An enzyme immunoassay for mouse epidermal growth factor utilizing a liquid phase double-antibody system[1]

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Summary. An enzyme immunoassay for mouse epidermal growth factor (EGF) involving a liquid phase double-antibody system was developed. The EGF-β-galactosidase conjugate prepared was stable for at least 8 months. By this method, EGF was detectable at a concentration as low as 20 pg per tube. The concentrations of EGF in various tissues of mice are also presented.
Key words. Epidermal growth factor; enzyme immunoassay; mouse.

Epidermal growth factor (EGF) is a single chain polypeptide of 53 amino acids first isolated from the male mouse submandibular gland[3] and subsequently from human urine[4]. EGF is well-known as a potent mitogen for a variety of cells in vivo and in vitro[5,6], and its biological activities have been studied extensively[7]. For example, EGF accelerates incisor eruption and eyelid opening when given to newborn animals, and inhibits gastric acid secretion and promotes the healing of corneal epithelium of adult animals. It also acts as a trophic factor for gastrointestinal mucosa[8].

Although the mouse submandibular gland is well-known as a rich source of EGF, it is also detectable, but in low concentrations, in various other tissues and fluids of animals[9]. Until now, determinations of EGF levels have been made by radioimmunoassay[9-11] or radioreceptor assay[12]. In this study, we developed an enzyme immunoassay for mouse EGF in a liquid phase double-antibody system.

Materials and methods. EGF was purified from the submandibular gland of ICR male mice by the method of Savage and Cohen[13]. The purified preparation was characterized as a single band by polyacrylamide gel electrophoresis. Anti-EGF rabbit serum was purchased from Collaborative Research, Waltham, MA; goat anti-rabbit IgG, from Cappel Laboratories, Malvern, PA. β-Galactosidase (EC 3.2.1.23., from *Escherichia coli*) was from Boehringer Mannheim, West Germany. N-Hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl)-maleimide (CHM) was purchased from Zieben Chemical Co., Tokyo.

Preparation of EGF-β-galactosidase conjugate. EGF (0.5 mg dissolved in 0.5 ml of 0.1 M phosphate buffer, pH 7.0) was incubated with N-hydroxysuccinimide ester of CHM (0.6 mg in 20 µl of dimethylformamide) for 90 min at 30 °C. The reaction mixture was then subjected to gel filtration on a Sephadex G-25 column (1 × 26 cm) equilibrated with 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA. The fractions containing EGF were pooled and incubated with β-galactosidase (0.3 mg dissolved in 0.1 M phosphate buffer, pH 7.0) for 30 min at 30 °C. To the solution, 30 µl of 0.1 M β-mercaptoethylamine was added, and the mixture was further incubated for 10 min at room temperature.

The reaction mixture was subjected to gel filtration on a Sephacryl S-300 column (1.6 × 45 cm) equilibrated with 0.1 M phosphate buffer, pH 7.0, and fractions containing β-galactosidase were collected and combined. To the solution, bovine serum albumin, $MgCl_2$, and NaN_3 were added as stabilizers to give a final concentration of 0.1, 0.01 and 0.1%, respectively. The EGF-β-galactosidase conjugate thus prepared was stored at 4 °C until used.

Enzyme immunoassay. 100 µl of standard EGF solutions or samples was mixed with 100 µl of anti-EGF serum previously diluted 1:500,000 with 0.05 M phosphate buffer, pH 7.3, containing 0.1 M NaCl, 0.1% bovine serum albumin, 0.1% EDTA, and 0.1% NaN_3 (buffer A). After the mixture had been kept for about 1 h at 4 °C, 100 µl of EGF-β-galactosidase conjugate diluted 1:10,000 with buffer A was added. The mixture was incubated for 24 h at 4 °C. Then 50 µl each of 10-fold diluted goat anti-rabbit IgG serum and 100-fold diluted normal rabbit serum was added (dilution was made in buffer A). The mixture was further incubated for 24 h at 4 °C.

The precipitate was collected by centrifugation at 3000 × g for 10 min and then washed twice with 1 ml of 0.02 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.1% bovine serum albumin, 0.01% $MgCl_2$ and 0.1% NaN_3 (buffer B). To the precipitate, 0.2 ml of 0.02 M O-nitrophenyl-β-galactopyranoside dissolved in buffer B containing 0.35% 2-mercaptoethanol was added as enzyme substrate. The suspension was incubated for 18 h at 37 °C, and the enzyme reaction was terminated by the addition of 1 ml of 0.1 M glycine-NaOH buffer, pH 10.3. The absorbance of the product, O-nitrophenol, was measured at 420 nm.

Results and discussion. The figure shows a standard curve for EGF, and dilution curves generated by submandibular gland extracts with the enzyme immunoassay presented here. The binding of EGF-β-galactosidase conjugate to anti-EGF was dose-dependently decreased by the addition of increasing amounts of free EGF in the range from 10 to 1000 pg per tube. About 100 pg of EGF showed a 50% inhibition of binding. Moreover, the slopes of dilution curves generated by extracts of male and female submandibular glands were parallel to those of the standard curve.

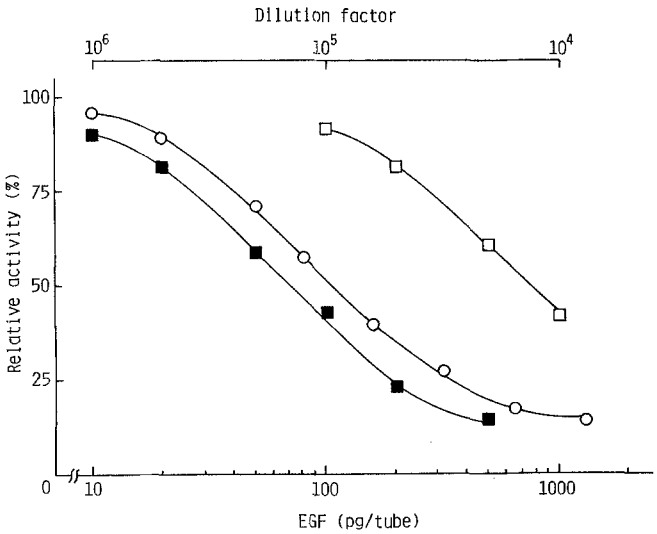
The table shows the concentrations of EGF determined by this method in various tissues of mice. As was expected, the concentration of EGF in the submandibular gland was exceedingly high, being about 10-fold higher in the male than in the female. Relatively high quantities of EGF, although extremely low as compared with the submandibular gland level, were detected in the sublingual gland, parotid gland, and kidney. There was also a significant sex difference in EGF concentrations in these organs, except in the kidney.

In this study, we determined EGF levels in the gastrointestinal tract using animals whose submandibular glands had been removed. Our preliminary experiments showed that the assay of EGF in the gastrointestinal tract gave higher values in the intact animals than in the sialoectomized animals, although in other

EGF concentrations in various tissues of male and female mice

Tissues	EGF concentration (ng/mg wet tissues)	
	Male	Female
Submandibular gland	1320 ± 7.8	141 ± 9[c]
Sublingual gland	0.776 ± 0.641	0.212 ± 0.106[b]
Parotid gland	0.234 ± 0.066	0.043 ± 0.011[a]
Heart	0.050 ± 0.013	0.039 ± 0.013
Lung	0.012 ± 0.005	0.010 ± 0.006
Liver	0.013 ± 0.004	0.011 ± 0.004
Kidney	0.125 ± 0.027	0.142 ± 0.021
Spleen	0.009 ± 0.002	0.007 ± 0.004
Stomach*	0.058 ± 0.019	0.023 ± 0.011
Duodenum*	0.016 ± 0.005	0.007 ± 0.004
Jejunum*	0.008 ± 0.003	ND
Ileum*	0.008 ± 0.005	ND

Tissues were removed from male and female ICR mice (10 weeks old) and homogenized with 9 vols of saline. The homogenates were centrifuged at 10,000 × g for 20 min, and the supernatants used as samples for enzyme immunoassay of EGF. * EGF concentrations were determined in mice after removal of submandibular gland (5 days prior to sacrifice). Each value shows the mean ± SE of 5–8 animals. [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$ as compared with male values. ND, not detectable.



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