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IMMUNITY AGAINST GENITAL INFECTION BY *HISTOPHILUS OVIS* IN RAMS

B. C. JANSEN and MARIANNA HAYES, S.A. Wool Board, P.O. Box 1378, Pretoria 0001

ABSTRACT

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Rams have been immunized against an infection of their genitalia by *Histophilus ovis*. An alum-precipitated antigen and an antigen plus Freund's complete adjuvant proved equally effective.

An injection of live *H. ovis* into the epididymal tissue proved to be a better method of challenging immunity than an injection into the vas deferens.

It was shown that cell-mediated immunity, as evidenced by tests for lymphocyte transformation, the presence of a macrophage migration inhibition factor and a delayed hypersensitivity skin reaction did not play a role in the resistance, nor did specific IgG antibodies have any protective influence. It was shown that neutrophils play a cardinal role in the immunity against *H. ovis* infection in so far as they phagocytize and destroy the organisms and are attracted to them by chemotaxis in immune animals.

INTRODUCTION

According to several reports in the literature, *Histophilus ovis* exerts its pathogenic effects in sheep by invading the body tissues. Rahaley & White (1977), for instance, isolated the organism post-mortem from abscesses in the supraspinous bursa, atlanto-occipital joint capsule and in the subcutaneous tissue of the brisket of sheep. The same sheep showed focal hepatic necrosis, nephritis, myocarditis, lesions in the central nervous system and pneumonia, and *H. ovis* could be isolated from all these lesions. The same organism was isolated from suppurative synovitis lesions and pyaemia in lambs in New Zealand (Kater, Marshall & Hartley, 1962), New South Wales (Hughes, Hartley, Haughey & McFarlane, 1964) and Western Australia (Dennis, 1974). *H. ovis* was also isolated from suppurative epididymitis in rams (Dodd & Hartley, 1955; Claxton & Everett, 1966). Van Tonder (1977) recovered *H. ovis* from epididymitis in rams in South Africa. Rahaley (1978) produced bacterial thrombosis and vasculitis in the liver, lungs, kidneys and heart by injecting lambs either intravenously or intranasally with *H. ovis*. To protect sheep against an infection by *H. ovis*, one would therefore have to provide them with either a cell-mediated or humoral immunity effective against the invasion of their tissues by this organism.

The current study was consequently aimed at investigating the immunity produced in sheep by injecting an antigen prepared from *H. ovis* in combination with different adjuvants. The resulting resistance was challenged by injecting a live *H. ovis* suspension into the epididymal tubule (Jansen, 1980) or into the epididymis, since the emphasis was on protecting rams against genital infection by *H. ovis*. The results showed that it was possible to protect rams by 2 injections of antigen plus Freund's complete adjuvant or by 3 injections of alum-precipitated antigen.

MATERIALS AND METHODS

Bacterial strain and culture methods

H. ovis Strain 0782* was used for all the experimental work. Ten per cent horse blood tryptose agar** was used as culture medium and all cultures were incubated for 48 h in 10 % CO_2 at 37 °C.

Preparation of antigen

The growth of *H. ovis* on surface cultures in Petri dishes was washed off with sterile 0,8 % NaCl solution and the suspension adjusted to 1 % of packed cells. A final concentration of 0,1 % formaldehyde was added to half of the *H. ovis* suspension before it was returned to the incubator for overnight incubation at 37 °C. This procedure rendered the suspension sterile on culture. A sterile solution of potassium alum was then added to the formalized *H. ovis* suspension to a final concentration of 0,9 % alum and the volumes were so arranged that a dose of 2 mℓ of the final product contained 0,5 mℓ of the original *H. ovis* suspension.

The other half of the original *H. ovis* suspension was subjected to ultrasonic vibration to disrupt the bacterial cells. An amplitude of 6 micron for 60 s repeated 5 times was used, and the suspension was sterile on culture after this treatment. Freund's complete adjuvant (FCA)* was then added, so that a final dose of 2 mℓ contained 0,5 mℓ of the sonicated bacterial suspension.

Experimental animals

A sufficient number of fifteen-month-old Merino rams was tested for freedom from bacterial infection of their genitalia by examining 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, 1977) and with Giemsa stain and examined for the presence of inflammatory cells and bacteria. Cultures were prepared on 10 % horse blood tryptose agar and incubated in 10 % CO_2 for 48 h at 37 °C.

The rams, found free from bacterial infection except for their preputial cavities, were kept intensively on regularly cleaned concrete floors and used for further experimentation.

Injections of antigen were given at 3-week intervals. Twelve rams received 3 injections of alum-precipitated antigen and 18 of the group received 2 injections of antigen plus FCA.

Their immunity was challenged 2 weeks after the last injection.

Challenge procedure

The bacteria from a 24 h culture of *H. ovis* were washed from the surface of the medium in Petri dishes, using Hank's balanced salt solution (HBSS) (Kruse & Patterson, 1973) and diluted such that a challenge dose contained about $0,5 \times 10^6$ organisms in 0,05 mℓ.

Seventeen of the rams were challenged by injecting the organisms into the epididymal tubule after an operation to expose the vas deferens as it emerges from the epididymis (Jansen, 1980). Thirteen rams were injected directly into the tissue of the cauda epididymidis through the scrotal skin. Three unvaccinated control rams were included for each challenge route.

Ten days after challenge, the rams were killed and their entire genital tract removed and transferred to the laboratory for a detailed examination of its component parts. Bacterial cultures from every part were prepared with sterile precautions. Specimens were collected for histopathological examination, fixed in 10 % buffered formalin and the sections stained with haematoxylin and eosin.

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

** Biolab Chemicals, 4 Bernard St., Colbyn, Pretoria 0083

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* Difco Laboratories, Detroit, Michigan, USA

Tests for cell-mediated immunity

The lymphocyte transformation test was applied according to the method described by Kristensen, Kristensen & Lazary (1982). Briefly, blood was collected from immune rams with preservative-free heparin as anticoagulant at a concentration of 50 IU/mℓ. The lymphocytes were separated from the other blood cells by diluting the whole blood with an equal volume of HBSS and layering it directly on to Ficoll-Hypaque with a density of 1,077 g/mℓ in sterile centrifuge tubes. The diluted blood: Ficoll-Hypaque ratio was between 3:1 and 4:1. After centrifugation at 800–1000 × g in a swing-out head for 30 min at room temperature, the lymphocytes were layered in the interphase between the Ficoll-Hypaque and plasma. The cell disc was collected with a sterile Pasteur pipette and resuspended in HBSS for washing. The cells were washed twice and then resuspended in Roswell Park Memorial Institute 1640* (RPMI) solution, supplemented with 100 units of penicillin, 100 μg streptomycin per mℓ and 0,2 % $NaHCO_3$. Fifteen per cent heat-inactivated foetal calf serum was added to the cell suspension. The total number of cells was counted with a haemocytometer and the percentage of live cells was determined by the trypan blue exclusion method (Jain & Jasper, 1967). The viability was invariably about 90 %. The cell suspension was then diluted with RPMI 1640 solution to a density of 2×10^6 cells/mℓ.

The antigen used in the test consisted of the lipopolysaccharide extracted from *H. ovis*, according to the method applied by Jansen, Hayes & Knoetze (1983). The tests were done at each of the following antigen concentrations: 10,0; 5,0; 2,5; 1,25 and 0,625 μg/mℓ.

Plastic plates with round-bottomed wells were used for the assays, and the following ingredients were distributed into the appropriate number of wells:

200 $\mu\ell$ of lymphocyte suspension.

20 $\mu\ell$ of antigen solution.

As a control 20 $\mu\ell$ of phytohaemagglutinin** containing 40, 30, 20, 10 and 5 μg/mℓ was introduced into separate wells instead of the antigen.

All tests were done in triplicate.

The mixtures were incubated in a humid incubator with a 5 % CO_2 atmosphere for 3 days at 37 °C. At this stage 1 μCi of tritiated thymidine (specific activity 6,7 Ci/mmol) in a volume of 10 $\mu\ell$ was added to each well and the incubation continued for 18 h. At this stage the viability of the lymphocytes was checked again. Finally the cells were harvested with a Titertek Cell Harvester D–001*** onto Flow Skatran type 125/05 filter discs****. The radio-activity was measured with a beta scintillation counter, using toluene with 0,1 % 2,5-diphenyl oxasole and 0,03 % p-bis (0-methylstyryl) benzene as scintillation fluid.

For the determination of the presence of a macrophage migration inhibitory factor (MIF), the method described by Rocklin (1976) was followed. Lymphocytes were collected from immune rams by using the method described above. They were suspended in HBSS in a concentration of 10×10^6 cells/mℓ.

Macrophages were collected from the peritoneal cavity of adult mice by killing them and then injecting 3 mℓ of HBSS at 4 °C into the peritoneal cavity. After light massage of the abdomen, the fluid was withdrawn and the cells were harvested by spinning at 400 *g* for 5 min. The harvested cells were washed once with HBSS and resuspended in HBSS in a concentration of 30×10^6 cells/mℓ.

Equal volumes of the macrophage and lymphocyte suspensions were mixed to prepare a cell suspension for filling the capillary tubes. The filled capillaries were centrifuges at 250 × g at room temperature for 5 min and then cut to the correct length. The capillaries containing the column of cells were then placed in the chambers of Sterilin Q 132* plastic plates. One mℓ of antigen solution containing 2,5 and 1,25 μg lipopolysaccharide/mℓ plus 3 mℓ HBSS containing 15 % inactivated foetal calf serum was added to separate chambers. The plates were then incubated overnight at 37 °C.

Control tests were done with lymphocytes from non-immune rams.

Skin testing for delayed hypersensitivity was performed by injecting intradermally in immune rams 0,1 mℓ of the sonicated suspension of *H. ovis* described above. The injection site was shaved and the skinfold thickness measured before injection. At 24 and 48 h after injection the site was examined for redness, increase in skinfold thickness and induration. Skin biopsies were taken simultaneously and fixed in 10 % buffered formalin, and the sections were stained with haematoxylin and eosin.

Neutrophil chemotaxis

The granulocyte fraction was isolated from ovine blood, according to the method described by Carlson & Kaneko (1976). The granulocytes were suspended in HBSS containing 15 % inactivated foetal calf serum in a concentration of 10×10^6 cells/mℓ. Neutrophils represented 89 % of these cells. The viability of the granulocyte suspension was greater than 94 % as was measured by the trypan blue exclusion technique.

The chemotaxis test was done by the agarose method described by Nelson, Quie & Simmons (1975). As attractant, the sonicated suspension of *H. ovis* used for preparing the FCA vaccine and a lipopolysaccharide extract of *H. ovis* containing 10 μg of antigen per mℓ were used.

The Petri dishes containing the agarose and the reagents were incubated for 4 h at 37 °C in a 7 % CO_2 moist atmosphere. The cells were then fixed with the agarose in place by the addition of 5 mℓ of absolute methanol and leaving them at room temperature overnight. After fixation the gel was removed and the cells stained with Giemsa stain.

The area of migration of the neutrophils around the wells was recorded by dark-field microphotography at 80 × magnification.

Detection of specific immunoglobulin in the serum of immune rams

An enzyme-linked, immunosorbent assay method, as described by Voller, Bidwell & Bartlett (1976), with the minor modifications indicated below, was used for the detection of IgG antibodies against *H. ovis* in the serum of immune rams. A lipopolysaccharide, 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 (prepared by the method of Sutherland, 1978) for the extraction of the polysaccharide. This antigen, made up

* Gibco Laboratories, Grand Island, New York 14072

** Miles Laboratories, P.O. Box 141, Goodwood 7460

*** Bios Laboratory, P.O. Box 784064, Sandton 2146

**** Skatran, Norway

* Sterilab, P.O. Box 92084, Norwood 2117

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 polyvinyl 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 were bled 10 days later. The IgG was recovered from the rabbit serum by the same methods as were 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 $\mu\ell$ 3 M NaOH and the absorbances measured in a Titertek Multiskan**.

All wash procedures were carried out in a Multiwash** apparatus.

Determination of the inhibitory action of immune serum on H. ovis

Wells were punched in tryptose-blood-agar plates, with sterile precautions. The wells were filled with serum from immune rams. After the contents of the wells had diffused into the medium, the wells were refilled with serum. This process was repeated 5 times to ensure a high concentration of serum in the medium immediately around the wells. Then a diluted liquid culture of *H. ovis* was thinly spread over the entire surface of the medium and incubation in a 10 % CO_2 atmosphere continued for 24 h at 37 °C.

The cultures were then examined for zones of inhibition around the wells.

RESULTS

Tests for cell-mediated immunity

The lipopolysaccharide extract of *H. ovis* did not produce a convincingly positive lymphocyte stimulation effect when brough into contact with lymphocytes derived from rams that had been vaccinated and proved immune by challenge with live organisms. Furthermore, it was not possible to demonstrate the presence of a macrophage migration inhibitory factor in the immune rams. The delayed hypersensitivity skin test was also negative.

IgG antibodies in the serum of vaccinated rams

Rams vaccinated with 1 or 2 injections of alum-precipitated antigen showed positive IgG titres against *H. ovis,* according to the enzyme-linked immunosorbent assay, although they were not immune to the challenge with live organisms. All immune rams had positive titres.

Inhibition of growth of H. ovis *by immune serum*

Fresh serum from immune rams had no inhibitory effect on the growth of *H. ovis*.

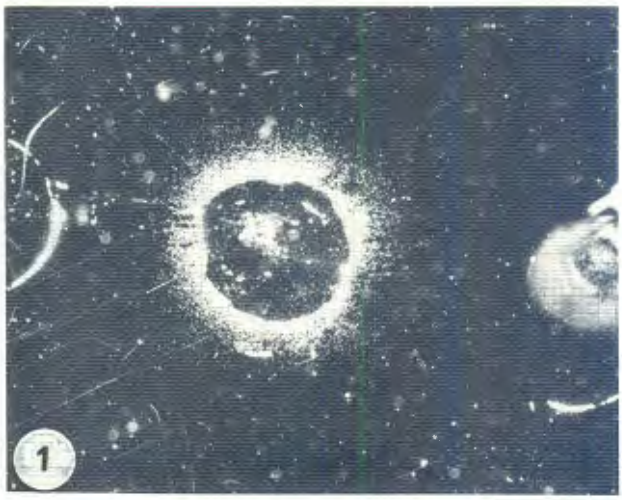

FIG. 1 Light area around well represents spontaneous migration of neutrophils: × 80.

Tests for neutrophil chemotaxis

Fig. 1 shows the extent of spontaneous migration from the well by the neutrophils during the test period. Fig. 2 shows the greater degree of attraction of the neutrophils towards the well containing the sonicated *H. ovis* suspension than in the other directions. The lipopolysaccharide extract showed a chemotactic effect similar to the one by the disrupted whole cell preparation. The antigen had no chemotactic effect on neutrophils of non-immune sheep.

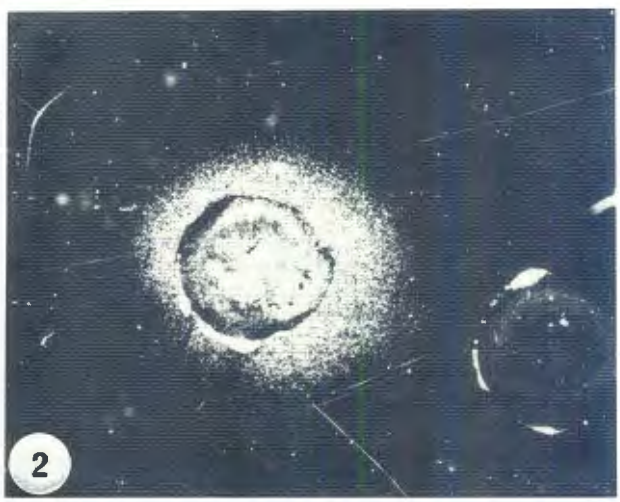

FIG. 2 Light area around well represents increased migration of neutrophils towards well containing antigen: × 80.

Histological sections, prepared from the ampullae vesiculae seminales and epididymis of an immune ram killed 36 h after challenge into the epididymis, showed

* PHARMACIA

** Taeuber & Corssen, P.O. Box 372, Johannesburg 2000

massive infiltration of neutrophils into the mucosa and lamina propria of the ampulla and vesiculae seminales and into the tissue of the epididymis (Fig. 3).

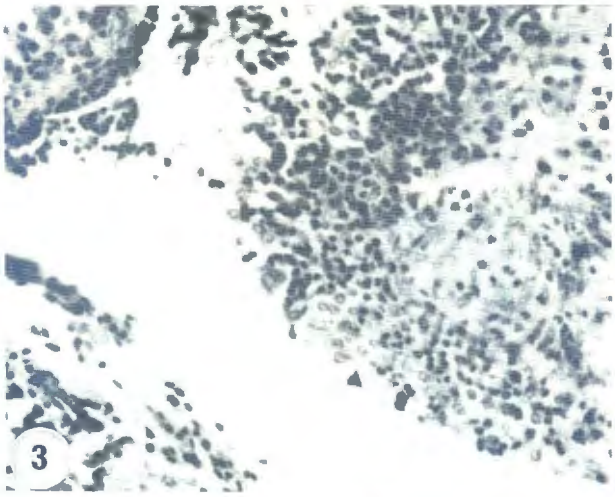

FIG. 3 Massive infiltration of neutrophils into mucosa and lamina propria of ampulla: × 450.

Immunity in rams vaccinated with antigen plus FCA

Thirteen rams were challenged by injecting the dose of *H. ovis* into the vas deferens after its surgical exposure. In 5 of these rams the ampullae, vesiculae seminales and epididymis appeared normal and were culturally negative for *H. ovis* on examination after slaughter. In the other 8 rams there was an area of oedema around the operation site and abscesses of varying size were distributed in the oedematous tissue. *H. ovis* could be isolated from the oedematous tissue in the rams so affected, and in 2 of them the ampullae and vesiculae saminales were also culturally positive.

In all of the 5 rams challenged by an injection into the epididymis, the epididymis, ampullae and vesiculae seminales appeared normal and were culturally negative for *H. ovis*. In 2 of these rams a 2 mm focus of pus-like material was present in the epididymal tissue at the point where the challenge dose had been deposited. The contents of the focus was sterile on culture and on histological examination it proved to be an accumulation of sperm resulting from a rupture of the ductus epidiymidis. This rupture could have been caused by the introduction of the challenge dose or the activity of the organism before it was overwhelmed by the body defences.

Immunity in rams vaccinated with alum-precipitated antigen

It was soon clear that rams given 2 injections of alum-precipitated antigen were insufficiently protected against challenge with live organisms. Consequently, the resistance produced by 3 injections of alum-precipitated antigen was investigated.

In all 4 rams, challenged by injection of *H. ovis* into the vas deferens, the ampullae, vesiculae seminales and epididymides were enlarged and culturally positive for *H. ovis,* while there were necrotic foci containing live organisms in the oedematous tissue around the operation site.

All 8 rams, challenged by injection into the epididymis, were fully protected. Their epididymides and accessory glands appeared normal macroscopically and histologically, and were culturally negative. Two of these rams showed pockets of spermatozoa, the same as those referred to above.

All the control rams showed a severe purulent epididymitis and an infection of the ampullae and vesiculae seminales.

Discussion

From the results obtained in this series of experiments it is clear that the ability of a ram to resist an infection of its genitalia with *H. ovis* is vested in its neutrophils. Not only are the organisms phagocytized and destroyed by ovine neutrophils (Jansen *et al.,* 1983), but the neutrophils of immune rams are also actively attracted towards *H. ovis* or its polysaccharide. The active participation of neutrophils in counteracting an infection by *H. ovis* is manifested by the large-scale infiltration of neutrophils into the tissues of the epididymis and the accessory glands in an immune ram challenged by the injection of the organism into the epididymis.

Cellular immunity, as evidenced by lymphocyte stimulation, the presence of MIF or delayed hypersensitivity, does not seem to play any role.

The presence of specific IgG antibodies in immune serum does not have any inhibitory effect on the multiplication of *H. ovis in vitro,* and rams showing antibody titres are not necessarily protected.

Challenging a ram's immunity by injecting *H. ovis* into the epididymis proved to be superior to an injection into the vas deferens. Some of the immune animals challenged by the former method showed at most a small cavity filled with spermatozoa at the injection site. The cavity was presumably the result of tissue destruction by the bacteria before they were overwhelmed by the body defences.

Several of the rams that should have been immune, according to the results obtained with the animals challenged by injection into their epididymides, showed a severe reaction when challenged via the vas deferens. An extensive oedema arose around the operation site, and the bacteria deposited inside the vas deferens apparently entered the oedematous tissue through the relatively thin wall of the vas deferens before they could be destroyed by the defence mechanism of the immune ram. After entering the oedematous tissue the bacteria could multiply in the devitalized oedematous swellings in a manner undistrubed by any body defences. The bacteria multiplying in this site could serve as a source for continued re-infection of the accessory glands.

The results of these experiments also showed that the immunity provided by 2 injections of antigen plus FCA was equivalent to the one provided by 3 injections of alum-precipitated antigen. Since it was found, however, that the FCA vaccine was inclined to cause unsightly, painful, hard swellings and sometimes abscesses, alum-precipitated vaccine is preferred.

It may justifiably be argued that the method of challenge is rather severe, but it certainly identifies the animals that are solidly immune. It also gives one the assurance that, if an animal can withstand such a severe artificial challenge, it will be resistant to any natural infection where the challenge is such milder. The vaccination of rams with an alum-precipitated antigen therefore seems to be an acceptable, effective means of protecting them against invasion of their genitalia by *H. ovis* and, more than likely, also against infection of their body tissues by this organism. A vaccine suitable for field use however, would, require further research into production methods, optimum dosage, duration of immunity and related matters.

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