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Radioimmunoassay of Human Epidermal Growth Factor in Human Breast Cyst Fluid*

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Abstract—*Epidermal growth factor (EGF) was assayed radioimmunologically in three secretions of the human mammary gland: milk* (n = 16), *colostrum* (n = 8) *and breast cyst fluid* (n = 139). *The immunoreactivity (immunoreactive EGF) detected in these three fluids corresponds directly to monomeric EGF, as can be shown by the complete cross-reaction with the standard and the identical behaviour in chromatography on Sephadex G50. The mean concentration of EGF is significantly greater in breast cyst fluid* (241 ± 143 ng/ml) *and in colostrum* (197 ± 56 ng/ml) *than in milk* (107 ± 22 ng/ml). *The range of individual values in cyst fluid is large,* 5-945 ng/ml. *A possible role of EGF as a paracrine or autocrine factor in the pathology of cystic dysplasia of the mammary gland and in the increased risk of malignant transformation is hypothesised.*

INTRODUCTION

BREAST cystic disease is associated with a risk of malignant transformation. Haagensen *et al.* [1] and Azzopardi [2] have shown that women with breast cysts have a two- to four-fold greater risk of breast cancer than matched controls. It is known that human milk contains large quantities of epidermal growth factor, which is responsible for the mitogenic activity of milk [3]. EGF, also called urogastrone, is a small polypeptide mitogen (mol. wt 6000) which promotes the proliferation of various cells from several tissues, in particular mammary epithelial cells [4-9].

Our radioimmunoassay of human epidermal growth factor enabled us to identify the growth factor in human breast cyst fluid and to compare the concentrations detected in breast cyst fluid, colostrum and milk.

MATERIALS AND METHODS

Antigen and antiserum

Radioimmunoassay was performed using human EGF_{1-52} both as standard and ^{125}I-labelled tracer. EGF_{1-52} differs from the native EGF_{1-53} in the lack of the carboxy-terminal arginine amino acid. Pure human EGF_{1-52} isolated from human urine [10] and rabbit anti-human EGF_{1-52} were generously provided by Dr H. Gregory, ICI Ltd, Macclesfield, U.K.

Labelling and purification of tracer

EGF_{1-52} was labelled with ^{125}I by the following modification of the method of Greenwood *et al.* [11]. To 25 μl of 50 mM phosphate buffer, pH 7.5 (labelling buffer), containing 2.5 μg of EGF_{1-52} were added 0.5 mCi ^{125}I and 10 μl of a freshly prepared solution of chloramine T (2 mg/ml) in labelling buffer. The reaction was arrested 20 sec after the addition of chloramine T by the addition of 10 μl of a solution of sodium metabisulfite (2 mg/ml) in labelling buffer.

The reaction mixture was immediately applied to a 0.8 × 30-cm column of Sephadex G 15 resin equilibrated and eluted with a 50-mM phosphate buffer, pH 7.5, containing 5 mg/ml bovine serum albumin (incubation buffer) (flow rate: 20 ml/hr; fractions: 1 ml). The labelled EGF_{1-52} emerged in the void volume of the column.

Aliquots of labelled EGF_{1-52} were stored at -20°C. Before each assay the tracer was repurified on a 0.8 × 40-cm column of Sephadex G 50 equilibrated and eluted with incubation buffer (flow rate: 20 ml/hr; fractions: 1 ml). The elution profile characteristically showed two radioactive peaks, the first in the void volume and the second

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in the internal volume of the column. The first peak consisted of degraded forms of labelled EGF_{1-52}. The second was immunoreactive and undegraded labelled EGF_{1-52}.

The specific activity of the tracer, evaluated by the autodisplacement method, was 110 μCi/μg EGF_{1-52}.

Incubation

The incubation volume of 0.4 ml contained 100 μl of incubation buffer, 100 μl of incubation buffer containing standard EGF_{1-52} (10, 25, 50, 100, 250 and 500 pg, and 1, 2.5, 5 and 10 ng) or unknown, 100 μl of tracer (about 20,000 cpm/100 μl) and 100 μl of antiserum diluted to 1/8000 in incubation buffer.

The mixtures were incubated at 4°C for 24 hr, after which 500 μl of a solution of anti-γ-globulin immunoadsorbant diluted 1/75 in incubation buffer containing 0.5% (v/v) Tween 20 was added (double antibody solid phase method) [12]. The solutions were placed on a shaker for 1 hr and subsequently 2 ml of incubation buffer containing 0.5% Tween 20 were added. The precipitate was collected by centrifugation and counted.

Specificity of the radioimmunoassay

The behaviour of human formylmethionine EGF_{1-53} obtained by genetic engineering (human F-met-EGF, AMGen Biological) was studied and the cross-reactions of growth hormone (STH A grade, Calbiochem), somatomedin C (kindly supplied by Dr Chatelain, Edouard Herriot Hospital, Lyon), bovine insulin (Novo Research Institute), human insulin (Novo Research Institute) and mouse epidermal growth factor (receptor grade, collaborative research) with EGF_{1-52} were evaluated.

Biological fluids

One hundred and thirty-nine samples of breast cyst fluid, eight samples of colostrum, 16 samples of milk and a pool of urine were assayed at the same time.

Breast cyst fluid was aspirated from the cysts, immediately centrifuged to remove cellular material and stored at -20°C until assay. Clinical, radiologic and therapeutic investigations as well as cytologic analysis were performed and no associated neoplasia was detected. Milk from women 2-8 days after delivery was manually transferred into a sterile tube at 9 a.m. during the second feed and was stored at -20°C until assay. Samples were centrifuged for 20 min at 10000 *g* and the aqueous phase, withdrawn from below the floating lipid layer, was assayed.

For the purposes of this study, milk produced during the 72 hr after delivery was considered as colostrum and that produced after the first 72 hr as mature milk.

Dilution curves of the various biological fluids were prepared comparing their cross-reactions with standard EGF_{1-52}.

Samples of breast cyst fluid, milk and concentrated urine (10 ml lyophilized) were fractionated on a 0.8 × 100-cm column of Sephadex G 50 'Superfine' resin equilibrated and eluted with incubation buffer (flow rate: 5 ml/hr; fractions: 0.9 ml). Eluate fractions were assayed comparing the size of the immunoreactive material with standard EGF_{1-52}.

RESULTS

Assay characteristics

Figure 1 shows the competitive binding curve for the standard $hEGF_{1-52}$. F-met-EGF_{1-53} produced complete displacement of labelled EGF_{1-52} from antibodies raised against EGF_{1-52}. The competitive binding curves obtained with EGF_{1-52} and F-met-EGF_{1-53} were not significantly different.

As shown in Fig. 1, human urine, breast cyst fluid and milk produced a complete displacement of labelled EGF_{1-52} whereas growth hormone, somatomedin C and bovine insulin failed to displace labelled EGF_{1-52} from the antibody. Furthermore, cross-reactions were observed with mouse EGF and human insulin only at concentrations 3000- and 600-fold higher respectively.

The smallest amount of EGF_{1-52} capable of reducing significantly the binding of labelled EGF_{1-52} to antibody in the absence of unlabelled EGF_{1-52} was 8 pg. The intra-assay coefficient of variation at various points on the standard curve was less than 10% for amounts of unlabelled EGF_{1-52} between 25 pg and 10 ng, and the inter-assay coefficient of variation was less than 20% for amounts of EGF_{1-52} between 25 pg and 5 ng.

Assay in biological fluids

Amounts of EGF detected in the biological fluids are summarized in Table 1.

EGF concentration detected in the pool of urine was 47 ng/ml.

Breast cyst fluid concentrations of EGF varied from 5 to 945 ng/ml, and the mean (± S.D.) concentration was 241 ± 143 ng/ml. Figure 2 illustrates the distribution of EGF concentrations in the 139 samples of breast cyst fluid. The mean (± S.D.) concentrations in colostrum and milk were 197 ± 56 and 107 ± 22 ng/ml respectively. A significant difference was seen in the mean values detected in colostrum and breast cyst fluids and those detected in milk ($P < 0.0005$).

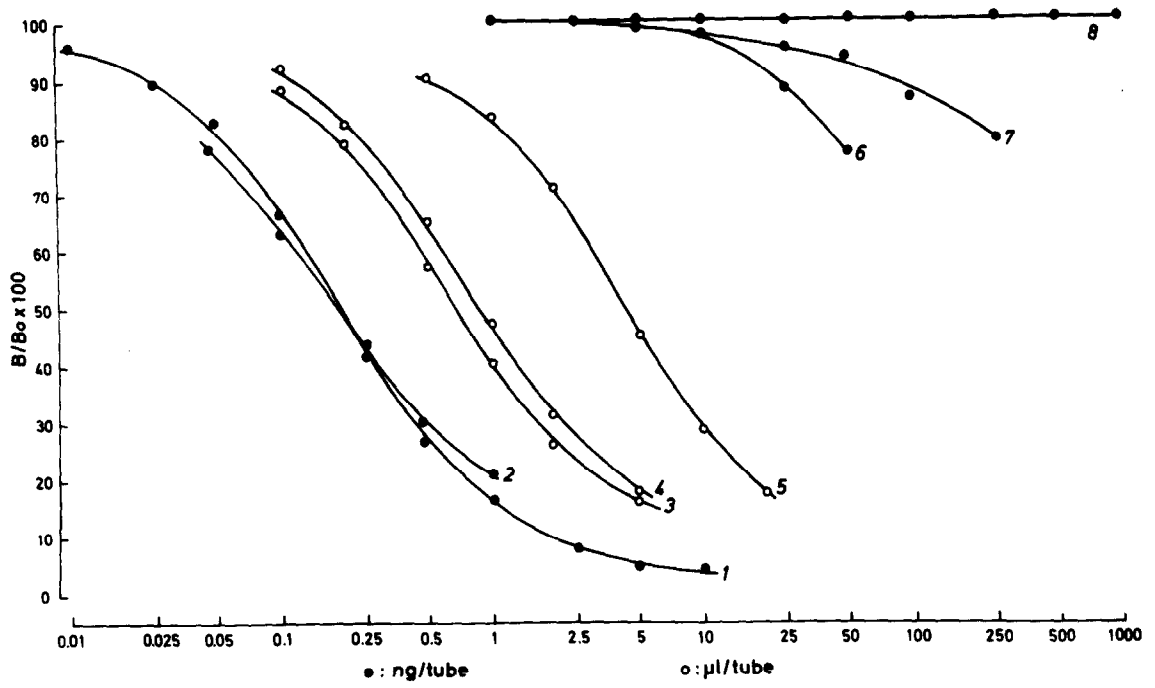

Fig. 1. Competitive binding curves generated by human EGF_{1-52} (1), human F-met EGF_{1-53} (2), breast cyst fluid (3), milk (4), urine (5), human insulin (6), mouse EGF (7) and STH, somatomedin C and bovine insulin (8) in $hEGF_{1-52}$ radioimmunoassay. (•), (○), $B/B_0 \times 100$, percentage of trace binding.

Molecular size of immunoreactive EGF

Sephadex G50 'Superfine' gel chromatography elution profiles of immunoreactive EGF in urine, breast cyst fluid and milk are shown in Fig. 3. The major peak of immunoreactivity occurred at the point at which pure standard EGF_{1-52} was eluted (K_d = 0.4, mol. wt = 6000). Small amounts of immunoreactive material of higher molecular weight were also observed in each biological fluid.

DISCUSSION

Radioimmunoassay of epidermal growth factor has been available since 1972 [13–17].

Our methodology is sensitive, precise and very specific. There is no cross-reaction with growth hormone or somatomedin C, and mouse EGF, which is very similar in amino acid sequence, has a cross-reaction of only 0.03%. This species specificity has already been observed by other authors [14, 18]. Furthermore, this assay can identify EGF_{1-53}, which has in addition a formylmethionine in the *N*-terminal position, as well as the EGF_{1-52}, which does not contain the carboxy terminal arginine.

With our assay we have detected EGF in urine, colostrum, milk and human breast cyst fluid. The argument that the assayed immunoreactivity corresponds directly to monomeric EGF is supported by the fact that the cross-reaction is complete between standard EGF_{1-52} and the various dilutions of the biological fluids. Likewise, after filtration on Sephadex G 50, the elution volume was shown to be identical for the immunoreactivities in the biological fluids and in the standard preparation of EGF. It has also been found that less than 5% of the immunoreactivity is eluted in the higher molecular weight zone. These findings are consistent with the data published by Hirata and Orth [19, 20] and Beardmore *et al.* [21].

Our data on the EGF concentration detected in the pool of urine (47 ng/ml) are consistent with the observations of other authors. Indeed, Gregory *et al.* [14] found a concentration of EGF of 30 ng/ml in a pool of urine and EGF concentrations varying from 6 to 150 ng/ml in individual samples. This range of concentrations

Table 1. Comparison of EGF concentrations (ng/ml) *in biological fluids (mean ± 1 S.D.; values are compared to EGF concentrations in milk)*

Pool of urine	Colostrum — collected 1–3 days after delivery (n = 8)	Milk — collected 4–8 days after delivery (n = 16)	Breast cyst fluid (n = 139)
47	197 ± 56*	107 ± 22	241 ± 143*

*Statistical difference ($P < 0.0005$) evaluated by t test.

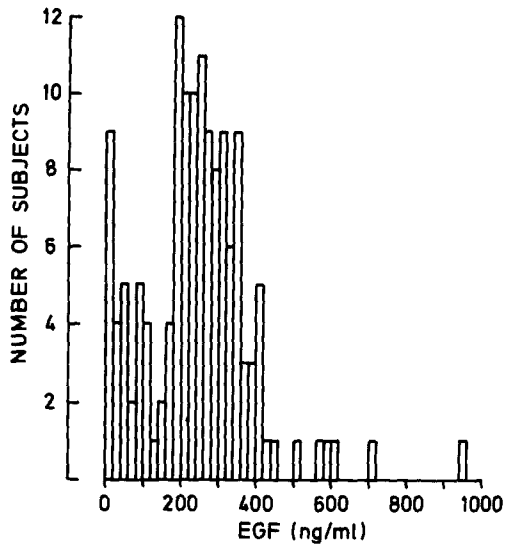

Fig. 2. Distribution of EGF concentrations in 139 breast cyst fluids.

in urine has been confirmed by Starkey and Orth [15], who described amounts of EGF varying from 29 to 272 ng/ml, with a mean of 88 ng/ml.

The work of Carpenter [3] and later of Beardmore *et al.* [21] has already shown that milk and colostrum contain important quantities of EGF. Our results confirm that the concentrations of EGF are greater in colostrum than in milk and our mean values are very similar to those found by Starkey and Orth [15] and Beardmore *et al.* [21].

To our knowledge, EGF has not been detected previously in breast cyst fluid. EGF was found in each sample, and in 78% of the samples the values were greater than 145 ng/ml, which is the maximal concentration found in milk. Furthermore, the mean value of the concentrations of EGF in breast cyst fluid was greater than that in milk and colostrum.

At this time there are no data to explain the existence or the varying concentration of EGF in breast cyst fluid. If it is postulated that the source of EGF is the mammary gland, it is then possible that EGF is a factor involved in paracrine or autocrine regulation of cellular proliferation. Moreover, the production of EGF could depend on the hormonal equilibrium and in particular on androgenic stimulation, as in the mouse submaxillary gland [13, 22].

Because of the demonstrated mitogenic activity of EGF [23–25], particularly in normal mouse mammary epithelium [4–9], in two breast cancer cell lines: MCF-7 [26, 27] and T-47 D [26, 28], and in primary cultures of fibroadenome [29], this growth factor might play a role in the partial proliferative pathology of cystic disease of the breast. Indeed, numerous papers [26–28] have shown that doses of EGF ranging from 0.01 to 60 ng/ml stimulate *in vitro* the multiplication of

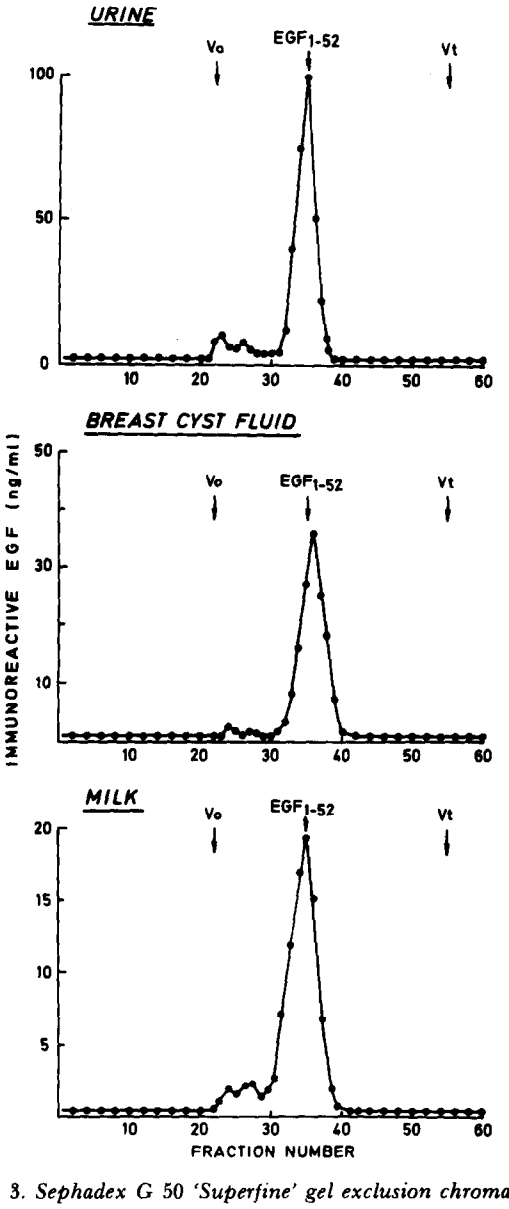

Fig. 3. Sephadex G 50 'Superfine' gel exclusion chromatography of concentrated urine, breast cyst fluid and milk. (•) EGF concentrations in eluates; V_0, void volume (blue dextran); v_t, total volume ($Na^{125}I$); EGF_{1-52}, elution volume of ^{125}I-labelled $hEGF_{1-52}$.

T-47 D and MCF-7 cells assayed by the [^{3}H]thymidine incorporation test.

The concentrations we assayed (5–945 ng/ml) in the breast cyst fluid were equal to or greater than those that stimulate the proliferation of these cancer cells, and the wide range of values is not likely to be related to the inter-assay coefficient for all the samples were assayed at the same time.

We are currently attempting to correlate the concentrations of EGF with cell proliferation, functional differentiation, cyst volume and relevant steroid concentrations. So far, we have emphasized that the higher are the EGF level, the lower the concentration of α-lactalbumin, which is a factor of functional differentiation [Collette *et al.*, personal communication].

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