J. Reprod. Fert. (1986) **78**, 337–345

Printed in Great Britain

A determinant of M_r 34 000 expressed by hamster epididymal epithelium binds specifically to spermatozoa in co-culture

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Summary. A murine monoclonal antibody raised against hamster cauda epididymal spermatozoa was shown to recognize an M_r 34 000 component of epididymal epithelium. Antigen was localized by immunocytochemistry on the surface and in the apical cytoplasm of principal cells in the proximal corpus epididymidis but not in the caput or initial segment regions. Spermatozoa from the corpus epididymidis expressed antigen on their post-acrosomal plasma membrane and annulus.

Epididymal principal cells from the proximal corpus region when cultured *in vitro* bound antibody on their apical surface for at least 5 days. Spermatozoa from the caput epididymidis co-cultured with epithelium expressed antigen after incubation for 8 and 24 h. These results suggest that a surface change to epididymal spermatozoa during maturation *in vivo* may also be elicited during in-vitro culture.

Introduction

Investigations using polyclonal antiserum or monoclonal antibodies have demonstrated conclusively that antigens (glycoproteins?) secreted by the mammalian epididymal epithelium are transferred onto the surface of spermatozoa during passage along the epididymis (see Moore, 1983, 1984; Eddy *et al.*, 1985). Some of these antigens may play a role in the acquisition of sperm fertilizing ability since antibodies (or Fab fragments) to specific epididymal components block fertilization *in vitro* and *in vivo* (Moore, 1981; Moore & Hartman, 1984; Ellis *et al.*, 1985). Such epididymal factors probably play a role in the development of sperm motility, for example forward motility protein (see Hoskins *et al.*, 1979), and in the formation on the sperm surface of specific receptors that recognize complementary residues on the zona pellucida and oolemma (Saling, 1982; Sullivan *et al.*, 1985).

To dissect out the biochemical factors involved in mammalian sperm maturation we have established an in-vitro culture technique for hamster epididymal epithelium in which the original association of epithelial cells is initially retained (Moore *et al.*, 1986). To identify functional principal cells in this heterologous culture a monoclonal antibody, raised against hamster spermatozoa from the cauda epididymidis, was used as a marker. In this paper we describe the characterization and localization of antigen on epididymal epithelium and spermatozoa *in vivo* and during in-vitro culture.

Materials and Methods

Monoclonal antibody. The secretory activity of epididymal epithelium was monitored with a monoclonal antibody (C5) produced in mice against hamster cauda epididymal spermatozoa according to techniques described previously (see Moore & Hartman, 1984), but on which we have not as yet reported. By enzyme-linked immunosorbent assay this antibody was shown not to cross-react with heart, liver, brain or lung tissue of the golden hamster, or to spermatozoa from rat, rabbit and man. Antibody was used unpurified in hybridoma culture supernatant.

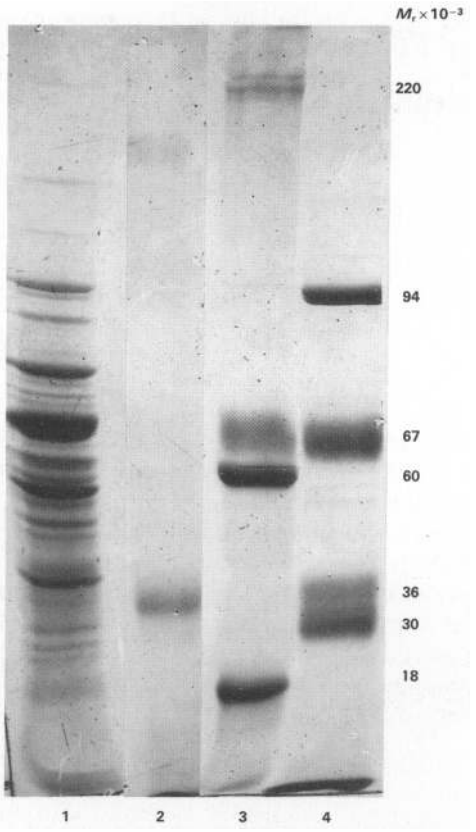

Fig. 1. SDS/polyacrylamide gel electrophoresis of protein extract of the corpus epididymidis (lane 1), C5 immunoprecipitated protein (lane 2), and marker proteins (lanes 3 and 4). The antigen to C5 ran as a single band at M_r 34 000. Marker protein molecular weights as given.

Molecular weight determination of antigen. Each proximal corpus epididymidis from two adult male hamsters was dissected free of fat, homogenized in 2 ml phosphate-buffered saline and centrifuged at 2000 *g* for 5 min. The supernatant was removed and an equal volume of 2% (w/v) deoxycholate added. This preparation was centrifuged at 30 000 *g* for 45 min and then subjected to an immunoprecipitation procedure with C5 antibody (or control antibody against non-sperm component) according to the methods of Phillips *et al.* (1981) using fixed *Staphylococcus* cells. Final supernatants were analysed by electrophoresis on 11·5% SDS/polyacrylamide gels as described previously (Ellis *et al.*, 1985).

Culture of epididymal epithelium and incubation of spermatozoa. Epididymal epithelium from the proximal corpus epididymidis, and spermatozoa from the caput epididymidis were cultured as described by Moore *et al.* (1986).

Immunocytochemistry. For light microscopy, glutaraldehyde-fixed frozen sections of hamster epididymis from prepubertal and adult males were stained with C5 antibody by the PAP technique after the method of Heyderman & Monaghan (1979).

To visualize the binding of C5 antibody to spermatozoa an immunofluorescent technique was used on paraformaldehyde-fixed cells (Moore *et al.*, 1985). Controls consisted of hybridoma culture supernatant without antibody or with antibody against a sperm acrosome component (18·6) produced in the testis (Moore *et al.*, 1985).

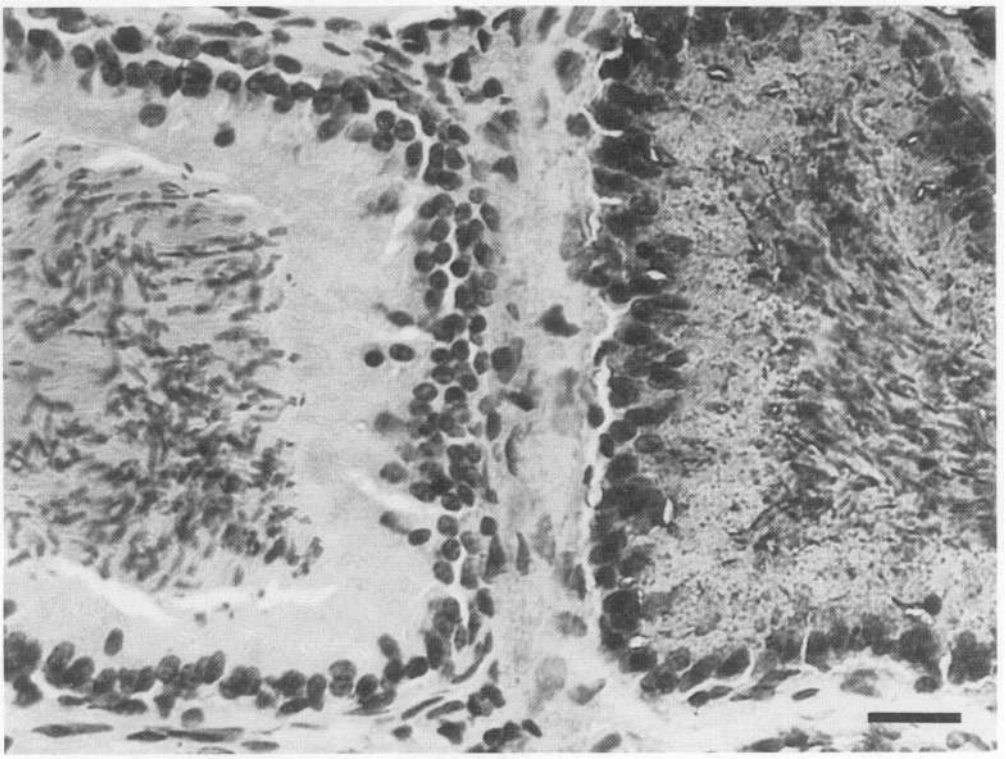

Fig. 2. A micrograph of a section of frozen-fixed epididymis from the junction of the distal caput and proximal corpus regions after immunocytochemical staining with C5 antibody and counterstaining with Mayer's solution. On the left, a section of tubule in the distal caput unstained; on the right, a section of tubule from the proximal corpus with PAP reaction product in the apical cytoplasm and on microvilli. Bar = 100 μm.

For electron microscopy, samples of spermatozoa or epithelium were fixed in 2·0% glutaraldehyde in 0·05 M-phosphate buffer (pH 7·3, 330 mosmol by addition of sucrose) and stained with C5 antibody by the techniques of Heyderman & Monaghan (1979), modified for resin sections, before being processed for electron microscopy as reported by Moore *et al.* (1986).

Results

Molecular weight determination of antigen recognized by C5 monoclonal antibody

After immunoprecipitation from the protein extract of the corpus epididymidis, the epitope recognized by C5 antibody ran as a single band when electrophoresed on a polyacrylamide gel and was ascribed a molecular weight of 34 000 compared with known marker proteins (Fig. 1). Control antibody failed to precipitate a band.

Immunocytochemistry

Epididymal localization of C5 determinant. On frozen-fixed sections of adult hamster epididymis C5 monoclonal antibody was shown by immunoperoxidase staining to recognize a determinant on

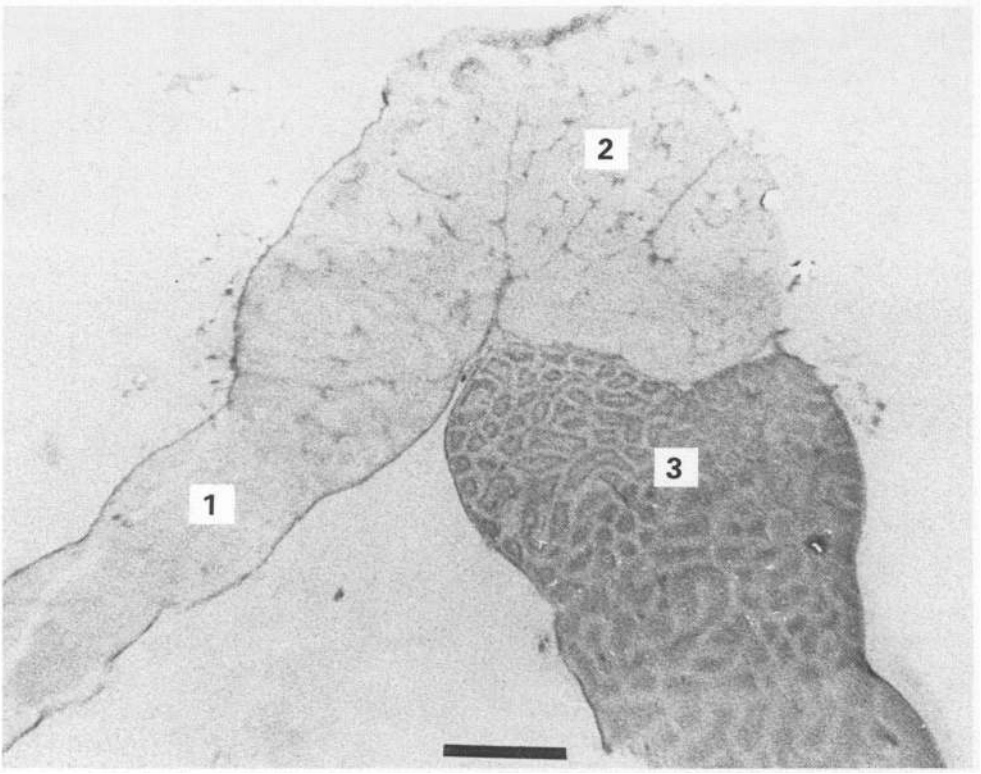

Fig. 3. A low-power micrograph of a section of proximal epididymis of an adult male following localizations of antigen against C5 antibody. Stain was limited to the proximal corpus (3) first on epithelium and more distally on spermatozoa. Only non-specific staining of connective tissue was present in the initial segment (1) or caput (2) regions. No counterstain. Bar = 1 mm.

the apical surface and within the supranuclear cytoplasm of principal cells in the proximal corpus region (Fig. 2). The epithelium within the distal caput region remained unstained, expression of C5 determinant being acutely confined at the proximal boundary of the corpus region by connective tissue (Fig. 3). Immunoperoxidase staining of the epithelium decreased down the corpus epididymidis, while staining of the luminal content (i.e. spermatozoa) increased. Localization on sections of epididymis from prepubertal hamsters indicated that C5 was present on principal cells before spermatozoa entered the lumen (Fig. 4).

The immunofluorescent technique demonstrated that spermatozoa from the initial segment or caput region of the epididymis did not express C5 determinant and were unstained. In contrast, spermatozoa from the proximal corpus displayed fluorescence (i.e. C5 determinant) over the post-acrosomal region of the heads and annulus (Fig. 5). Electron microscopy revealed that this staining was limited to the plasma membrane (Fig. 6).

Localization of C5 determinant on cultured epididymal epithelium. Immunoperoxidase labelling of cell cultures was used to identify principal cells and their secretory activity in culture. At the light microscope level, principal cells were labelled with brown reaction product indicating the presence of antigen (Fig. 7). Plaques of cells originating from an attached ball of tissue were consistently stained for the first 5 days in culture, as were some single cells attached to non-staining basal or fibroblast cell types. After 5 days in culture, specific staining decreased and by 9 days regressed to control levels. Cell preparations cultured for 14 days, or more, consisted mainly of fibroblasts

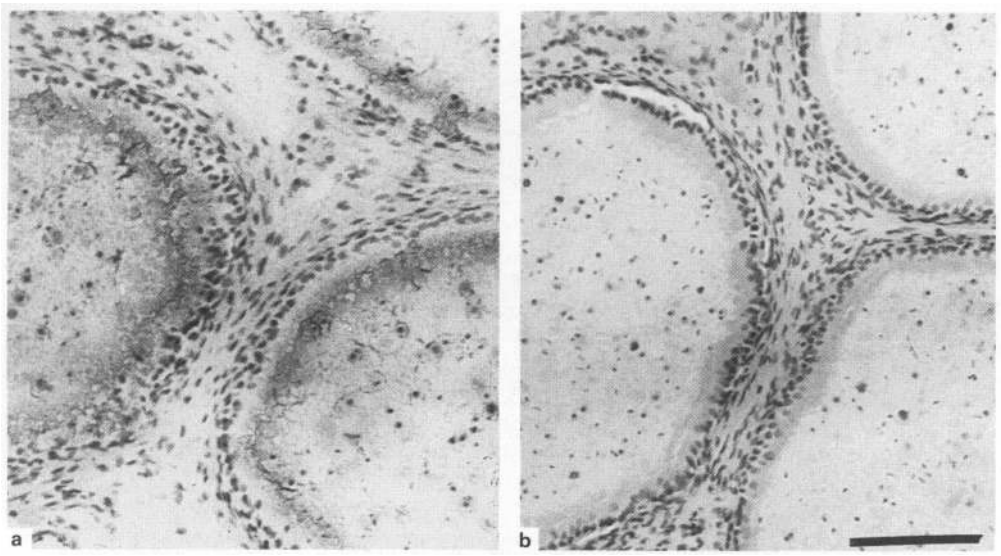

Fig. 4. Micrographs of a section of frozen-fixed epididymis from the corpus region of a prepubertal male (a) after immunocytochemical staining with C5 antibody and (b) control without first antibody. In (a) reaction product was present on the apical border of epithelium even in the absence of spermatozoa. Counterstained. Bar = 0·5 mm.

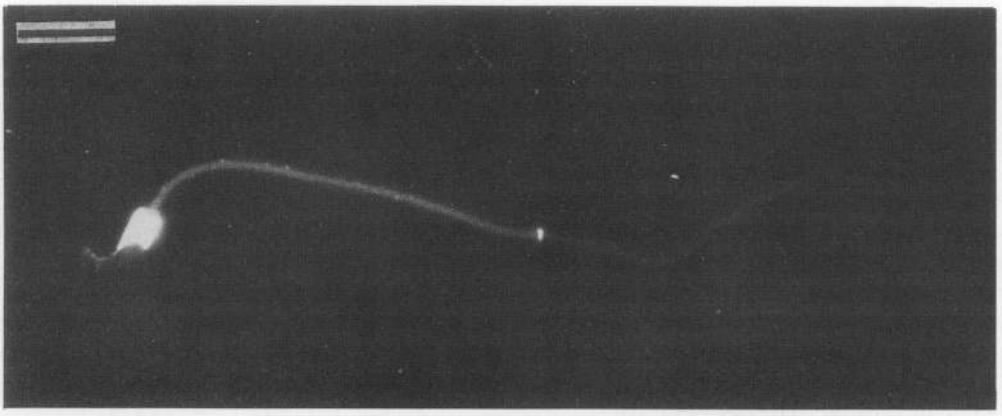

Fig. 5. Immunofluorescent localization of the determinant to C5 antibody on a spermatazoon from the corpus epididymidis. Fluorescence was on the post-acrosomal region and annulus. Bar = 10 μm.

which stained with a second antibody (anti-mouse IgG) in a low and non-specific manner without C5 monoclonal antibody.

Ultrastructural observations of reaction product indicated that antibody was binding exclusively to the apical cell surface of principal cells (Fig. 8). Individual variation in the expression of antigen on epithelium was apparent by the lack of reaction product on some cells whose immediate neighbour displayed strong staining.

Localization of C5 determinant on spermatozoa co-cultured with epithelium. A significantly greater proportion of spermatozoa from the caput epididymidis, incubated for 8 and 24 h with

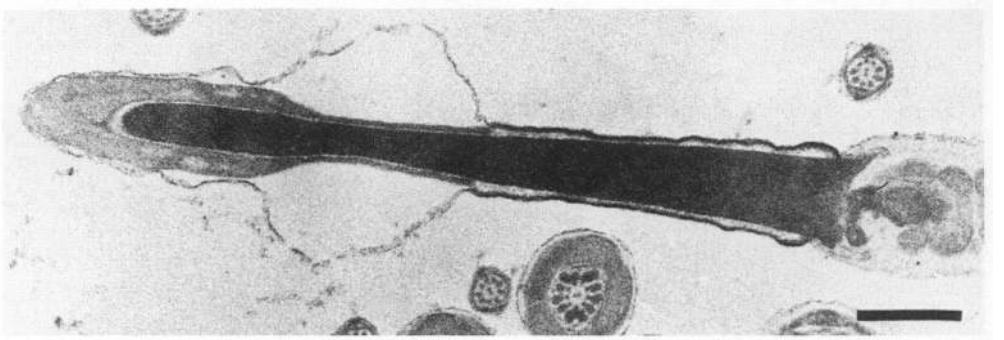

Fig. 6. Electron micrograph of a section of hamster spermatozoa from the corpus epididymidis after immunocytochemical localization for C5 determinant. Electron dense reaction product was present on plasmalemma mainly over the post-acrosomal region although some staining in the equatorial region was also displayed on some spermatozoa. The membrane of the anterior acrosome and tail remained unstained. Bar = 1μm.

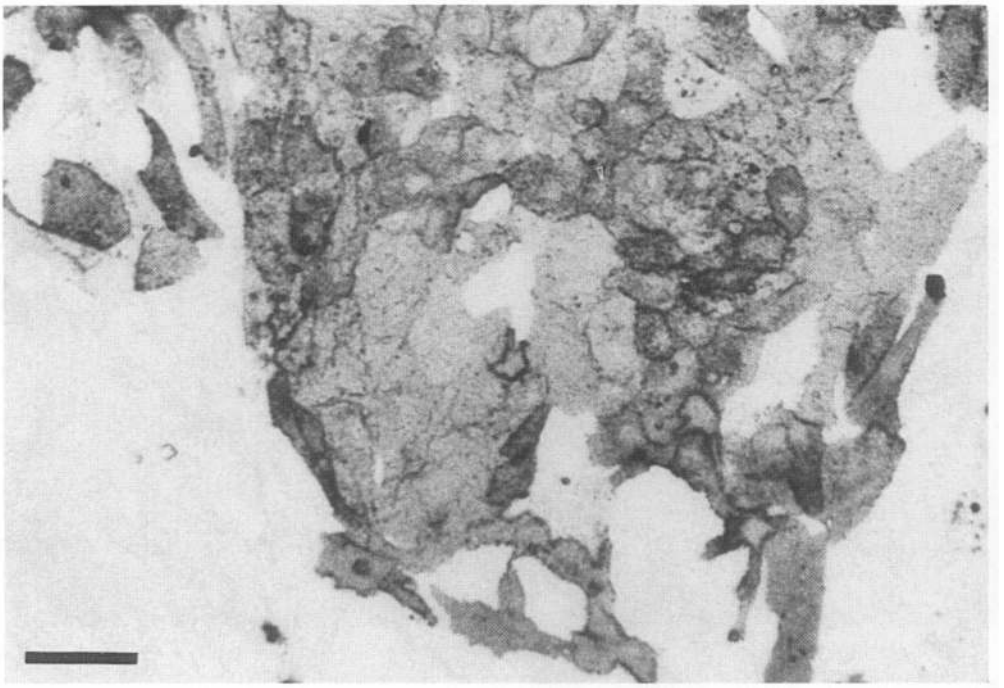

Fig. 7. Light micrograph of principal cells stained with C5 antibody after 5 days in culture. Bar = 50 μm.

epithelium from the corpus epididymidis cultured with androgen expressed C5 determinant as localized by immunofluorescence compared with spermatozoa incubated with cultures without androgen (Table 1).

Discussion

The cultures of epididymal epithelium prepared by our methods clearly contained a heterologous population of cells representing the normal constituents of the epithelium and the underlying peri-

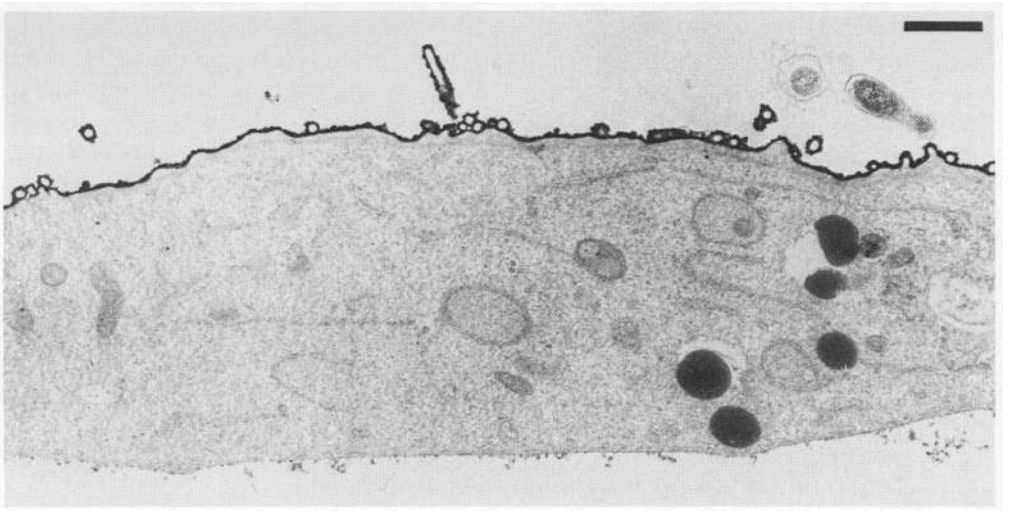

Fig. 8. An electron micrograph of a section of flattened principal cells stained with C5 antibody. Reaction product was confined to the apical membrane and microvilli. No counterstain. Bar = 1 μm.

Table 1. Proportion of spermatozoa from the caput epididymidis displaying immunofluorescence to C5 antibody after incubation with epididymal epithelium from the corpus epididymidis cultured with and without androgen

Incubation (h)	Proportion* of spermatozoa displaying immunofluorescence (%)	
	+Androgen	−Androgen
4	Not detectable	Not detectable
8	22 ± 7 (faint)	Not detectable
24	†34 ± 4 (medium)	2 ± 1 (very faint)

*Mean of 3 experiments; 100 spermatozoa examined each time. Fluorescence was faint or of medium intensity compared with spermatozoa recovered from the corpus epididymidis (50% of spermatozoa without acrosomes at 24 h).

†Significantly different from controls (*t* test, $P < 0.05$).

tubular cell layers. While epithelium could be identified by gross morphology when it surrounded tissue spheres, a biochemical marker was desirable for cells plated out across culture dishes. In the epididymis, principal cells have been implicated as the main cell type interacting with spermatozoa (see Moore, 1984), therefore a monoclonal antibody (C5) was used as both cell marker and probe of continued secretory function.

Localization of antigen recognized by this antibody, within the intact epididymis, was restricted to regions of epithelium and spermatozoa distal to the caput epididymidis. A limited distribution of epididymal secretory product or metabolite has been reported by others (Lea *et al.*, 1978; Moore, 1980; Brooks, 1981; Prakash & Moore, 1982; Jones *et al.*, 1985) and illustrates the regional functional activity of the epithelium often corresponding to a particular segment or duct circumscribed by connective tissue. How this fine control mechanism may operate is unknown.

Localization of C5 determinant on the apical border of the epididymal epithelium of the prepubertal male (when only a limited amount of cell debris was present in the epididymal lumen) indicated that, in the corpus region, the presence of C5 determinant in the cytoplasm of principal cells (in adult males) was due to synthesis rather than endocytosis from spermatozoa. This result was confirmed by the absence of C5 determinant on caput spermatozoa but its presence over the post-acrosomal plasma membrane and on the annulus of spermatozoa from the proximal corpus region.

The binding of C5 antibody to cultured cells indicated that many were clearly of epithelial origin (rather than peritubal) and that secretion of product continued for at least 5 days, as also reported by Klinefelter *et al.* (1982). On frozen sections of epididymis, immunostaining was apparently present in the apical cytoplasm of principal cells but stain at the ultrastructural level was observed only on the apical surface of cultured cells. Since there is no doubt that antigen was present, this discrepancy was considered unlikely to be an artefact of frozen sections. It may, however, reflect an inability of antibody to reach intracellular sites after fixation, or be representative of expression of antigen only on the apical cell membrane of cultured principal cells. It is hoped that these differences can be resolved by further investigations using cryo-ultramicrotomy.

The appearance on spermatozoa from the caput epididymidis of previously undetected antigen after co-culture with epithelium would indicate that secretions can be transferred to spermatozoa under in-vitro as well as in-vivo conditions (Moore, 1981). Furthermore, the limited binding of determinant to the post-acrosomal region of the sperm head and to the annulus suggests a specific coupling to the sperm surface. These membrane changes were concomitant with the induction of forward motility in a proportion of spermatozoa (Moore *et al.*, 1986). A forward motility protein (M_r 37 000) has been claimed to be essential for the development of sperm motility (Hoskins *et al.*, 1979). Although the molecular weight of 34 000 for the C5 determinant would suggest that it is not forward motility protein, the specific binding to the annulus might well induce changes in the flagellum movement. Further studies are now in progress to test this hypothesis.

This study was supported by grants from the Ford Foundation, the Medical Research Council, the Agriculture and Food Research Council and by a donation from Elf-Aquitaine (UK) plc.

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Received 18 November 1985