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TESTOSTERONE-INDUCED ACCUMULATION OF EPIDERMAL GROWTH FACTOR IN THE SUBMANDIBULAR SALIVARY GLANDS OF MICE, ASSESSED BY RADIOIMMUNOASSAY*

MICHAEL L. ROBERTS†

Department of Dental Medicine and Surgery, University of Melbourne, Parkville, Victoria, 3052, Australia

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Abstract—A radioimmunoassay has been developed for the polypeptide epidermal growth factor. This assay has been used to measure the content of the factor in mouse submandibular salivary glands and to confirm the discrepancy between the amounts in male and female salivary glands. Following a series of testosterone injections, female mice demonstrate markedly elevated levels of this growth factor in their submandibular glands.

SALIVARY glands of rodents have been reported to contain a number of factors, apparently unrelated to the exocrine functions of the gland, and which possess biological activities in other parts of the body. Two of these are nerve growth factor[1] and epidermal growth factor.[2] The latter is a single chain polypeptide,[3] molecular weight 6400, which possesses *in vivo* and *in vitro* biological activity in a number of systems.

Epidermal growth factor (EGF) produces precocious eyelid opening and incisor eruption in neonatal mice,[2] stimulates the nucleic acid and/or protein synthesis in a variety of cells *in vitro*,[4,5] and has recently been shown to cause a hypertrophic healing response when applied to the wounded cornea of a rabbit.[6]

These effects of EGF suggest a possible modulating role of the polypeptide in growth and development. Such possible function may be elucidated by the accurate measurement of EGF in blood and tissues during embryogenesis and organogenesis. However, the presently available bioassay techniques for EGF, involving the tooth eruption or eye-opening responses, are inappropriate for this type of study as they are prohibitively time consuming, lack precision and are of suspect specificity when unpurified samples are assayed.

This communication describes the development of a radioimmunoassay for EGF and the use of this assay to measure the concentration of EGF in the submandibular salivary glands of male and female mice. Sexual dimorphism in the submandibular glands of mice has long been recognized.[7] Consequently the effects of testosterone on the EGF content of the submandibular glands of female mice was investigated using this assay.

During the progress of this work, a double antibody assay for EGF has been described and used to detect an immunologically cross-reacting material in sera from pregnant women.[8]

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† Senior Research Officer, National Health and Medical Research Council of Australia.

MATERIALS AND METHODS

Purification of EGF. EGF was isolated from the salivary glands of adult male C57Bl/ten mice by the procedure of Savage and Cohen.[9] The purified polypeptide produced the appropriate responses of precocious eyelid opening and incisor eruption in neonatal mice.

Development of anti-EGF antibodies. A modification of the procedure described by Boyd and Peart[10] was used to immunize the rabbits. 1 mg of EGF was mixed with 50 mg animal charcoal in 2 ml of distilled water. After 30 min, 8 mg of sodium dinaphthylmethane disulphonate ("Belloid TD", Geigy Ltd) was added, the suspension was ultrasonically dispersed for 1 min and then emulsified with an equal volume of complete Freund's adjuvant (Commonwealth Serum Laboratories). Four rabbits were immunized, each with one quarter of the above emulsion, by multiple subcutaneous injections. This procedure of immunogen preparation and injection was repeated at fortnightly intervals for 12 weeks. Each fortnight the animals were bled from an ear vein into heparinized tubes and plasma obtained by centrifugation at 1200 *g* for 15 min at room temperature.

Antibodies to EGF were detected by serially diluting the plasma with 0·1 M Tris–HCl pH 7·5 containing 0·2% gelatin and 0·1% sodium azide (henceforth called the RIA buffer) and incubating 0·5 ml of each dilution with 0·1 ml of iodinated EGF (8000 cpm) at 4° for 18 hr. The antibody-bound labelled polypeptide and free labelled EGF were separated by the dextran-coated charcoal method.[11]

Iodination of EGF. The lactoperoxidase technique[12] was used to label the EGF with ^{125}I (Radiochemical Centre, Amersham).

Iodinated peptide was separated from free iodide by passage of the iodination mixture through a Sephadex G-25 column (0·7 × 25 cm) equilibrated and eluted with RIA buffer. One ml fractions were collected and those containing iodinated EGF were divided into small aliquots and stored at −20°.

Radioimmunoassay technique. All assays were set up in disposable plastic tubes, 9 × 75 mm.

The incubation mixtures for the RIA consisted of 0·5 ml of a standard amount EGF or the unknown sample, in RIA buffer, 0·1 ml of anti-EGF anti serum, suitably diluted with RIA buffer, 0·1 ml ^{125}I-labelled EGF (8000 cpm) in RIA buffer. The dilution of anti-serum to be used was that which, upon serial dilution, had shown a binding of 30 per cent of the ^{125}I-labelled EGF.

Following incubation at 4° for 18 hr, the antibody-bound and free labelled EGF were separated by the dextran-coated charcoal method, and the radioactivity of each fraction determined by the use of a well-type crystal scintillation counter.

Calculation of the proportion of total radioactivity bound to antibody in the presence of known amounts of EGF allowed the construction of calibration curves. These curves were used to determine the EGF content of unknown samples.

Triplicate incubations were prepared for each level of EGF standard and for each unknown sample.

Preparation of salivary gland extracts. Submandibular salivary glands were removed from eleven male and ten female C57Bl/10 mice, following cervical dislocation, and weighed immediately.

The glands were homogenized in a small volume of ice-cold distilled water using a glass Ten Broeck type tissue grinder.

The homogenate was diluted to 20 ml with distilled water and stored at −20° prior to radioimmunoassay.

The EGF content of the salivary glands was determined by thawing the homogenates, diluting them with RIA buffer and including this diluted homogenate in radioimmunoassay.

Effect of testosterone on EGF content of female mouse submandibular glands. Five adult female C57Bl/10 mice were injected daily with testosterone propionate (Calbiochem, U.S.A.) at a dosage of 6 mg/100 g body wt. The testosterone was dissolved in olive oil to give a concentration of 10 mg/ml.

Four adult female C57Bl/10 mice were injected daily with an equivalent volume of olive oil.

All injections were given subcutaneously.

On the eighth day the mice were starved for 7 hr, and then killed by cervical dislocation. The submandibular glands were removed immediately, weighed, homogenized as previously described and assayed for EGF by radioimmunoassay.

RESULTS

Three of the four rabbits had developed high titres of anti-EGF antibodies following three injections of EGF-charcoal complex. These antibodies were suitable for use in a radioimmunoassay for EGF in salivary glands. The sensitivity of the assay was 50 pg.

Submandibular salivary glands of both male and female mice contained a material which binds to the anti-EGF antibody and the immuno-reactivity of the gland homogenates behaves in a similar fashion to purified EGF upon dilution (Fig. 1).

The submandibular glands of male mice contained 0·78 μg EGF/mg wet wt (S.D. ± 0·79 μg/mg).

The glands from the female mice contained much less EGF, the mean concentration being 0·06 μg/mg wet wt (S.D. ± 0·06 μg/mg).

The submandibular glands of female mice injected daily with testosterone were

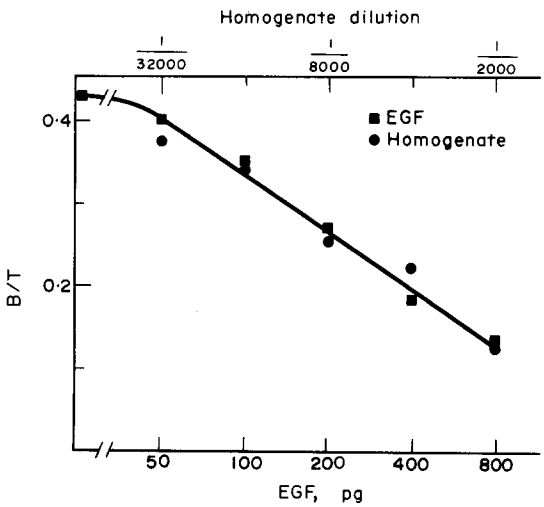

FIG. 1. Dilution curve obtained with salivary gland homogenate compared to a calibration curve prepared using purified EGF standards.

heavier (mean 80·5 mg, S.D. ± 9·3 mg) than those from mice of equivalent body weight injected with the oily vehicle (mean gland wt 65·5 mg, S.D. ± 5·2 mg).

Testosterone injections also caused an increase in the EGF concentration in the salivary glands. The glands of mice injected with the androgen had a mean EGF concentration of 0·45 μg/mg wet wt (S.D. ± 0·17 μg/mg) while those from mice injected with oil had a mean EGF concentration of 0·09 μg/mg wet wt (S.D. ± 0·07 μg/mg). This difference was significant when analysed by the Student *t*-test ($P < 0·02$).

DISCUSSION

The development of this radioimmunoassay will greatly facilitate the study of the possible roles of EGF in normal physiology and in development. Radioimmunoassays for EGF will overcome the lack of precision and the large time commitment involved in the bio-assay of this polypeptide. The immunological assay will also be much less susceptible to non-specific effects than the biological assays. The observed similar response of the immunoreactivity of the crude gland homogenate and purified EGF to dilution indicates the absence of gross non-specific effects in the present assay. The presence of cross-reacting, biologically inactive fragments of the EGF in gland homogenate must be considered as a source of radioimmunoassay inaccuracy and experiments are at present being carried on to examine any contribution of such fragments to the reported levels.

The sexual variation reported for submandibular gland concentrations of EGF[13] have been confirmed and quantitated by the present assay. As with a number of other substances which show differences in concentration in the male and female submandibular glands, the EGF content of the female gland can be increased by testosterone treatment. Although this effect could be produced by alterations in the rate of synthesis, secretion or inactivation of the polypeptide in the salivary gland, experiments on other actions of the androgen on submandibular glands[14] suggest that the increases observed here are probably due to the stimulation of EGF synthesis.

In addition to the assay described above, a solid phase radioimmunoassay for EGF has been developed recently[15] and the EGF level in submandibular salivary glands as assessed by this assay are similar to those found in the present study.

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