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Potential involvement of C_3 complement factor in amphibian fertilization

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Abstract

We have assessed the potential involvement of C_3, the third complement factor, and its receptor in *Bufo arenarum* fertilization. We show that a polyclonal antibody against a *B. arenarum* C_3-like factor (C_3Ba) reacts specifically with components of the extracellular matrix (ECM) of coelomic eggs and the cell membrane of uterine eggs. Interestingly, we have identified a 163 kD protein immunoreactive with a monoclonal antibody against the CD11b α chain of the human C_3 receptor on the cell membrane of the animal pole of uterine eggs, the site of entry of the sperm, but not in coelomic eggs (CR3*Ba*). Treatment of coelomic eggs with a pars recta oviductal-like protease, trypsin, induced the translocation of C_3Ba from the ECM to the cell membrane. Furthermore, inhibition of CR3*Ba* by trypan blue, as well as inhibition of C_3Ba by anti-C_3Ba on uterine eggs impaired fertilization, whereas identical treatment on sperm cells did not alter percentage fertilization. Our results suggest, (A) that changes in the localization of C_3Ba from the ECM to the cell membrane may be triggered by trypsin-like proteases during passage of eggs through the oviduct; and (B) that C_3Ba/CR3*Ba* may be involved in *B. arenarum* fertilization.

Keywords: Complement; Fertilization; *Bufo arenarum*

1. Introduction

It is well established that the extracellular matrix (ECM), also called vitelline envelope, of amphibian coelomic eggs is comprised of tightly packed filaments (Mariano et al., 1984). During its journey through the uppermost segment of the oviduct named pars recta, structural changes on the ECM induced by trypsin-like enzymes (Grey et al., 1977; Miceli et al., 1978; Yoshizaki and Katagiri, 1981) turn the eggs susceptible to the action of acrosome proteases, thus allowing fertilization. Furthermore, it is known that acrosome reaction occurs when the sperm contacts outer-layers of the egg (Cabada et al., 1989). Despite numerous studies toward elucidating the mechanisms involved in egg–sperm recognition, its specie-specificity and fusion, it is still not clear how these events occur. We have suggested previously the involvement of serum proteins present in oviductal fluid and ECM of eggs in the recognition and fusion between the egg and the sperm in

Abbreviations: IgG, immunoglobulin G; kD, kilodalton; NMS, normal mouse serum; NRS, normal rabbit serum; PBS, phosphate buffered saline; RT, room temperature; SDS, sodium dodecyl sulfate.

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Bufo arenarum (Sueoka et al., 1997; Llanos et al., 1998). More precisely, we have considered over a decade ago (Mansilla, 1986) the possibility of the presence of complement in the ECM of *B. arenarum* oviductal eggs. This was based on the observation of lysis of the ECM of oviductal eggs by purified antibodies against pars recta secretions, potentially complement-mediated. One of the serum components C_3, the third complement factor, is a pivotal element of the complement system in the classical and alternative pathway (Lambris, 1988; Lambris et al., 1994) both described in amphibian (Fujii et al., 1985; Fernandez, 1986; Grossberger et al., 1989; Kunath-Muglia et al., 1993; Llanos et al., 1999). Studies from our laboratories have recently shown the presence of a C_3b-like protein in *B. arenarum* serum (Llanos et al., 1999). C_3 binds specifically to CD11b/CD18 complex, an α/β heterodimeric glycoprotein which belongs to the β2 integrin family also named CR3 in mammals (Wright et al., 1987). CR3 is expressed on the surface of human circulating monocytes, granulocytes, NK cells and in subsets of macrophages. C_3-specific ligands have also been shown in *Xenopus laevis* macrophages (Sekizawa et al., 1984) and in hamster eggs (Anderson et al., 1993). Involvement of complement has been suggested in various aspects of mammalian reproduction including estrual cycle regulation of complement activity in follicular fluid (Oliphant et al., 1984; Fahmi and Hunter, 1985), presence of complement regulatory proteins in human eggs (Fenichel et al., 1995), gamete interaction (Anderson et al., 1993), and acrosome reaction (Cabot and Oliphant, 1978; Suarez and Oliphant, 1982; Price et al., 1984). Furthermore, a protein named membrane cofactor protein identified in the inner membrane of human sperm cells with the ability of binding C_3b has been thought to be involved in mammalian fertilization (Cervoni et al., 1992; Anderson et al., 1993). The fact that *B. arenarum* pars recta secretions contain trypsin-like proteases hormone-regulated during sexual cycle (Fernandez et al., 1984, 1997), capable of activating the complement cascade, in analogy with hormone-regulation of complement activity in bovine (Fahmi and Hunter, 1985) and rabbit (Oliphant et al., 1984) follicular fluid, suggested further a potential association between these phenomenon in *B. arenarum*. Therefore, the goals of this study were, (i) to determine if C_3- and CR3-like factors are present in eggs and/or sperm cells in *B. arenarum*; (ii) to assess the biological consequences of the inhibition of C_3 and CR3 analogs in *B. arenarum* fertilization.

2. Materials and methods

2.1. Animals

Mature *B. arenarum* toads were collected in Tucuman, Argentina, between May and October and housed at 25°C.

2.2. Gametes

2.2.1. Eggs

In order to obtain coelomic eggs, the pars recta portion of the oviduct of anesthetized animals was tied at both ends, at the ostium and at the initiation of pars pre-convoluta. After surgery, ovulation was induced by subcutaneous injection of homogenates of homologous pituitaries. Seven to twelve hours later, the eggs were removed from the peritoneal cavity and placed in Ringer solution containing 10 mM Tris pH 7.6 (Ringer–Tris). In order to obtain uterine eggs, occlusion of the pars recta was omitted. After 7–12 h injection with homologous pituitary, eggs were removed from the ovisac and dejellied as described previously (Cabada et al., 1989).

2.2.2. Sperm cells

Sperm cells from *B. arenarum* were obtained by maceration of testes in Ringer–Tris containing 0.25 M sucrose. The cell suspension was then filtered through a 30-μm mesh nylon screen and centrifuged at $1600 \times g$ for 5 min at 4°C. The supernatant was discarded and the cell pellet resuspended in Ringer solution and counted in an hemocytometer under microscope (Cabada et al., 1989).

2.3. Immunodot-blot procedure

We have reported previously (Llanos et al., 1999), the isolation of a C_3 analog from *B. arenarum* serum using yeast cell wall extract zymosan (Zy) and subsequent preparation and purification of antiserum (anti-C_3*Ba*). The C_3-specificity of the protein A-Sepharose chromatography purified anti-C_3*Ba* polyclonal antibody was performed by

immunodot-blot procedure as follows (Llanos et al., 1999): 15 μg zymosan-bound C_3Ba (Zy-C_3), activated Zy as negative control, *B. arenarum* serum (*Ba*S-C_3^+) or *B. arenarum* serum depleted of C_3Ba with Zy (*Ba*S-C_3^-) were loaded onto a nitrocellulose membrane (Millipore Corp., Bedford, MA). The membrane was blocked subsequently with phosphate buffered saline (PBS) containing 2% bovine serum albumin (PBS-BSA) and then incubated with anti-C_3Ba. Protein A-Sepharose purified pre-immune rabbit serum (Llanos et al., 1999) was included as negative control (NRS). After three washes with PBS containing 0.05% Tween-20 (PBST) the membrane was incubated with anti-rabbit immunoglobulin G (IgG) (1:1000 in blocking solution) labeled with biotin and then with extravidin–peroxidase according to Sigma Co. (St. Louis, MO). Following three washes with PBST, color was developed with 0.1 mg/ml 3,3′-diaminobenzidine (3,3′,4,4′-tetra-aminobiphenyl) and 0.01% H_2O_2 in 0.1 M Trizma base pH 7.2.

2.4. Protein extraction from the cell surface of uterine eggs

Vitelline envelopes were removed with forceps from dejellied eggs. The peeled eggs were then incubated with Ringer–Tris pH 8.0 containing 1%

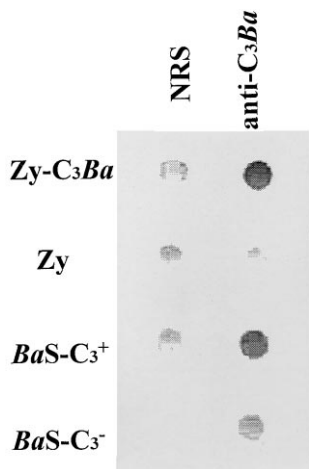

Fig. 1. Specificity of anti-C_3Ba antibody evaluated by immunodot-blot procedure. Zy-C_3Ba (activated Zy bound to C_3Ba), activated Zy (Zy), *B. arenarum* serum (*Ba*S-C_3^+) and *B. arenarum* serum C_3-depleted with activated Zy (*Ba*S-C_3^-) were loaded onto nitrocellulose membrane. Purified anti-C_3Ba (11.2 μg/ml) was used as primary antibody. Purified normal rabbit serum (NRS) was used as negative control.

Nonidet-P 40 (Sigma) and 2 mM PMSF for 20 min at room temperature (RT). The incubation medium was recovered and centrifuged at 7000 × *g* at 4°C during 10 min. The supernatant was thereupon dyalized against 50 mM Tris–HCl pH 6.8 at 4°C overnight. Samples were then loaded in a 7.5% polyacrylamide gel electrophoresis in non-reducing conditions and transferred to nitrocellulose membrane as described below.

2.5. Polyacrylamide gel electrophoresis

Protein components (40 μg) extracted from the plasma membrane of uterine eggs were mixed with a nondenaturing sample buffer (50 mM Tris pH 6.8, 10% glycerol, 2% w/v sodium dodecyl sulfate (SDS) and 0.001% bromophenol) to detect C_3Ba, whereas 100 μg total *B. arenarum* blood was mixed with a denaturing sample buffer (0.05% v/v β-mercapto-ethanol, 50 mM Tris pH 6.8, 10% glycerol, 2% w/v SDS and 0.001% bromophenol) to detect CR3. Samples were subsequently boiled in a 100°C water bath for 10 min and loaded in a polyacrylamide gel. The stacking gel consisted of a 5% (w/v) acrylamide in 130 mM Tris pH 6.7 containing 0.1% SDS and a 7.65% separating gel made in 360 mM Tris pH 8.7 containing 0.1% SDS (Laemmli, 1970). The running buffer was comprised of 25 mM Tris, 250 mM glycine pH 8.3 and 0.1% SDS. Electrophoretic fractionation was carried out on a Bio-Rad apparatus at a constant current of 15 mA (all ingredients were from Bio-Rad Lab, Hercules, CA). Samples were loaded in duplicate, one stained with Coomassie blue and the other processed for Western blotting as described below.

2.6. Western blotting procedure

Following electrophoresis, proteins were electrotransferred onto a nitrocellulose membrane (Millipore Corp.). The filters were then blocked with 5% non-fat dry milk in PBST, then incubated overnight at 4°C with 40 μg/ml rabbit anti-C_3Ba antibody or 1 μg/ml mouse anti-human CR3 antibody. Bands were visualized with biotinylated anti-rabbit or anti-mouse IgG (1:1000 in blocking solution) followed by extravidin–peroxidase complex and 0.1 mg/ml 3,3′-diaminobenzidine (3,3′,4,4′-tetra-aminobiphenyl) and 0.01% H_2O_2 in 0.1 M Trizma base pH 7.2 (Sigma).

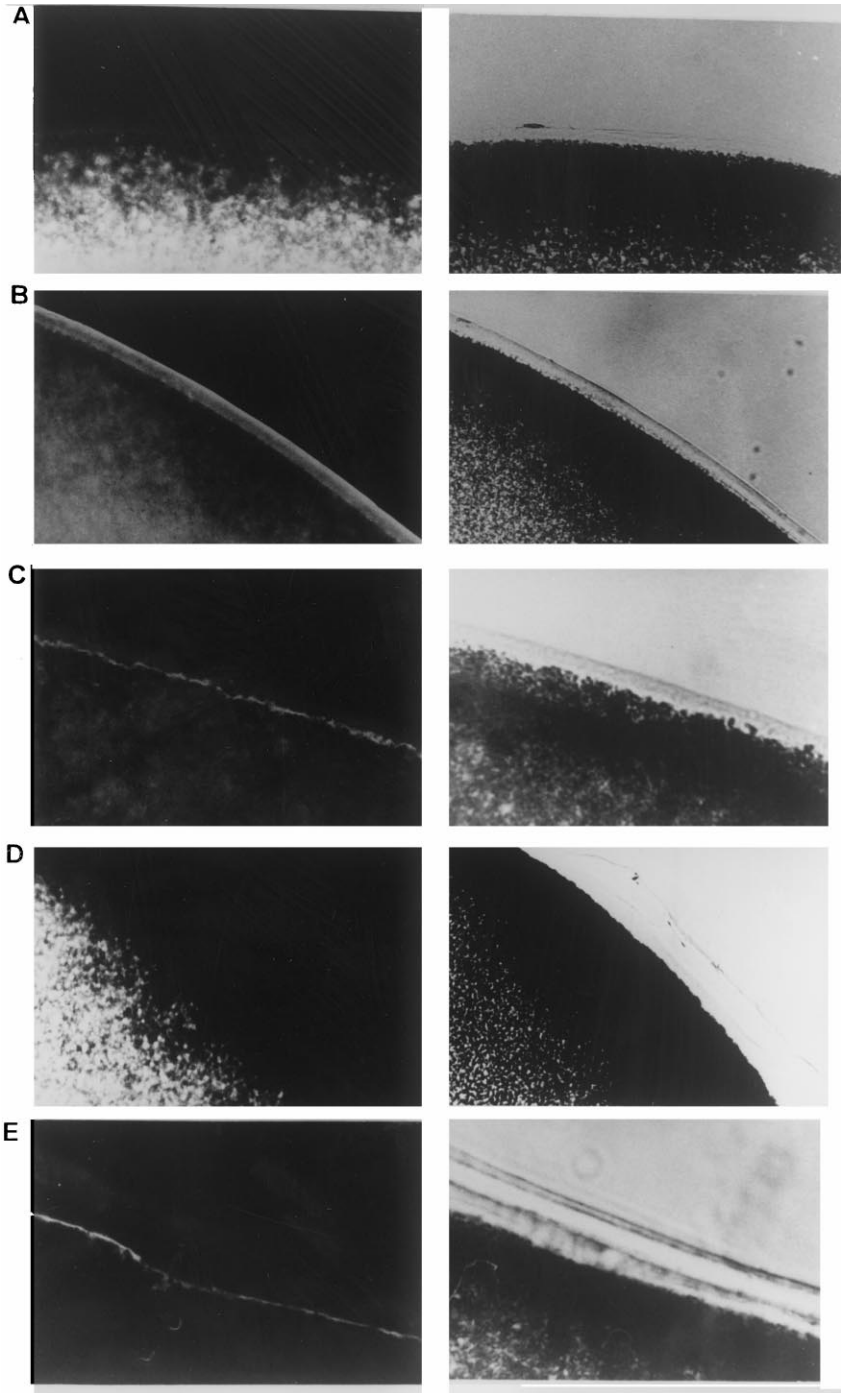

Fig. 2. Immunofluorescence to C_3*Ba* on oocytes. Left: immunofluorescence. Right: view under optical microscope. (A) Coelomic oocyte treated with NRS. (B) Coelomic oocyte treated with anti-C_3*Ba*. (C) Trypsinized coelomic oocyte treated with anti-C_3*Ba*. (D) Uterine oocyte treated with NRS. (E) Uterine oocyte treated with anti-C_3*Ba*.

2.7. Immunofluorescence procedure

Coelomic and uterine eggs were fixed in a solution comprised of 3.7% formaldehyde, 0.25% glutaraldehyde and 0.2% Triton X-100 in PBS pH 7.4 overnight at RT. Samples were then embedded in Hystowax (Merck, Whitehouse Station, NJ). Deparaffinized sections were rinsed with PBS and

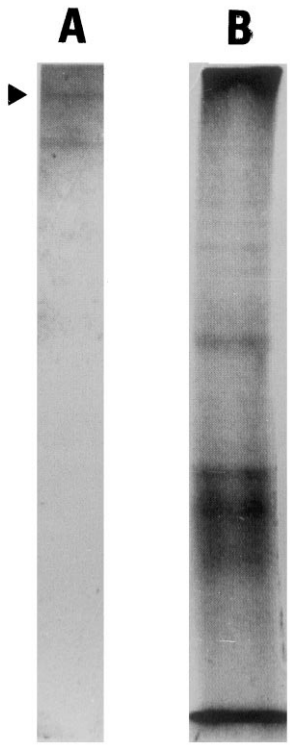

Fig. 3. C_3Ba present on the surface of the cell membrane of uterine eggs. (A) Western blotting showing a band reactive with anti-C_3Ba at 190 kD. (B) Coomassie blue staining of a duplicate sample.

blocked with PBS-BSA for 30 min at RT. Subsequently, anti-C_3Ba (40 μg/ml) prepared in our laboratories (Llanos et al., 1999), anti-CR3 from Sigma (40 μg/ml), or protein A-Sepharose purified preimmune (Llanos et al., 1999) normal rabbit serum (NRS) diluted in blocking solution was added. After 1 h at RT, samples were washed with PBST and incubated with FITC-labeled anti-mouse (to detect CR3*Ba*, Fig. 6) or anti-rabbit (to detect C_3Ba, Fig. 2) IgG from Amersham (Arlington Heights, IL). Following 1-h incubation at RT, samples were thoroughly

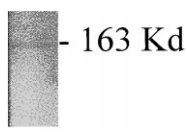

Fig. 4. Western blot to CR3*Ba* in *B. arenarum* blood using anti-human CR3 monoclonal antibody. A band at 163 kD is observed.

rinsed with PBS and mounted in 50% glycerol-PBS. Slides were examined under a Nikon fluorescence microscope and photographed with a Kodak Tri-X Pan (ASA 400) camera.

Immunofluorescence procedure to detect reactivity with anti-CR3 antibody in leukocytes (Fig. 5) was performed in human and *B. arenarum* blood as follows: blood was collected in tubes containing heparin-EDTA. Cells were centrifuged at $320 \times g$ and supernatant was discarded. Cells were then resuspended in PBS-BSA, blocked with PBS-BSA and treated with anti-CR3 antibody (20 μg/ml, Sigma) in PBS-BSA (2×10^7 cells per ml) or with purified normal mouse serum (NMS) provided by Sigma as negative control. After 1-h incubation at 4°C, cells were thoroughly washed, then incubated with secondary anti-mouse FITC-labeled IgG for 1 h. Cells were then washed and viewed under microscope as described above.

2.8. Fertilization assay

Briefly, uterine eggs were placed in plastic culture plates containing trypan blue (1, 25, 50, 75, 100 and 1000 μM), anti-C_3Ba (15, 30, 60 and 120 μg/ml), purified pre-immune serum or vehicle (Ringer solution) for 1 h at RT. After thoroughly washing with Ringer solution, eggs were inseminated with 1×10^6 sperm cells per ml in 10% Ringer at RT. Following 30-min incubation, eggs were washed with Ringer and viewed under stereoscopic microscope. Percentage fertilization was assessed in stage of morula.

3. Results

3.1. Specificity of anti-C_3Ba antibody

To evaluate the specificity of the anti-C_3Ba antibody, immunodot-blot procedure was performed as described in Section 2. Fig. 1 shows high reactivity of anti-C_3Ba (11.2 μg/ml) with Zy bound to C_3Ba (Zy-C_3Ba) and *B. arenarum* serum (*Ba*S-C_3^+) whereas little reactivity was found with activated Zy alone (Zy) or with *B. arenarum* serum pre-adsorbed with activated Zy (C_3-depleted serum; *Ba*S-C_3^-). Control with protein-A Sepharose purified pre-immune NRS showed little reactivity.

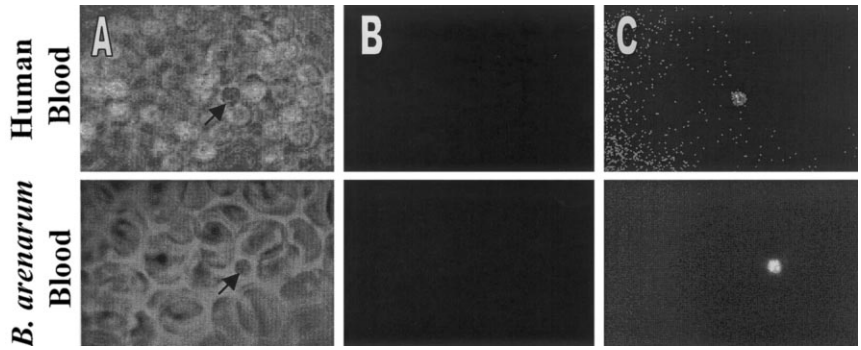

Fig. 5. Immunofluorescence. The results indicate that the monoclonal antibody against human CR3 cross-reacts with proteins present in *B. arenarum* leukocytes. Human blood was used as positive control. (A) View under optical microscope. (B) View under fluorescence microscope of cells treated with NMS as negative control. (C) Cells treated with anti-CR3. The arrow points at leukocytes.

3.2. Evaluation of C_3Ba localization in eggs and sperm cells

We have evaluated the presence of C_3Ba in eggs and sperm cells by immunofluorescence procedure as described in Section 2. Fig. 2 shows the results obtained by immunofluorescence (left) and the corresponding profile observed by optical microscope (right). Fig. 2A and D correspond to coelomic and uterine eggs, respectively, incubated with purified pre-immune NRS as negative controls. Anti-C_3Ba reacted specifically with proteins localized on the ECM of coelomic eggs (Fig. 2B) whereas in uterine eggs, the fluorescence was visualized in the cell membrane (Fig. 2E). In view of the previous observations (Cabada et al., 1978; Miceli et al., 1978, 1987; Mansilla-Whitacre et al., 1992) showing ultrastructural changes in the ECM of coelomic eggs in contact with pars recta oviductal fluid containing trypsin-like proteases, we have treated coelomic eggs with 1 mM trypsin for 30 min at RT to address the possibility that oviductal trypsin-like proteins may be responsible for the changes on C_3Ba localization from the vitelline envelope to the cell membrane. Interestingly, Fig. 2C shows C_3Ba in the cell membrane of coelomic eggs after treatment with trypsin resembling the pattern of reactivity found in uterine eggs (Fig. 2E). The vitelline envelope was partially separated with a needle to enable proper visualization of fluorescence in the cell membrane as described by Nadai et al. (1996). In order to confirm the identity of the immunoreactive protein, we extracted the protein components of the cell membrane of uterine eggs with Nonidet-P 40 as described in Section 2, and performed Western blotting in non-denaturing conditions. A band reactive with anti-C_3Ba was visualized at 190 kD (Fig. 3A), similar to that reported for C_3b (Llanos et al., 1999). Fig. 3B shows the Coomassie

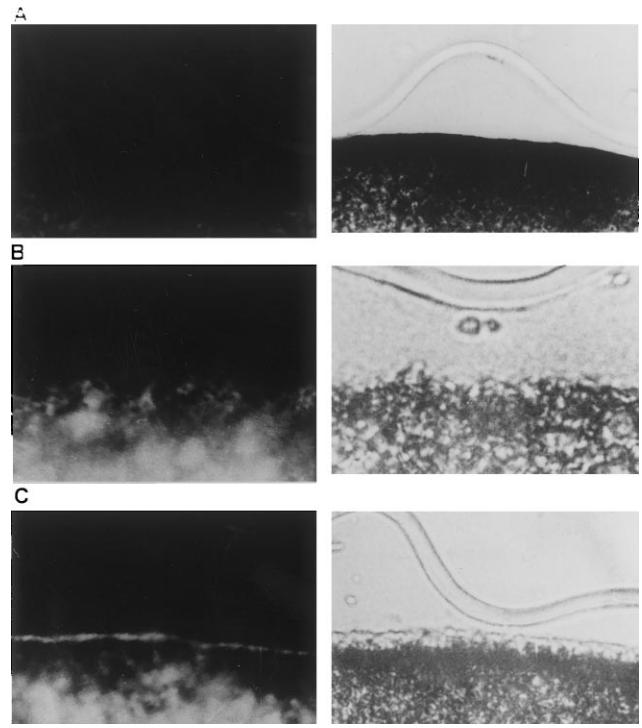

Fig. 6. Immunofluorescence to CR3*Ba* in uterine oocytes. Left, immunofluorescence; right, view under optical microscope. (A) Incubation with NMS. (B) View of the vegetative pole of an oocyte treated with anti-CR3 antibody. (C) View of the animal pole of an oocyte treated with anti-CR3 antibody.

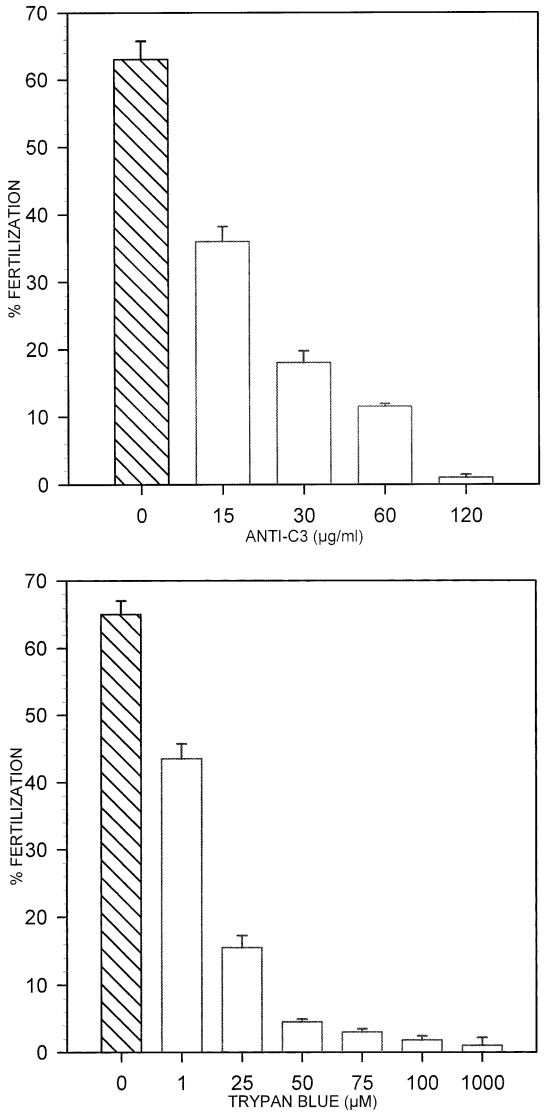

Fig. 7. Effect of anti-C_3*Ba* antibody or trypan blue on percentage fertilization. (A) Percentage fertilization was inhibited by treatment of uterine oocytes with increasing concentrations of anti-C_3*Ba* antibody. (B) Incubation of uterine oocytes with increasing concentrations of trypan blue inhibited percentage fertilization in a dose-dependent manner. Controls with NRS (0 µg/ml anti-C_3*Ba* or 0 µM trypan blue) show high percentage fertilization.

blue staining of a duplicate sample. The 190 kD band is not visible with Coomassie blue suggesting a low relative amount of this protein in the sample.

We have found absence of reactivity to anti-C_3*Ba* antibody in intact sperm cells, sperm cells following acrosome reaction or in testicular tissue by immunofluorescence procedure (data not shown).

3.3. Assessment of CR3Ba in eggs and sperm cells by immunofluorescence

In mammals, C_3b binds specifically to its receptor CR3 (CD11b/CD18) during processes involving cell–cell interaction and phagocytosis. We have used a monoclonal antibody that recognizes the CD11b α chain of the human CD11b/CD18 complex. We have determined that this antibody also reacts with a band of similar size (163 kD) in *B. arenarum* leukocytes (Fig. 4). Because of the large size of *B. arenarum* erythrocytes, which unlike human erythrocytes contain nuclei, classical cell-separation procedures were not suitable. Therefore, both Western blotting (Fig. 4) and immunofluorescence procedures (Fig. 5) with anti-CR3 antibody were performed using total blood. Fig. 6 shows the localization of immunoreactivity with anti-CR3 antibody in uterine eggs (CR3*Ba*). Results obtained by immunofluorescence are in the left and the corresponding profile observed by optical microscope in the right. CR3*Ba* was found only in the animal pole (Fig. 6C) whereas absence of reactivity was observed in the vegetative pole (Fig. 6B). Fig. 6A corresponds to treatment with secondary antibody alone as negative control. Immunofluorescence to CR3*Ba* on coelomic eggs and sperm cells showed negative results (data not shown).

3.4. Inhibition of C_3Ba and CR3Ba on eggs impairs fertilization

Eggs were incubated with increasing concentrations (15–120 µg/ml) of anti-C_3*Ba* antibody, washed thoroughly with Ringer and incubated with sperm cells as described in Section 2. Antibodies to C_3*Ba* induced inhibition of fertilization in a dose-dependent manner (Fig. 7A). Affinity purified NRS did not exert any inhibitory activity. We have then attempted to evaluate the effect of inhibition of CR3 on fertilization by using the monoclonal antibodies against human CR3. Although inhibition could be observed with this approach in three separate experiments, the results were not quantitatively reproducible. Since C_3*Ba* may be bound to its receptor CR3*Ba* in the cell membrane of uterine eggs encumbering epitopes potentially reactive to the antibody, we have used an alternative strategy to inhibit CR3. This strategy was based on previous reports on the ability of trypan blue to inactivate CR3 in the cell

membrane of polymorphonuclear cells impairing complement-mediated phagocytosis (Guckian et al., 1978). Furthermore, incubation of uterine eggs with trypan blue (1–1000 μM) prior to insemination impaired fertilization in a dose-dependent fashion (Fig. 7B). In contrast, treatment of sperm cells with trypan blue in either 10% Ringer or in 1 × Ringer solution did not alter the morphology and/or motility of sperm cells (cells were trypan blue negative indicating high cell viability). Sperm cells treated with trypan blue and non-treated control cells washed with Ringer and resuspended in 10% Ringer were equally effective fertilizing uterine eggs (data not shown).

4. Discussion

The presence of complement factors in the reproductive tract has been described assertively in mammals (Cabot and Oliphant, 1978; Oliphant et al., 1984; Price et al., 1984; Cervoni et al., 1992; Anderson et al., 1993). The participation of complement in the acrosome reaction in rabbit (Cabot and Oliphant, 1978) and human sperm (Anderson et al., 1993) has been suggested previously. Inversely, the ability of spermatozoa to activate the complement cascade has also been suggested (Suarez and Oliphant, 1982). Cabot and collaborators (Cabot and Oliphant, 1978) suggested the possibility that C_3 complement factor activated by components of follicular fluid may be involved in triggering the acrosome reaction in rabbit via alternative cascade. Most interestingly, it was later observed that human sperm cells that had undergone the acrosome reaction express a unique tissue-specific variant of C_3-receptor named membrane cofactor protein (CD46) (Cervoni et al., 1992; Anderson et al., 1993) but not CR3 (Fusi and Bronson, 1991), and that damaged or dead sperm activate the alternative complement pathway and bind C_3b. These observations plus additional findings showing that specific antibodies to CR3 bind to the human egg plasma membrane and inhibit fertilization (Anderson et al., 1993) lead the authors to suggest the possibility that C_3

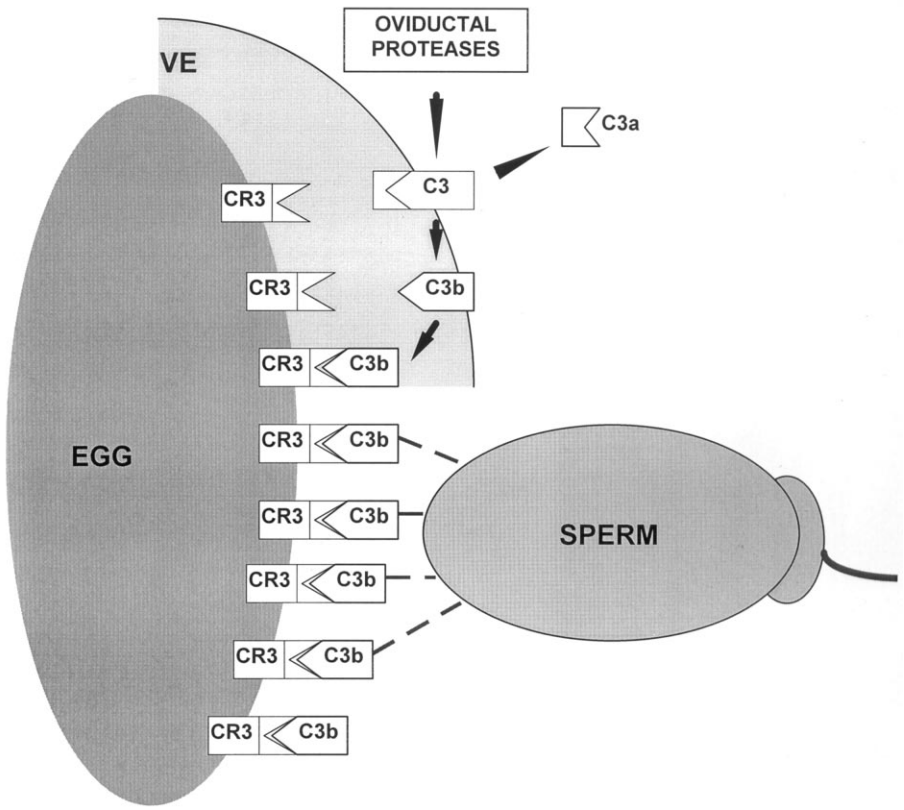

Fig. 8. Proposed mechanism of the potential involvement of C_3Ba/CR3*Ba* in *B. arenarum* fertilization.

may be involved in sperm–egg interaction in mammals. Moreover, complement regulatory proteins have been described in human eggs (Fenichel et al., 1995). The results shown in this report indicate that C_3Ba and CR3*Ba* may be involved in *B. arenarum* fertilization. C_3Ba is clearly present on the ECM of coelomic eggs and the cell membrane of the animal pole of uterine eggs (Figs. 2 and 6). Treatment of coelomic eggs with trypsin changed the localization of C_3Ba to the cell membrane suggesting the possibility that trypsin-like proteins in the oviduct, such as those described in pars recta secretions of *B. arenarum*, may play a fundamental role in activating C_3 as suggested by Cabot in a mammalian system (Cabot and Oliphant, 1978). Moreover, the molecular weight of C_3Ba extracted from the cell membrane of uterine eggs resemble that of human C_3b (190 kD). C_3Ba and CR3*Ba* were not found in sperm cells. Fig. 8 represents a potential mechanism by which C_3Ba and CR3*Ba* may participate in *B. arenarum* fertilization. We suggest that C_3Ba is one of multiple serum components that incorporate into the ECM of coelomic eggs during development (Sueoka et al., 1997; Llanos et al., 1998). Trypsin-like proteases from pars recta secretions may activate C_3Ba inducing the translocation of a C_3b analog to cell membrane binding specific ligands (CR3*Ba*). Because human monocytes, macrophages and polymorphonuclear cells expressing complement receptors recognize and phagocyte particles coated with C_3b (Wright et al., 1987), we foresee the possibility that *B. arenarum* eggs may recognize and engulf the sperm in a similar fashion. In this regard, the observations of Koehler and collaborators (Koehler et al., 1991) showing that zona free hamster and human eggs are capable of binding and engulfing *Candida albicans* without triggering lysosomal activity are highly relevant. Conversely, it has been shown (D'Cruz et al., 1992) that C_3-bound human sperm can be phagocyted by polymorphonuclear leukocytes. In fact, the same research team (D'Cruz and Haas, 1995) have later shown that a C_3 receptor is the primary adhesive glycoprotein involved in neutrophil-mediated immune injury to human sperm. Our results showing that inhibition of C3*Ba* by anti-C3*Ba* and CR3*Ba* by trypan blue on *B. arenarum* eggs impaired fertilization strongly suggest the participation of C_3/CR3 analogs in fertilization in this amphibian specie.

Acknowledgements

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