The origin and function of cement gland secretion in *Pomphorhynchus laevis* (Acanthocephala)

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SUMMARY

Cement gland protein in male and inseminated female individuals of an acanthocephalan parasite of fish, *Pomphorhynchus laevis* (Müller, 1776), was localized by immunohistochemistry using an antibody specific for cement protein. Male *P. laevis* possess 3 pairs of round to oval cement glands ranging from 0·5 to 0·9 mm in length and from 0·3 to 0·7 mm in width. Each gland has an outer portion containing nuclear fragments and other cellular organelles surrounding a space for storage of gland products. Very little work has been carried out on the nature of the cement gland secretions. We have previously reported that the major component of cement is a protein with molecular weight of 23 kDa; in fresh glands it is white in colour. Immunohistochemical studies herein reported were carried out using a polyclonal antibody raised against purified *P. laevis* p23 cement protein (anti-p23PL). Localization of p23 cement protein at the light microscope level, by means of the anti-p23PL antibody, shows that p23 is present within the cytoplasmic layer of the gland as well as in the gland duct lumen. Interestingly, the p23 cement protein was also identifiable at the posterior ends of females retaining the cap. Positivity to anti-p23PL antibody was obtained not only in the external part of the copulatory cap, but also within the vaginal tract and at the base of the uterine duct. Thus, we report herein the first photographic evidence that the copulatory cap is not a simple gonopore lid but it is really an intravaginal plug.

Key words: acanthocephalan, cement glands, copulatory cap immunohistochemistry.

INTRODUCTION

Among some taxa of invertebrates, such as the Insecta and Acanthocephala, several behavioural and physiological adaptations of males may help to reduce the effectiveness or diminish the occurrence of second inseminations of the same female (Parker, 1970). One of these adaptations is a mating plug, produced by the male accessory gland secretions, which is usually formed after insemination to form a plug within the female genital apparatus. Such a plug will delay or prevent the introduction of sperm by another male.

In the Acanthocephala, cement gland secretion of the males is presumed to contribute to the formation of the copulatory cap ('mating plug') which is often noticed on the posterior end of female individuals and sometimes also on the male genital region as well as on other parts of the body (Abele & Gilchrist, 1977; Parshad & Crompton, 1981; Moore & Bell, 1983; Richardson, Martens & Nickol, 1997).

Very little work has been done on the nature of the cement gland secretions. The cement has been considered to be a mucilaginous material (Van Cleave, 1949), a proteinaceous substance (Haley & Bullock, 1952), a mixture of glycogen and galactogen material (Rengaraju & Das, 1976) or lipid substance (Asaolu, 1981). Recently, based on electrophoretic separation of material from the cement glands of entire male *Pomphorhynchus laevis* and from the isolated glands, we assumed that a protein of 23 kDa could be the main component of cement secretion (Dezfuli *et al*. 1998).

In order to demonstrate directly that p23 protein is the main component of the cement gland secretion, we purified this protein and produced a polyclonal antibody in rabbits by subcutaneous injection. This antibody was purified by affinity on membranes carrying the p23 protein, and used for immunostaining male and female individuals of *P. laevis*. Production of an antibody against p23 cement protein has proved useful to investigate the cement production site within the genital apparatus of males and the mode of formation of the copulatory cap after insemination of the female.

MATERIALS AND METHODS

Specimens of chub (*Leuciscus cephalus*), ranging in length from 180 to 370 mm, were sampled on several occasions by electrofishing in Lama stream, north of Padua, Italy. The fish were taken to the laboratory and on dissection many adult *Pomphorhynchus laevis* were recovered. Male acanthocephalans were dissected and the cement glands were isolated.

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Purification of the p23 protein from the cement glands

Three hundred cement glands from male *P. laevis* were isolated and electrophoretic separation of proteins was performed by SDS–PAGE (Laemmli, 1970) using the Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Milan, Italy) according to the manufacturer's instructions. The glands were solubilized in SDS lysis buffer (62·5 mM Tris–HCl, pH 6·8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0·1% bromophenol blue), sonicated for 15 sec, boiled for 5 min and centrifuged at 13000 ***g*** for 3 min. Samples of supernatant fluid and molecular weight markers (High Molecular Standard Mixture plus Dalton Mark VII-L, Sigma Chemical Co., St Louis, USA) were applied to a 10% continuous polyacrylamide gel and electrophoresed at 200 volts, until the tracking dye reached the bottom of the gel. Proteins were visualized by Coomassie R250 stain (Harlow & Lane, 1988).

Using gel electrophoresis, a band of 23 kDa was quantified by densitometry, excised from the gel, and pulverized in a tissue grinder. The acrylamide-protein mixture was divided into 5 aliquots; each aliquot was used in the immunization of 2 rabbits (1 male, 1 female, strain HY/CR, Charles River, Italy). Five subcutaneous injections of about 100 μg of p23 protein per rabbit were necessary to induce the maximal immune response against this protein.

The first immunization was performed by 1:1 dilution of the acrylamide–protein mixture containing the p23 protein with Freund's complete adjuvant. This solution was used for subcutaneous injection in 4 different points in the lateral side of the rabbit. Later immunizations were performed 1 month after the first one, and at 15 day intervals. For the later immunizations, an equal volume of the protein and Freund's incomplete adjuvant were combined. Rabbit blood was withdrawn from the ear circular vein, left for 1 h at 4 °C in 50 ml centrifuge tubes and then spun for 5 min at 3000 ***g***. The serum was used for the purification of antibodies raised against the p23 protein of *P. laevis* (herein termed as anti-p23PL).

For the antibody purification, the cement glands of 20 male acanthocephalans were denatured (see above), loaded onto a 10% polyacrylamide–0·1% SDS gel and then the gel was electroblotted on a nitrocellulose membrane (Hybond C, Amersham) and the proteins stained with Ponceau Red. The band containing the p23 protein was cut off the membrane and used for affinity purification of the antibody anti-p23PL. The membrane was put in a blocking solution (5% non-fat dried milk, 20 mM Tris–HCl, pH 8, 0·9% NaCl, 0·2% Tween-20) for 60 min. After a brief incubation in washing solution (20 mM Tris–HCl, pH 8, 0·9% NaCl, 0·2% Tween-20), the membrane was incubated for 90 min at room temperature with the serum from rabbits immunized against p23 on a shaking table. Then, the antibody was eluted from the membrane by addition of 100 mM glycine, pH 2, and the eluted solution was adjusted to pH 8.

Immunohistochemistry of the cement glands

Twenty-five mature males and 8 females were fixed in Bouin's solution and then embedded in paraffin wax with a melting point of about 58 °C and 7 μm thick sections were cut. After dewaxing, sections were blocked with 3% hydrogen peroxide for 5 min. After 2 washes with physiological solution (PBS), sections were incubated at room temperature in blocking buffer (1·5% normal goat serum in PBS) for 30 min. After 2 washes with PBS, the sections were incubated with a solution containing the primary antibody (1% BSA in PBS, 1:1000 diluted anti-p23PL antibody) for 30 min at room temperature. As a negative control, adjacent sections were incubated in the same solution in the absence of the anti-p23PL antibody. All the sections were washed 3 times in PBS. The protein/anti-p23PL antibody was revealed using Super Sensitive kit Multilink Immunodetection System (BioGenex, San Ramon, CA, USA). After staining with diaminobenzidine (DAB), the chromogen substrate specific for peroxidase, the sections were stained with haematoxylin and then washed in water. The sections were then dehydrated, mounted and examined under bright-field illumination.

All immunohistological tests were performed 3 times on whole mounted male and female *P. laevis*. Light micrographs were obtained with a Leitz photomicroscope.

RESULTS

Male *P. laevis* have 6 cement glands, round to oval in shape, ranging in length from 0·5 to 0·9 mm and in width from 0·3 to 0·7 mm. The 3 pairs of glands are arranged in 3 different levels in tandem. Each gland has a cortical glandular zone (Fig. 1A) about 20 μm in thickness, bearing numerous nuclear fragments (see Dezfuli *et al*. 1998) (Fig. 1A). The diameter of nuclei ranges from 10 to 14 μm. Each nucleus has a lobed shape with an irregular border. The glandular zone or cytoplasmic layer surrounds a space ('vesicular region') for accumulation of the cement secretion (Fig. 1A).

Immunohistochemistry of male and female acanthocephalans

Sections of mature males and females were subjected to the immunostaining procedure to ascertain the localization of cement protein within the acanthocephalan body. Some longitudinal sections of the posterior end of a male in which 3 cement glands are

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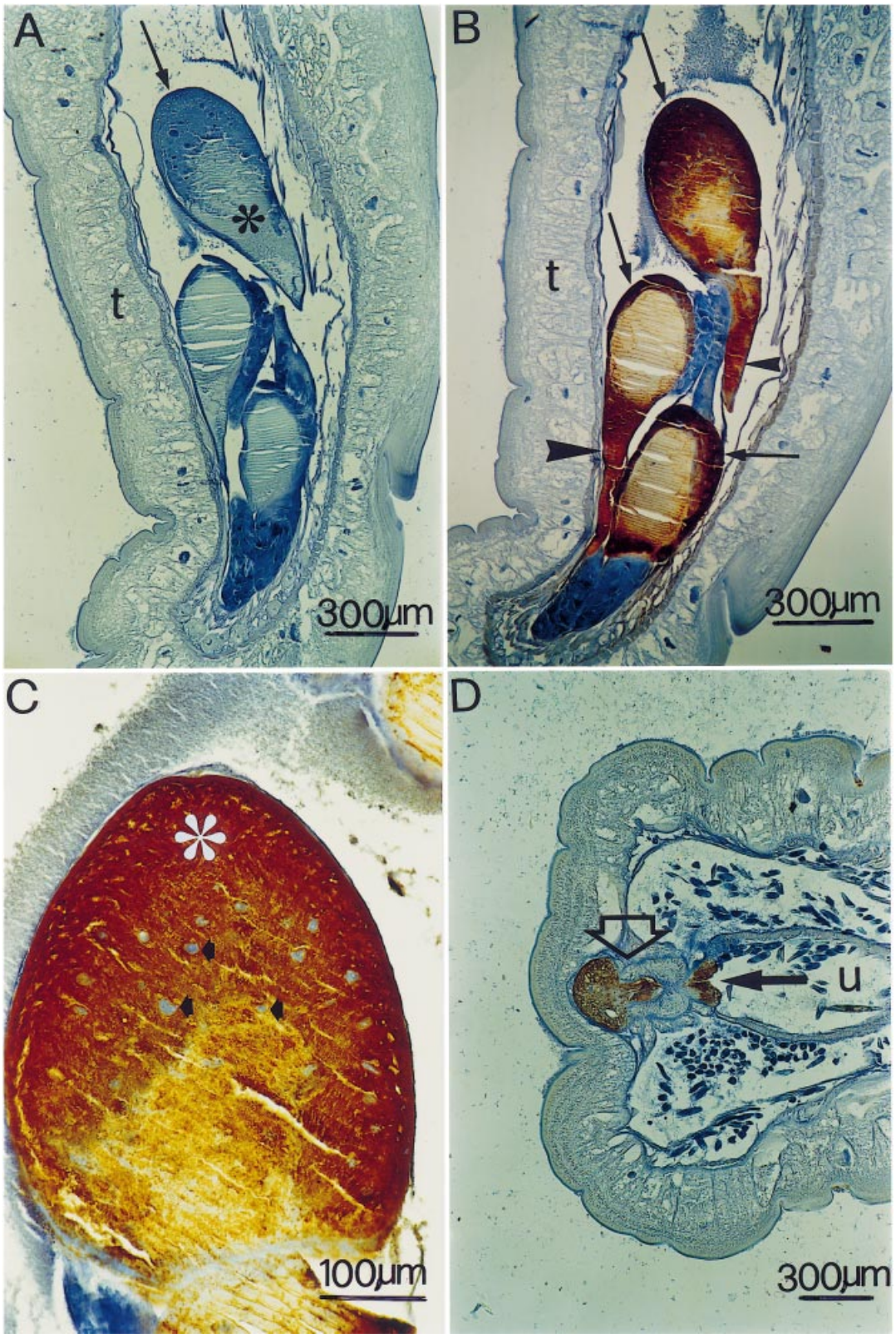

Fig. 1. (A) Micrograph showing the posterior extremity of a male *Pomphorhynchus laevis* used as control, note the cement glands. Cortical zone (arrow) and vesicular zone (asterisk) are evident. t, Tegument. (B) Male acanthocephalan incubated with antibody anti-p23PL. Occurrence of cement (dark brown) mainly in the cortical zones (arrows) of the cement glands as well as in gland ducts (arrow heads) can be seen. t, Tegument. (C) High magnification of a single gland, note the presence of nuclei (arrows) stained in blue within the cortical zone filled with cement (white asterisk). (D) Micrograph shows the posterior end of a female. Antibody anti-p23PL revealed the penetration of cement from the vaginal lumen (empty arrow) to the posterior lumen of the uterus (arrow). u, Uterus.

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evident were incubated in the absence (Fig. 1A, negative control) or in the presence (Fig. 1B) of a solution containing the affinity-purified anti-p23PL antibody at 1:1000 dilution. Previous immunohistological experiments were performed in order to determine the optimal working dilution of this antibody (data not shown). As is clearly evident in Fig. 1B, the antibody revealed the presence of p23 only in the cortical cytoplasmic region and vesicular zone and gland-associated duct (Fig. 1B, brown). In contrast, in the absence of anti-p23PL antibody, all acanthocephalan structures and organs are blue-stained (Fig. 1A), confirming the specificity of the produced antibody.

Under higher magnification of a single gland, specific immunoreactivity was observed. The cortical glandular region was stained dark brown; within it were found many nuclear fragments stained in blue (Fig. 1C). Furthermore, the nuclei from the cortical area migrate to the 'vesicular region' and during this process they degenerate (Fig. 1C, nuclear fragments stained in blue).

Immunohistochemistry was performed also on female individuals, some of which retained a copulatory cap. In this group, the antibody anti-p23PL reacted only with the posterior ends of inseminated females (Fig. 1D), mainly at the copulatory cap level. In this group of females, cement was observed on the surface of the genital pore, as well as inside the vagina and uterus.

DISCUSSION

Conflicting observations exist on the nature of cement in Acanthocephala. The cement has been considered to be mucilaginous material (Van Cleave, 1949), a proteinaceous substance (Haley & Bullock, 1952), or a mixture of glycogenic and galactogenic materials (Rengaraju & Das, 1976). The result of a comparative histochemical investigation of the distribution of alkaline phosphatase activity in 23 species of acanthocephalans was published by Bullock (1958). With reference to the cement glands, the author obtained positive results for only 2 species. The cement was stained an intensive blue-black colour with Heidenhain's iron haematoxylin and it was supposed to contain protein (Parshad & Crompton, 1981). In contrast, Asaolu (1981) suggested it is possible that the secretory granules (dark-drops) seen in the cement glands and their ducts are actually lipids.

Recently, electrophoretic analysis of cement glands of *Pomphorhynchus laevis* showed the existence of a protein with molecular weight of 23 kDa (Dezfuli *et al*. 1998). It has been widely assumed that the copulatory cap in Acanthocephala is formed by the secretion(s) of cement glands; unequivocal confirmation in the case of *P. laevis* based on the present immunohistochemical survey has now been obtained.

During this survey we noticed the presence of p23 cement protein in the posterior lumen of the uterus of female *P. laevis* as well as on the external surface of the gonopore. This study has documented for the first time the depth of cement penetration within the genital tract of female acanthocephalans. We can now conclude that the 23 kDa protein is the product of cement glands that forms the copulatory cap. The copulatory caps are often noticed around the posterior ends of female worms (Van Cleave, 1949; Lawlor *et al*. 1990) and sometimes on male bodies (Abele & Gilchrist, 1977; Parshad & Crompton, 1981; Parveen, 1990; Richardson *et al*. 1997).

Concerning sexual congress and copulation, in the fish *Lepomis cyanella* experimentally infected with the acanthocephalan *Leptorhynchoides thecatus*, 2 male worms were observed *in copula* (Richardson *et al*. 1997). These authors suggested that male–male copulation is indiscriminate mating and could occur occasionally. On the other hand, such events between male *Moniliformis moniliformis* were interpreted in the context of parental investment and sexual selection (Abele & Gilchrist, 1977). These authors proposed that the evolution of the cement gland and sexual behaviour was considered to be the result of sexual selection. Cement and capping behaviour both have a function in preventing the escape of sperm, and most likely evolved in response to sexual selection and help to avoid subsequent insemination. The copulatory cap minimizes losses of the male's spermatozoa from the female body, and might prevent insemination by other males (Crompton, 1985). In the context of sexual selection, the copulatory cap of *M. moniliformis* was thought to be analogous to a 'chastity belt' (Krebs & Davies, 1981). The occurrence of a copulatory cap or mating plug is common among some orders of insects and has the same putative functions as in the Acanthocephala (Parker, 1970; Aiken, 1992). Another function of cement which has been generally assumed is to assist in holding the partners together during copulation (Parshad & Crompton, 1981; Mehlhorn *et al*. 1988). This function is essential because insemination is dependent on the meticulous adjustment of the bursa around the genital region of the female (Van Cleave, 1949).

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