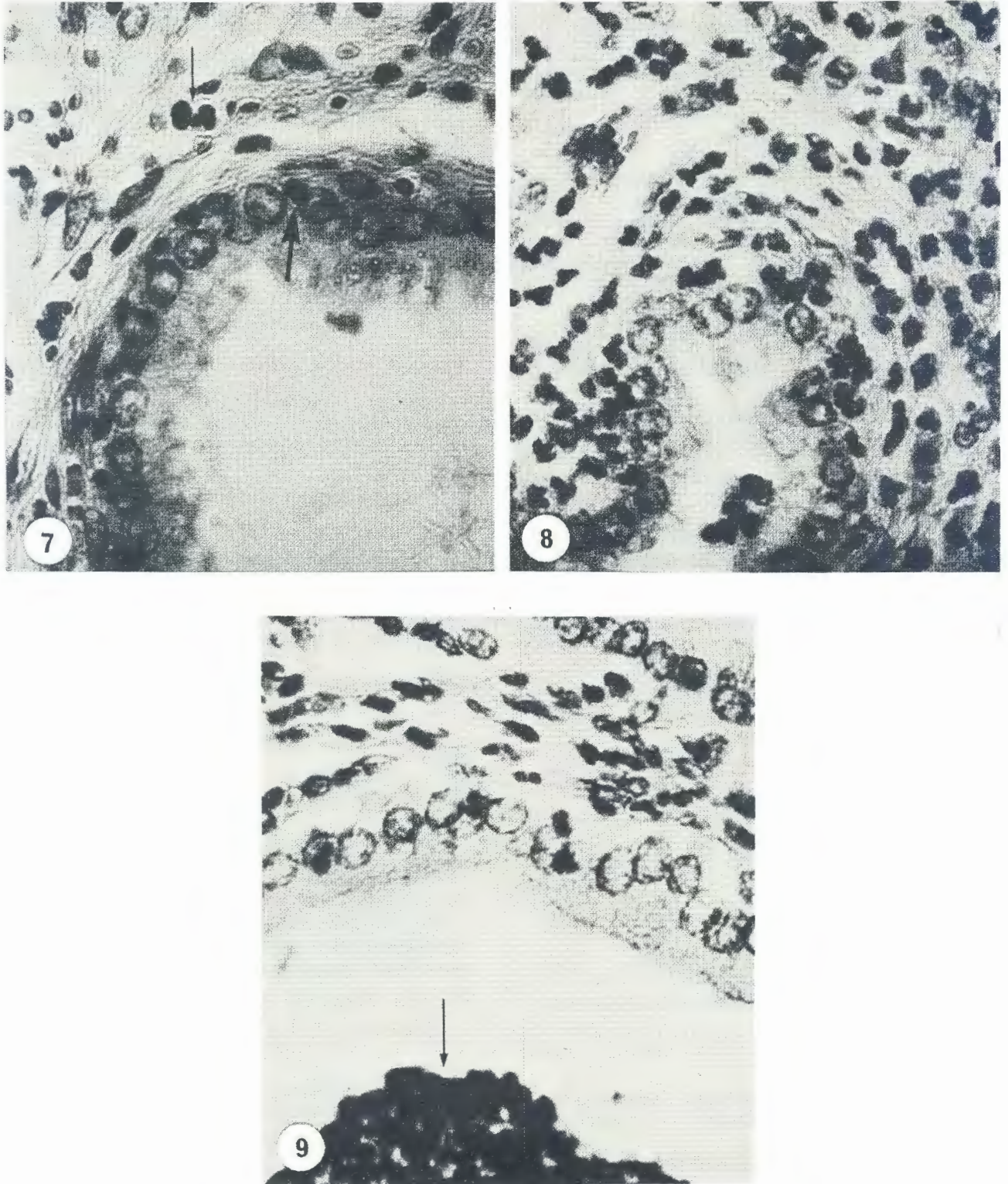


FIG. 7 Few isolated neutrophils in lamina propria of ampulla (small arrow). Single neutrophil migrating through normal epithelium (large arrow): $\times 1\ 200$

FIG. 8 Plenty of neutrophils in lamina propria and in epithelium of ampulla: $\times 1\ 200$

FIG. 9 Mass of neutrophils in lumen of ampulla: $\times 1\ 200$

The reaction of the ampulla to *H. ovis*

An injection of killed *H. ovis* into the abdominal section of the vas deferens of non-immunized rams, followed by their killing 24 h after the operation, produced the following histological picture in the ipsilateral ampulla: Only a few isolated neutrophils in the lamina propria and some migration through the epithelium at widely separated localities in the section. The epithelium appeared normal and no impression of any inflammatory reaction could be gained (Fig. 7).

The position was clearly different in 3 rams previously immunized against *H. ovis* and subsequently subjected to the same procedure. The histological sections from the ampulla ipsilateral to the injected vas deferens showed in its lamina propria accumulations of neutrophils among the cellular elements (Fig. 8). Numerous neutrophils migrated through the epithelium and accumulated in masses within the lumen of the ampulla (Fig. 9). The epithelial cells remained normal. Sections prepared from the ampulla contralateral to the injected vas deferens in the same animal showed a perfectly normal histological picture.

DISCUSSION

A major function of the neutrophil is the phagocytosis and destruction of invading micro-organisms. This function is performed in the genital tract by the neutrophils migrating through the epithelium lining the lumen of the tract to reach the bacteria migrating up the tract against the direction of flow of the semen. This migration of bacteria constitutes one of the essential elements in the pathogenesis of bacterial infections of the reproductive tracts of rams (Jansen, 1980 b). The migration of neutrophils through intact tubular epithelium can be seen in Fig. 2, and their presence in the lumen of the tubule in Fig. 3, in response to the injection of dead bacteria into the epididymal tubule.

The evidence for *in vivo* phagocytosis of *H. ovis* introduced into the lumen of the epididymal tubule given in Fig. 1 was confirmed in the *in vitro* situation and several of the factors influencing the process were elucidated. By exposing live *H. ovis* to blood granulocytes and, subsequently, by conducting viable counts, it was shown that immune and non-immune neutrophils performed equally well in phagocytizing and destroying the organisms. Neither immune nor non-immune serum effected any improvement in the performance of immune and non-immune cells. The presence of complement made no difference to the phagocytic and bactericidal effect of the neutrophils.

The action of the neutrophils being not impeded by seminal fluid means that the destruction of the bacteria can continue in the genital tract.

That the phagocytosis and destruction were real and were effected by neutrophils was proved conclusively by the electron microscopic studies. It could be seen clearly that the neutrophils performed this action, sometimes by incorporating clumps of bacteria into their cytoplasm.

An objection which might be raised against introducing the dead bacteria into the epididymal tubule via the vas deferens is that the operation wound and puncture of the vas deferens could obstruct the free normal flow of the contents of the tubule to the outside. It could be reasoned that the trapped foreign material would in any case elicit an influx of neutrophils and subsequent phagocytosis. This objection was overcome by introducing the bacteria into the abdominal section of the vas deferens and allowing them to flow through the ampulla along the normal course taken by the semen. There could thus be no obstruction to the flow of the material and the reaction of the body to it could be monitored.

By injecting dead *H. ovis* into the abdominal section of the vas deferens, it was shown that the non-immune ram responded rather poorly to the passage of the organisms through its ampulla by the appearance of a few neutrophils in the lamina propria and the epithelium of the ampulla. The immune animal, on the other hand, showed an intense reaction by the massive infiltration of neutrophils into the lamina propria and epithelium, forming large accumulations in the lumen of the ampulla.

The reason why dead organisms were injected into the tubule of the epididymis and the vas deferens was that we wanted to study the reaction of the neutrophils towards the bacteria without the possibility of the inflammation caused by live organisms complicating the picture.

By relating the experimental results to what presumably happens in nature, one can surmise that, if *H. ovis* enters the lumen of the urethra, accessory glands, vas deferens or of the tubulus epididymidis, some neutrophils would be attracted to them and migrate through the epithelium to phagocytize the bacteria and kill them. Neutrophils in the lumen of the genital tract are in effect outside the body and their reaction towards *H. ovis* can be compared with that of neutrophils in a test tube. The experimental results achieved show that neutrophils substantially reduce the number of viable *H. ovis* in their immediate environment by phagocytosis and destruction. The fact that the blood-testis and blood-epididymal barriers limit the ability of blood-borne macromolecules to enter the lumen of the tubules of the male reproductive tract (Setchell, Voglmayr & Waites, 1969; Howards, Jessee & Johnson, 1976; Setchell, 1967), and which was shown to apply to the IgG antibodies generated in the blood of rams by injections of *H. ovis* antigen, makes no difference in this instance. Phagocytosis and killing proceeded effectively in the absence of complement and antibodies.

It is doubtful whether the influx of neutrophils into the lumen of the genital tract would be sufficient to restrain the multiplication of the organisms entirely in a non-immunized animal. This doubt is reinforced by the results recorded in Table 5, which show that *H. ovis* can multiply rapidly in the environment provided by seminal fluid. If the destruction were sufficient, *H. ovis* would not be able to cause orchitis or epididymitis. The position is entirely different, however, in immunized animals which show a massive infiltration of neutrophils into the walls, epithelium and lumen of their ampullae when dead *H. ovis* are introduced into their lumen. Whether this reaction is adequate to protect a ram in nature, is the subject of a current investigation.

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