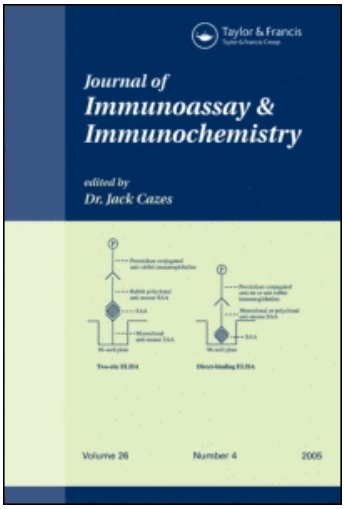

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Homologous Radioimmunoassay for Epidermal Growth Factor in Human Saliva

S. Dagogo-Jack[a]; S. Atkinson[a]; P. Kendall-Taylor[a]
[a] Endocrine Unit, Wellcome Research Laboratories, Royal Victoria infirmary, Newcastle on Tyne, UK

To cite this Article Dagogo-Jack, S. , Atkinson, S. and Kendall-Taylor, P.(1985) 'Homologous Radioimmunoassay for Epidermal Growth Factor in Human Saliva', Journal of Immunoassay and Immunochemistry, 6: 1, 125 — 136
To link to this Article: DOI: 10.1080/01971528508063025
URL: http://dx.doi.org/10.1080/01971528508063025

JOURNAL OF IMMUNOASSAY, 6(1&2), 125-136 (1985)

HOMOLOGOUS RADIOIMMUNOASSAY FOR EPIDERMAL GROWTH FACTOR IN HUMAN SALIVA

Dagogo-Jack S, Atkinson S, and Kendall-Taylor P.
Endocrine Unit, Wellcome Research Laboratories,
Royal Victoria infirmary,
Newcastle on Tyne NE1 4LP, UK.

ABSTRACT

Epidermal growth factor (EGF) is a small polypeptide with potent mitogenic activity. Its synthesis by mouse submaxillary gland is stimulated by certain hormones. To assess its physiological significance in man,we have developed a homologous radioimmunoassay for human epidermal growth factor (hEGF) in saliva. A satisfactory standard curve was readily obtained using either buffer or peptide-free saliva.

The mean IC50 was 436 ± 200 pM (mean ± SD) and sensitivity approximately 35 pM. The mean normal salivary hEGF in 63 males was 314.6 ± 21.7 pM (± SEM) and 354 ± 27.8 pM in 48 females. The difference between the means of the sexes was not significant. Assays of aliquots stored under different conditions showed hEGF in saliva to be stable and the method reproducible. Salivary hEGF secretion did not suggest diurnal rhythmicity and was unrelated to meals. (Key Words: epidermal growth factor, saliva, radioimmunoassay).

INTRODUCTION

Epidermal growth factor (EGF) belongs to a group of peptides collectively known as growth factors. These growth factors appear to modulate cell proliferation and differentiation by undergoing saturable interactions with specific receptors on target cells (1).

EGF, which was first isolated from the mouse submaxillary gland (2), has a molecular weight of 6000 daltons with 53 aminoacid residues. An analogous peptide, urogastrone, was isolated from human urine and subsequently shown to be a potent inhibitor of gastric secretion (3).

0197-1522/85/0601-0125$3.50/0

Urogastrone shares close structural similarity and functions with mouse EGF. In this paper the term human epidermal growth factor (hEGF) is used interchangeably with urogastrone.

EGF is a potent mitogen for cells from a variety of species; it also has complex endocrine interactions (4). Its synthesis in the mouse submaxillary gland is stimulated by thyroxine and testosterone; and inhibited by thyroidectomy, adrenalectomy and ovariectomy (5). Furthermore, EGF stimulates the proliferation of cultured thyroid cells in several species but appears to inhibit hormonogenesis (6,7).

Neither the source nor the physiological role of hEGF in man has been determined (8,9). This homologous radioimmunoassay for hEGF in saliva was developed for use in physiological studies and for the complementation of concurrent work on the role of epidermal growth factor in thyroid cell growth and metabolism (10).

MATERIALS AND METHODS

Antigen and Antiserum

Highly purified epidermal growth factor extracted from urine of normal adults and specific antiserum to hEGF were generous gifts from Dr. H. Gregory, Imperial Chemical Industries Ltd., Cheshire, England. The antiserum had been raised in the rabbit by multiple subcutaneous injections of 1 mg 10% pure urogastrone-EGF in complete Freund's adjuvant (11).

Iodination Procedure.

hEGF 2.5 µg was iodinated by the Iodogen method (12). The iodination mixture was immediately applied to a column (30 cm x 1.6 cm) of Sephadex G50 fine resin (Pharmacia, Uppsala, Sweden) and eluted

with 0.1M phosphate buffer pH 7.2 containing 0.1% BSA. The peak of radioactivity corresponding to ^{125}I-hEGF eluted at 1.8 x V_o and incorporated 45-70% of total radioactivity added. Specific activities of approximately 90 μCi/μg (20 Bqfmol^{-1}) were obtained. Labelled hEGF was diluted in assay buffer; aliquots were stored at -70°C, thawed only once and used within 8-10 weeks of preparation.

Saliva Samples

Unstimulated saliva samples were collected in 30 ml sterile plastic bottles and centrifuged at 1500 x g for 10 min to precipitate debris. The resultant clear supernatants were applied directly to the assay.

Peptide-free saliva

Activated charcoal ('Norit OL') was added to pooled saliva from normal adults to give a concentration of 100 mg/ml. After mixing for 4 hrs at room temperature on a magnetic stirrer, the suspension was centrifuged at 30,000 x g for 30 min. Fine charcoal particles remaining in the supernatant were removed by passage through two successive grades of sartorius membrane filters (Millipore S.A.) of 0.45 μm and 0.2 μm pore sizes. Peptide-free status was confirmed by adding a small amount of tracer ^{125}I-hEGF to the original pool of saliva. The efficiency of the method, as determined by recovery of ^{125}I-hEGF added to saliva pool, was 96%.

Subjects

Sixty-three males and forty-eight females were studied. These were mostly University students. The age range was 19 to 66 yr with a median of 21 years. The subjects were free from any known disease and had no past history of thyroid disorder.

Eight women were taking oral contraceptive pills but the rest of the subjects had no drug history.

Radioimmunoassay procedure.

The radioimmunoassay was carried out in 0.04M phosphate buffer containing 0.15M sodium chloride, 0.01M EDTA, 0.5% (w/v) bovine serum albumin and 0.1% (w/v) thiomersal, pH 7.2. Triplicate determinations were made in 4 ml plastic tubes (Sarstedt). Each tube contained either hEGF, standard (100µl) or saliva sample (100 µl); antiserum (100 µl) diluted in buffer to 1:24,000; and ^{125}I-hEGF (10,000 counts/min, approx.60 pM) in buffer (100 µl). The tubes were vortex-mixed and left at 4°C for 24h.

Bound and free moieties were separated using antibody linked to solid phase (SAC CEL, Wellcome Reagents, England). The precipitates, which contained the antiserum-bound fractions, were counted in a multi-channel gamma counter (NE1600, Nuclear, Chicago, USA). The ratio of antiserum specifically bound to ^{125}I-hEGF to total ^{125}I-hEGF added was calculated for each standard, expressed as a percentage of the initial binding, and plotted against the corresponding dose of standard.

Statistical analysis.

The significance of differences in mean values between groups was assessed by Student's t-test.

RESULTS

Sensitivity and specificity.

Equilibrium conditions were attained within 18 - 24 hours in this system. Figure 1 shows a representative dose-response curve for the homologous hEGF RIA. The limit of detection was 35pM.

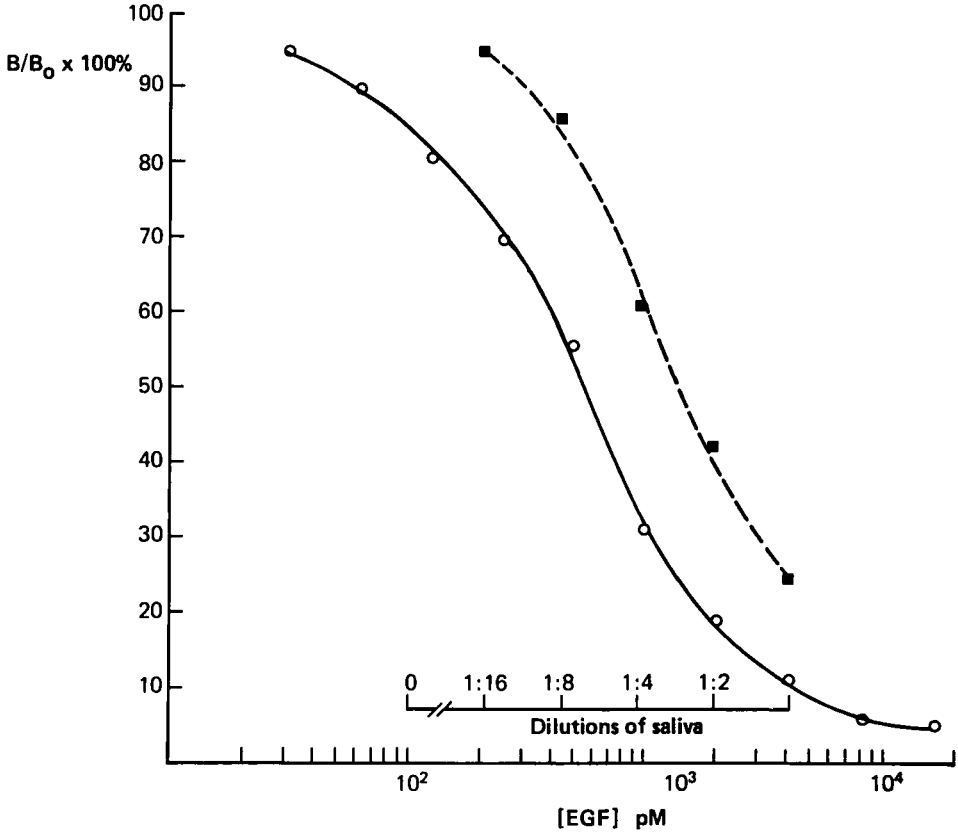

Figure 1. Dose response curves for hEGF standard (o——o) and dilutions of saliva (■---■) in the homologous radioimmunoassay. Each point represents the mean of triplicate determinations.

This could be improved further by altering assay conditions but the enhanced sensitivity was deemed unnecessary for the assay of hEGF in saliva. Dose-response curves using peptide-free saliva in place of assay buffer were superimposable on the standard curve. There was no detectable damage to the incubates that was attributable to intrinsic salivary factors.

The specificity of the antiserum had previously been established (11).

Reproducibility

Three unknown saliva samples were assayed for hEGF multiple times in the same batch. The mean (± SD) hEGF concentrations were 635.6 ± 54.4 pM (n=9), 204.4 ± 23.1 pM (n=9), and 256.9 ± 26.7 pM (n=8). The coefficients of variation were 8.5%, 11.2% and 10.3%, respectively.

Three solutions of hEGF standard were prepared in buffer to give concentrations of 4000 pM, 800 pM and 80 pM, respectively. These were stored at -40°C in aliquots and assayed repeatedly for hEGF over 13 consecutive assays. The means (± SD) were 3,700 ± 475.3 pM, 778 ± 100.7 pM, and 115.1 ± 24.5 pM respectively. The coefficients of variation were 12.9%, 12.9% and 21.3%, respectively. The mean specific binding, B°/T, was 13.5 ± 3.2% (± SD), and the IC50, 436 ± 200 pM (0.12 - 0.36 ng/tube) over the course of 20 consecutive assays. The corresponding value for nonspecific binding was 2.5 ± 0.3%. When pooled saliva samples (200 µl) replaced buffer in additional control tubes, the level of nonspecific binding observed was 2.7 ± 0.61% (mean ± SD, n=20). There was no significant difference between the mean nonspecific binding in buffer and that in pooled saliva.

Stability of salivary EGF

Six normal adults (3 males, 3 females) produced fresh saliva samples. After centrifugation, the supernatant from each was divided into four portions. One set was assayed immediately and the other three portions from each subject were aliquotted and left at either room temperature, +4°C or -40°C. Aliquots of stored samples were retrieved and assayed repeatedly over a period of ten days. There was no appreciable loss of hEGF immunoreactivity over the period of storage at the different temperatures.

Salivary hEGF in normal subjects

Fig. 2 shows the scatter of salivary hEGF values obtained from normal adults. The mean normal salivary hEGF in 63 males was 314.6 ± 21.7 pM (± SE) and 354 ± 27.8 pM in 48 females. The concentration of hEGF in saliva appears to have a normal distribution in the population studied. There is a wide spread in the range of normal values. The difference between the means for males and females was not significant.

Daily Variation in Salivary hEGF Concentration.

Salivary hEGF concentration was assayed in daily samples from seven normal adults (4 males, 3 females) produced at 10 am. The results, expressed as percentage change in hEGF concentration from the value on day 1, were plotted against the days of study (Fig.3). The mean 10 am salivary hEGF concentration for the group remained fairly stable although there was considerable daily variation in individual samples.

Diurnal rhythm

Five healthy volunteers (3 male, 2 females) collected saliva every 2 hours between 0800 and 2200, then 4 hourly until 0600 the following day. Precise note was made of when meals, snacks or beverages were taken. At the end of the collection, the saliva samples were assayed for hEGF. There was no consistent diurnal pattern and no discernible relationship between salivary hEGF concentration and meal times.

DISCUSSION

The standard bioassay for epidermal growth factor which is based on morphological events in immature mice, namely, stimulation

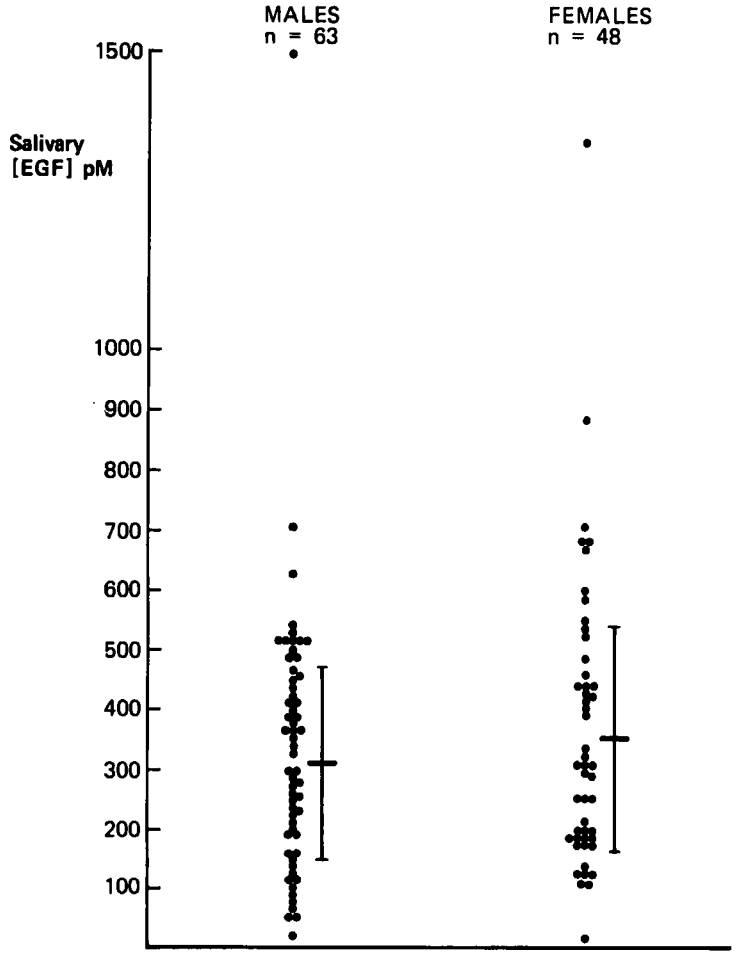

Figure 2. Salivary hEGF levels in normal subjects. The horizontal bars represent the mean hEGF values and vertical bars, one standard deviation. The difference between the means for males and females was not significant.

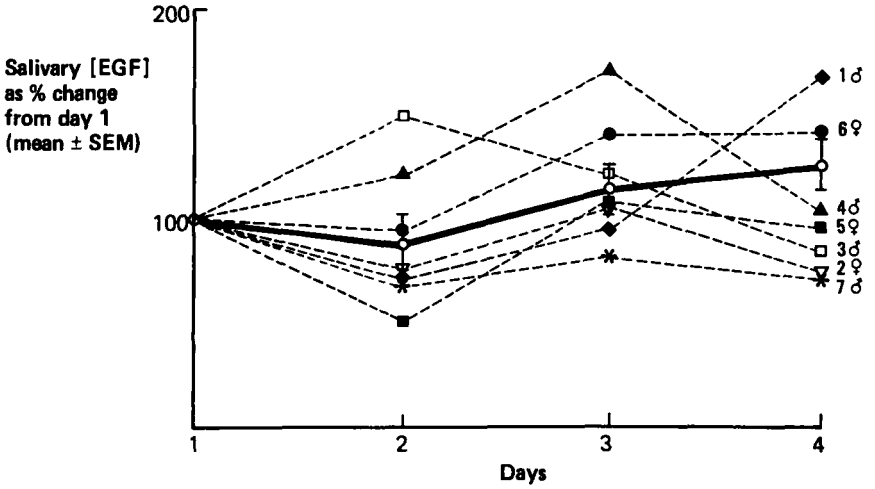

Figure 3. Daily variation in salivary hEGF in seven normal subjects. Fresh saliva samples produced daily at 10 am were assayed immediately over the four day period of study. The broken lines represent individual profiles, the solid line represents the mean variation for the group and the marginal figures represent the subjects.

of premature eye opening and incisor eruption, is both cumbersome and insensitive (2,3). Radioimmunoassay methods have subsequently been developed for measuring EGF in human body fluids, such as urine, milk, saliva, cerebrospinal fluid, pancreatic juice and plasma (12-15).

Plasma dilutions do not readily generate dose-response curves parallel to the EGF standard curve. Urinary EGF concentration would appear to reflect total EGF excreted from various possible sources; such measurements would, therefore, not address the question of specific sites(s) of synthesis of EGF in man. Saliva is a readily available body fluid with distinct advantages as an assay medium. Sampling involves no stress or discomfort to the subject and repeated samples can easily be obtained.

The range of salivary hEGF concentration in normal adults obtained by us (80 pM - 2 nM) differs from previously quoted values

of 5.6 to 16.8 ng/ml, that is, 1 to 2.7 nM (12). The disagreement is much more marked at the lower end than at the top end of the range. As this is the first exhaustive application of a homologous radioimmunoassay to the measurement of hEGF in saliva, the observed difference may merely reflect the increased sensitivity of our assay. The finding of marked variability in salivary EGF concentrations among normal subjects has also been observed in urinary EGF excretion studies (16). Such studies also showed significantly higher urinary EGF levels in females than males when results were expressed as a function of creatinine excretion. We found no significant difference in mean salivary EGF levels between the sexes.

That no relationship was seen between meal times and salivary EGF concentration was an important negative finding. This, together with the known stability of EGF in saliva, should simplify the collection and general handling of samples. However, samples produced round the clock by five subjects showed appreciable hour to hour variation in hEGF concentration. Although this finding is provisional because of the small population studied, it may be prudent to take multiple samples at different periods of the day; the ease of collection would make this feasible. Alternatively, subjects could be advised to produce samples at a fixed time of the day.

We have described and validated a new homologous radioimmunoassay for epidermal growth factor in saliva. This assay has enabled initial study of aspects of EGF physiology. It should prove to be useful for the investigation of patients with a variety of disorders.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council and North of England Cancer Research Campaign.

Correspondence to Dr. S. Dagogo-Jack.

REFERENCES

1. Hill, D.J., Holder, A.T., Seid, J., Preece, M.A., Tomlinson, S. and Miller, R.D.G. Increased thymidine incorporation into fetal rat cartilage in vitro in the presence of human somatomedin, epidermal growth factor, and other growth factors. J. Endocrinol. 1983; 96: 489-497.

2. Cohen, S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. J. Biological Chemistry. 1962; 237: 1555-1562.

3. Gregory, H. Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature. 1975; 257: 325-327.

4. Gospodarowicz, D., Greenburg, G., Bialecki, H. and Zetter, B.R. Factors involved in the modulation of cell proliferation in vivo and in vitro: the role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. In Vitro. 1978; 14: 85-115.

5. Gresik, E.W., Schenkein, I., van der Noen, H. and Barker, T. Hormonal regulation of epidermal growth factor in the submandibular gland of the adult mouse. Endocrinology. 1981; 109: 924-929.

6. Westermark, K., Karlsson, F.A. and Westermark, B. Epidermal growth factor modulates thyroid growth and function in culture. Endocrinology. 1983; 112: 1680-1686.

7. Ollis, C.A., Davies, R., Munro, D.S. and Tomlinson, S. The control of thyroid cell proliferation and function by epidermal growth factor (EGF) and thyrotropin (TSH), British Endocrine Societies 3rd Joint Meeting 1984; Abstract 124.

8. Elder, J.B., Williams, G., Lacy, B. and Gregory, H. Cellular localization of urogastrone/epidermal growth factor. Nature. 1978; 271: 466-467.

9. Daughaday, W.H. and Heath, E. Physiological and possible clinical significance of epidermal and nerve growth factors. Clinics in Endocrinology and Metabolism. 1984; 13: 207-226.

10. Atkinson, S., Smith, P.A. Taylor, J.J. and Kendall-Taylor, P. Specific epidermal growth factor receptors on porcine thyroid cell membranes. FEBS Letters. 1983; 53: 88-92.

11. Gregory, H., Holmes, J.E. and Willshire, I.R. Urogastrone levels in the urine of normal adult human. J. Clin. Endocrinol Metab. 1977; 45: 668-672.

12. Fraker, T.J. and Speck, J.C. Protein and cell membrane iodinations with a sparingly soluble chloroamide: 1,3,4,6-tetrachloro-3 , 6 -diphenylglycuril. Biochemical and Biophysical Research Communications. 1978; 80: 849-859.

13. Starkey, R.H. and Orth, D.N. Radioimmunoassay of human epidermal growth factor (urogastrone). J. Clin. Endocrinol. Metab. 1977; 45: 1144-1153.

14. Byyny, R.L., Orth, D.N. and Cohen, S. Radioimmunoassay of epidermal growth factor. Endocrinology. 1972; 90: 1261-1266.

15. Dailey, G.E., Kraus, J.W., and Orth, D.N. Homologous radioimmunoassay for human epidermal growth factor (urogastrone). J. Clin. Endocrinol. Metab. 1978; 46: 929-935.

16. Hirata, Y. and Orth, D.N. Epidermal growth factor (urogastrone) in human fluids: size heterogeneity. J. Clin. Endocrinol. Metab. 1979; 48: 673-679.