Enzyme immunoassay for mouse epidermal growth factor in a liquid phase double-antibody system

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Epidermal growth factor (EGF), a single chain polypeptide of 53 amino acids, was first isolated from male mouse submandibular glands¹⁾ and subsequently from human urine²⁾. This peptide is a potent mitogen and differentiating factor for a variety of cells *in vivo* and *in vitro*, and its biological functions have been studied extensively³⁻⁵⁾. For example, it accelerates incisor eruption and eyelid opening when given in newborn animals, and inhibits gastric acid secretion and promotes the healing of corneal epithelium of adult animals. It acts as a trophic factor for gastrointestinal mucosa⁶⁾.

EGF is present in extremely high concentrations in the mouse submandibular gland, and its synthesis in the gland is under the control of endogenous androgens⁷⁾. Low levels of EGF are detectable in various tissues and fluids of both mice and humans, and are based on determinations made by radioimmunoassay⁸⁻¹⁰⁾ or radioreceptor assay¹¹⁾. In this study, we developed an enzyme immunoassay for mouse EGF in a liquid phase double-antibody system.

Materials and Methods

Materials. EGF was isolated from the submandibular glands of adult male mice of the ICR strain by the method of Savage and Cohen¹²⁾. The isolated preparation gave a single protein band on polyacrylamide-gel electrophoresis¹³⁾. Anti-EGF rabbit serum was purchased from Collaborative Research, Waltham, MA. Goat anti-rabbit immunoglobulin G (IgG) was obtained from Cappel

Laboratories, Malvern, PA, and β -galactosidase (EC 3.2.1.23, from *Escherichia coli*) from Boehringer Mannheim, 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. The procedures are essentially based on the method reported by Yamamoto *et al.*¹⁴⁾ for the preparation of thyroid hormone- β -galactosidase conjugates. EGF (0.5 mg) was dissolved in 0.5 ml of 0.1 M phosphate buffer, pH 7.0, and the solution mixed with 20 μ l of N-hydroxysuccinimide ester of CHM (30 mg/ml, dissolved in dimethylformamide). The mixture was incubated for 90 min at 30°C, and then passed through a column (1 $\times$ 26 cm) of Sephadex G-25 equilibrated with 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA. The fractions containing proteins were combined and mixed with 60 μ l of β -galactosidase (5 mg/ml, dissolved in the above buffer). The mixture was incubated for 30 min at 30°C. After incubation, 30 μ l of 0.1 M β -mercaptoethylamine was added, and the mixture was further incubated for 10 min at room temperature.

The mixture was then applied to a column (1.6 $\times$ 45 cm) of Sephacryl S-300 equilibrated with 0.1 M phosphate buffer, pH 7.0, and eluted with the same buffer. The major fractions containing β -galactosidase were collected and combined. To the solution, bovine serum albumin, MgCl₂ and Na₂S₂O₃ were added as stabilizers to give a final concentration of 0.1, 0.01 and 0.1%, respectively.

The conjugate solution prepared was stored at 4°C, and it was stable for at least 6 months.

Enzyme immunoassay. One hundred μ l of tissue extract or standard solution containing various amounts of EGF was mixed with 100 μ l of anti-EGF serum previously diluted to 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 incubation for 1 h at 4°C, 100 μ l of EGF- β -galactosidase conjugate previously diluted to 1:10,000 with buffer A was added. The mixture was incubated for 24 h at 4°C. Then, 50 μ l each of 10-fold diluted goat anti-rabbit IgG serum and 100-fold diluted normal rabbit serum was added. These sera were diluted with buffer A. The mixture was further incubated for 24 h at 4°C.

The precipitate was collected by centrifugation at $3,000 \times g$ for 10 min and 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). One fifth ml of 0.02 M 0-nitrophenyl- β -galactopyranoside dissolved in buffer B containing 0.35% 2-mercaptoethanol was added to the precipitate, and the suspension incubated for 18 h at 37°C. After incubation, the enzyme reaction was stopped by adding 1 ml of 0.1 M glycine-NaOH buffer, pH 10.3, and the absorbance of the product, 0-nitrophenol was measured at 420 nm in a 1-ml microcuvette.

Results and Discussion

Fig. 1 shows a typical standard curve of enzyme immunoassay for EGF. The binding of EGF- β -galactosidase conjugate to anti-EGF antibody was dose-dependently decreased by the addition of increasing quantities of free EGF in the range from 10 to 1000 pg per tube. About 100 pg of EGF showed a 50 percent inhibition of binding. Several analysis showed no apparent alteration in the pattern of the standard curve. In addition, the slope of dilution curves generated by extracts of male and female submandibular glands was almost

Fig. 1 Standard curve for EGF (○), and dilution curves generated by male (▲) and female (△) extracts of submandibular glands. The glands were removed from 10-week-old ICR mice and homogenized with 9 volumes of saline. The homogenate was centrifuged at $10,000 \times g$ for 30 min. The supernatant obtained was serially diluted by saline and assayed for EGF concentrations.

parallel to that of the standard curve. The concentration of EGF in the mouse submandibular gland was about 10-fold higher in males than in females as reported previously⁸⁾. Several other peptides in the mouse submandibular gland, β -nerve growth factor, EGF-binding protein and esteroprotease A¹⁵⁾, had no competitive binding to the EGF antibody.

The enzyme immunoassay for EGF in a liquid phase double-antibody system presented here is sufficiently sensitive, since this method can detect as low as 20 pg EGF per tube, and thus the sensitivity seems to be higher than that of radioimmunoassay⁸⁻¹⁰⁾. The EGF- β -galactosidase conjugate prepared in this study contains less than 20 percent of free β -galactosidase, which can be easily removed by washing the precipitate of antigen-antibody complex. Moreover, the conjugate is stable for at least 6 months. There is no need for use of radioisotopes throughout the procedures.

In the present assay, we measured β -galactosidase activity with the use of 0-nitro-

phenyl- β -galactopyranoside as a substrate. If the enzyme activity is measured fluorophotometrically with the use of another substrate, 4-methylumbelliferyl- β -galactopyranoside, under the condition of a further dilution of both the conjugate and antibody, a higher sensitivity could be expected.

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