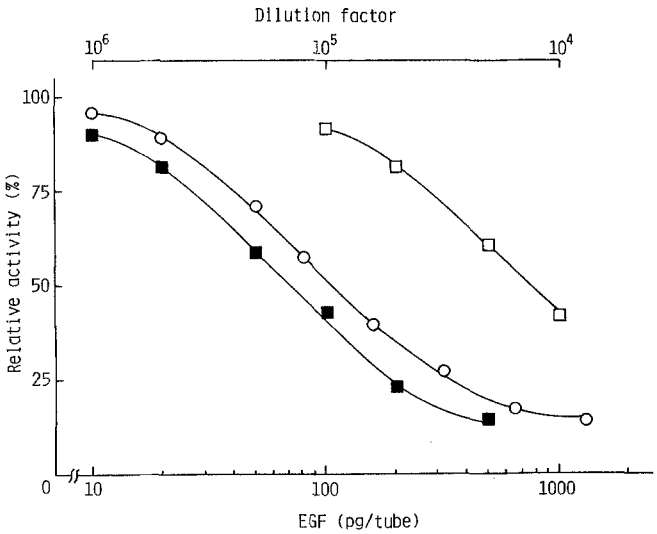

Standard curve for EGF (○) and dilution curves of extracts from male (■) and female (□) mouse submandibular glands. The glands were homogenized with 9 vols of saline and the homogenates centrifuged at 10,000 × g for 20 min. The resulting supernatants were used as extracts.

tissues there was no marked difference in concentration between the two groups. Since intact animals secrete EGF continuously from the submandibular gland and it is acid-stable[7], part of the secreted peptide may remain in a native form on mucous epithelia of the gastrointestinal tract.

The enzyme immunoassay for EGF utilizing the liquid phase double-antibody system described here is quite sensitive. This method can detect concentrations of the peptide as low as 20 pg per tube, and thus the sensitivity is almost the same as that of the widely used radioimmunoassay[9-11]. The EGF-β-galactosidase conjugate prepared in this study contained less than 20% of free β-galactosidase, which could be easily removed by the washing of the antigen-antibody complex precipitate. The conjugate was stable for at least 8 months at 4°C. Moreover, there is no need for the use of radioisotopes throughout the procedure.

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The membrane attack complex of *Xenopus laevis* complement

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Summary. Rabbit erythrocyte membranes lyzed by *Xenopus laevis* serum exhibited a typical ultrastructural complement lesion with an inner diameter of 80 ± 9 Å. The protein pattern associated with lyzed membrane is compared to a similar human preparation.
Key words. Xenopus laevis; complement lesions; membrane attack complex; rabbit erythrocytes.

The membrane attack complex (MAC) of complement is a self-assembly multimolecular complex which is capable of impairing biological membranes[3]. The ultrastructure and biochemical composition has been well investigated in the human[3,4]. Complement lesions have also been described in other mammals[5], but, only in a few recent reports in lower vertebrates, including nurse shark[6], rainbow trout[7] and cobra[8]. Little information has been gathered on the amphibian complement system. An antibody-dependent hemolytic system is well established[9-11] and an alternative pathway has also been suggested[12]. C_{1q} protein, isolated from the bullfrog[13], C_3[12] and C_4[14] from the clawed frog are, so far, the only components which have been characterized in amphibians.

We have undertaken a study to investigate the amphibian MAC. In this report the ultrastructure and biochemical composition of the lesions produced by *Xenopus laevis* serum are described, to provide additional information on the amphibian's complement system and its phylogenetic relationship to other vertebrates.

Material and methods. Anesthesized adults of *Xenopus laevis* were bled by cardiac puncture. The blood was allowed to clot for 1 h at room temperature and 3 h at 0°C and then centrifuged at 12,000 × g for 30 min. The serum was stored in 0.5-ml aliquots and frozen at −70°C. Before use the serum was dialyzed extensively in veronal-buffered saline (VBS) pH 7.5. Aliquots of the serum were de-complemented by treatment with Zymosan, according to Sekizawa et al.[12], or by heat inactivation at 56°C for 30 min. Samples of total serum were labeled with ^{125}I (Amersham, England) according to McConahey and Dixon[15]. Human serum was obtained from a normal individual by cubital vein puncture and stored like the *Xenopus* serum until use.

Rabbit erythrocytes (RRBC) were obtained by bleeding a rabbit from the ear vein; before use they were washed twice in isotonic VBS and then three times in VBS containing 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$ and 0.1% gelatine.

Erythrocyte membranes were prepared by incubating 40 λ of packed RRBC with 0.5 ml *Xenopus* or 0.5 ml human serum at 37°C in a water bath until complete hemolysis was apparent. Control erythrocyte membranes were obtained by osmotic lysis in distilled water. The ghosts were pelleted in a microfuge (Beckman model B) and washed three times in ethylene diamine tetraacetic acid Na_2-salt (EDTA) pH 8.0.

Electron microscopic examinations were made at once by drying a drop of erythrocyte membrane suspension on colloidion-carbon-coated 200 mesh copper grids and negatively staining them

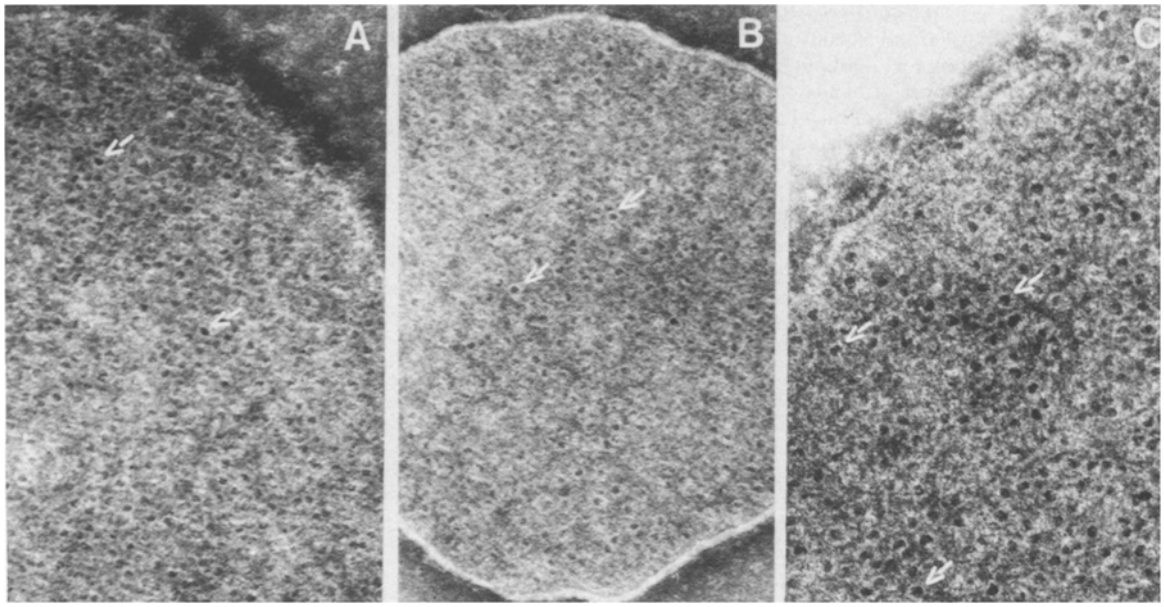

Figure 1. Rabbit red cell membranes lyzed by *Xenopus laevis* serum (panel B) and, for comparison, by human serum (panel A). In both cases, the arrows indicate the typical ring-like complement lesions. × 99,000. Panel C represents a selected image of the membrane lyzed by *Xenopus* serum. The arrows point to the holes. They appear as dark areas surrounded by an oligomeric circular structure. × 160,000.

with 2% sodium phosphotungstate pH 7.5. A Philips electron microscope was used and was operated at 60 kV. Micrographs were taken at a magnification of 99,000- and 132,000-fold.

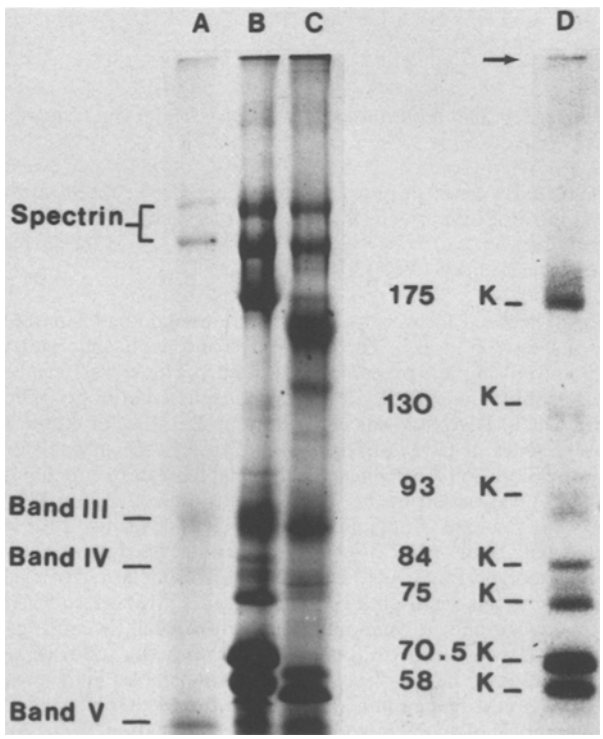

Figure 2. Figure 2 depicts the electrophoretic pattern in 5–10% gradient SDS-PAGE of SDS-dissociated rabbit erythrocyte membranes lyzed by *Xenopus* (track B) and human (track C) sera. Track A represents the SDS-dissociated rabbit erythrocyte membranes obtained by osmotic lysis in distilled water; the nomenclature of the resolved pattern is according to Cohen[16]. Track D shows the protein pattern of the ^{125}I-labeled *Xenopus* serum associated with the rabbit red cell membranes after lysis. The arrow indicates the high molecular weight protein which does not penetrate into a 5–10% gradient gel. Calculated molecular weights of each band are indicated at the left-hand side.

The Lämmli buffer system[16] was used for sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). 40 λ of erythrocyte membranes lyzed by unlabeled and labeled *Xenopus* sera were dissolved in 100 λ of sample buffer containing 3% SDS and boiled for 5 min at 100 °C. 10–30 λ of these preparations were analyzed on 5-10% gradient SDS-PAGE under non-reducing conditions. The rabbit erythrocyte membranes lyzed by human serum and by osmotic lysis were treated as below and used as control. Gels with iodinated samples were dried and exposed to Kodac X-Omat AR films for different periods of time. Ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase B (92,500) β-galactosidase (116,250) and myosin (200,000) (BIO-RAD) were used as molecular weight markers.

Results and discussion. Normal serum of *Xenopus laevis* was able to lyse rabbit erythrocytes. This property is lost after treatment with Zymosan or heat-inactivation, suggesting the involvement of complement in the hemolysis reaction.

As shown in figure 1 (panel B), the rabbit erytrocytes lyzed by *Xenopus* serum subjected to transmission electron microscopy were covered with typical complement-lesions indistinguishable from those caused by human serum used as control (panel A). The holes show a dark central portion surrounded by a clear ring with an inner diameter of 80 ± 9 Å. In regard to size they resemble nurse shark complement lesions (80 Å)[6] but differ from those caused by human (100–110 Å)[5], guinea pig (85-95 Å)[5] and cobra (72 ± 9 Å)[8] complement.

Under stronger magnification (panel C), the surrounding ring appears mostly single, sometimes incomplete, and constitutes an oligomeric structure. The analysis conducted with a 5–10% gradient SDS-PAGE of the proteins associated with the membrane of rabbit erythrocytes after lysis by *Xenopus* and human sera is shown in figure 2. Leaving aside the membrane proteins of the rabbit erythrocytes (track A), the *Xenopus* pattern (track B) appears to differ from the human pattern (track C) in number and molecular weight of the proteins associated with the lyzed membranes.

By using ^{125}I-labeled-*Xenopus* serum to exclude the membrane proteins of the rabbit erythrocytes, we can detect 7 distinct protein bands (track D): a band with a mol.wt of about 175,000, two slightly radioactive bands with a mol.wt of about 130,000 and 93,000, two bands with a mol.wt of about 84,000 and 75,000

and doublet band that has a much stronger radioactive intensity with a mol.wt of about 70,500 and 58,000.

Apart from the protein of mol.wt of about 58,000, this pattern appears to consist of a number of bands of the human MAC even though the molecular weights of each band are higher than the published mol.wt of isolated high purified human MAC components (C_{5b}, 173,000; C_7, 110,000; C_6, 99,000; $C_{8\alpha/\gamma}$, 93,000; C_9, 76,000; $C_{8\beta}$, 70,000)[3,4].

It is interesting to note the *Xenopus* high molecular weight band which did not penetrate in 5–10% gradient gel (arrow in fig. 2, track D) and was resistant to reduction by 2-mercapthoethanol and boiling in SDS (data non shown). It may represent, as in man[18], the polymerized C_9 (poly C_9) responsible for the oligomeric structures observed in the lyzed membranes at the electron microscope.

Structural information on highly purified *Xenopus* MAC, extracted from complement lyzed cells and from the fluid-phase, is needed to establish a more precise relationship with human MAC.

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Effect of phosphorylation of myosin light chains on interaction of heavy meromyosin with regulated F-actin in ghost fibers

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Summary. The binding of phosphorylated heavy meromyosin to regulated F-actin in ghost fibers at high Ca^{2+} concentration increases, and at low Ca^{2+} concentration decreases, the anisotropy of intrinsic tryptophan fluorescence of F-actin. The effect is opposite to the effect of the binding of dephosphorylated heavy meromyosin.

Key words. Phosphorylated heavy meromyosin; regulated F-actin; ghost muscle fiber.

The binding of soluble fragments of myosin, e.g. heavy meromyosin (HMM) and subfragment 1 (S-1) induces significant changes of F-actin conformation[1–7]. The Ca^{2+}-dependent changes of polarized fluorescence of intrinsic tryptophan residues and F-actin-bound 1:N^6-ethenoadenosine 5-diphosphate induced by the binding of heavy meromyosin and subfragment 1 were found to be related to the presence of intact 18-kDa light chains[8]. Recently, the effect of phosphorylation of 18-kDa light chains on the changes of polarized fluorescence of intrinsic tryptophan residues and unregulated F-actin-bound rhodamin-phalloidin in ghost fibers, induced by the binding of heavy meromyosin, was observed to be dependent on the concentration of free calcium ions[9–11].

In the present study changes of polarization of intrinsic tryptophan fluorescence of ghost-fibers F-actin containing the rebound troponin tropomyosin complex, induced by the binding of phosphorylated and dephosphorylated heavy meromyosin at low and high concentrations of calcium ions, were investigated. The anisotropy of tryptophan fluorescence was used as an indicator of F-actin conformational changes induced by the binding of phosphorylated and dephosphorylated heavy meromyosins.

Materials and methods. The study was carried out on myosin-free ghost single fibers of skeletal muscle of rabbit, containing more than 80% of actin (for determination of F-actin in ghost fibers see below). Phosphorylated and dephosphorylated HMM containing 98 ± 2% and 0–2% of total 18-kDa light chains in phosphorylated form, respectively, were prepared as described by Stepkowski et al.[12]. Phosphorylated and dephosphorylated myosin (10 mg/ml) prepared according to Stepkowski et al.[13] was dissolved in 0.5 M KCl, 20 mM phosphate buffer pH 7.0, 2 mM $CaCl_2$. Digestion was carried out at 22–24 °C for 4 min in the presence of trypsin-free α-chymotrypsin with enzyme substrate ratio 1:260. Phenylmethylsulphonyl fluoride (8.7 mg/ml in ethanol) was added to the final concentration of 0.36 mM to stop the reaction. The solution was dialyzed against 20 mM phosphate buffer pH 7.0, to precipitate undigested myosin and all proteolytic fragments insoluble at low ionic strength. The precipitate was removed by centrifugation. HMM was purified by precipitation with ammonium sulphate or by ultracentrifugation of heavy meromyosin complexed with actin, followed by dissociation of the acto-heavy meromyosin complex in the presence of 5 mM magnesium chloride and 5 mM pyrophosphate, and removal of actin by repeated ultracentrifugation[14].

HMM was checked by sodium dodecyl sulphate gel electrophoresis[15] (fig. 1d, e), whereas ghost fibers were checked on minislab gradient gels (5–20%) according to the method of Matsudaira and Burgess[16] (fig. 1a, b, c). The amount of actin in ghost fibers was calculated from densitometric measurements of electrophoretic patterns of parallel samples of ghost fibers used for sodium dodecyl sulphate electrophoresis in 6–8% gels[5,17]. The amount of phosphorylated 18-kDa light chains was calculated from densitometric data of the electrophoretic patterns of phosphory-