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Radio and Enzyme Immunoassays for Human Epidermal Growth Factor with Mouse Monoclonal Antibodies

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SUMMARY

We have developed a solid-phase radioimmunoassay (SP-RIA), and two "sandwich"-type ELISA with mouse monoclonal antibodies (MAbs), for the quantitation of human epidermal growth factor (EGF).

CB-EGF.1, an IgG1 MAb with high affinity for human EGF, is used in the SP-RIA as coating antibody at 20 μg/ml, and the samples are mixed with radioiodinated recombinant human EGF (rhEGF), and incubated for 3 hours at 37°C. The sensitivity of this assay is 2 ng/ml, with intra-, and inter-assay coefficients of variation below 12%, for a wide range of concentrations (top limit of 300 ng/ml).

The two "sandwich" ELISA assays were constructed using CB-EGF.2, an IgG1 MAb that recognizes both human and mouse EGF, as "capture" antibody, and horseradish peroxidase conjugated CB-EGF.1. If antigen and conjugate are incubated sequentially, the sensitivity of the system is 6 ng/ml, but if they are mixed and applied immmediately to the active solid phase, the sensitivity of the assay increases to 400 pg/ml.

The "sequential" ELISA shows an excellent correlation with the SP-RIA for the quantitation of rhEGF in samples coming from different steps of the production process of this molecule. Both ELISA systems have been preliminarily tested for the detection of EGF in human saliva and urine, rendering values that coincide with previously reported data.

RESUMEN

Utilizando anticuerpos monoclonales (AcM) de ratón para el factor de crecimiento epidérmico (EGF), hemos construido un radioinmunoensayo de fase sólida (RIA-FS) y dos sistemas ELISA tipo *sandwich*, para la cuantificación de esta molécula.

El RIA-FS está basado en la adsorción del AcM CB-EGF.1 (IgG1) al poliestireno, a 20 μg/ml, y la incubación de las muestras a estudiar con el EGF radioyodado, por 3 horas, a 37°C. La sensibilidad del ensayo es de 2 ng/ml, con coeficientes de variación intra e inter ensayo por debajo de 12%, para un amplio rango de concentraciones (límite superior de 300 ng/ml).

En los ELISA se empleó este mismo AcM, conjugado con peroxidasa, como anticuerpo de revelado, y el CB-EGF.2 (IgG1) en la fase sólida. Si el antígeno y el conjugado se incuban secuencialmente, la sensibilidad del sistema es de 6 ng/ml, pero si ambos son mezclados y aplicados simultáneamente a la fase sólida activa, la sensibilidad del ensayo se incrementa hasta 400 pg/ml.

El ELISA "secuencial" demostró una excelente correlación con el RIA-FS para la cuantificación de EGF humano recombinante, en muestras provenientes del proceso de producción y purificación de esta molécula. Ambos sistemas ELISA han sido ensayados en pruebas preliminares para su capacidad de detección de EGF humano en muestras de saliva y orina, arrojando valores que coinciden con datos reportados previamente en la literatura.

INTRODUCTION

Epidermal growth factor (EGF) is a single chain polypeptide consisting of 53 aminoacids, with potent proliferation stimulatory activity on numerous cell types *in vitro*; *in vivo*, EGF stimulates the growth of epithelial cells, including cornea, and could play some role in angiogenesis, regeneration of nerve, and tendon healing, among other functions (Cohen, 1987; Elliot, 1980; O'loughlin *et al.*, 1985; Goldsmith, 1983; Tsutsumi *et al.*, 1986).

Human EGF is identical to urogastrone, an antisecretory agent also found in urine, and has a remarkable homology in sequence with mouse EGF, being identical 37 of the aminoacid residues, with three disulfide linkages located in the same relative positions (Harper *et al.*, 1987). As expected, mouse EGF is recognized by the EGF receptor on human cells, and their biological activities are nearly undistinguishable (Cohen, 1987).

Recently, the availability of large amounts of EGF, obtained by recombinant DNA techniques (Oka *et al.*, 1985; Urdea, 1987), has promoted the clinical evaluation of this molecule as a wound-healing drug (Van Brunt and Klausner, 1988); some laboratories have also started to assay EGF as an antitumoral agent (Fonseca *et al.*, 1988), based on the emerging data that suggest that some cancers of epidermal origin express an unusual large amount of surface receptors for this growth factor (Macias *et al.*, 1986; Veale *et al.*, 1987).

In this context, the development of quantitative immunoassays for human EGF could be essential for both preclinical and clinical pharmacology. Some authors have recently reported EGF concentrations in saliva, serum, urine, milk, sweat, amniotic fluid, gastric juice, and seminal plasma, using radioimmunoassays, enzyme immunoassays, and time-resolved immunofluorimetric assays (Connolly and Rose, 1988; Pesonen *et al.*, 1987; Hirata *et al.*, 1980, 1982; Joh *et al.*, 1986). The progressive introduction of monoclonal antibodies (MAbs) in these assays will eventually increase their sensitivity and specificity, as has occurred in other cases (Budd and Smith, 1986).

In this paper we report the development of a solid-phase radioimmunoassay, and two "sandwich"-type ELISA, for the quantitation of human EGF, based on two IgG1 mouse MAbs previously generated by our group (Freyre *et al.*, 1989). Apart from their potential relevance for pharmacological studies, these systems are being used for the monitoring of the process of production of this molecule in our Center, complementary to the radioreceptor analysis (Macias *et al.*, 1985) currently employed for the determination of the biological activity of the final preparations.

MATERIAL AND METHODS

Monoclonal Antibodies

The generation and characteristics of the two IgG1 (k) mouse MAbs CB-EGF.1 and CB-EGF.2 have been described elsewhere (Freyre *et al.*, 1989; Gavilondo *et al.*, 1988); briefly, BALB/c mice were immunized intraperitoneally (i.p.) with highly pure recombinant human EGF (rhEGF), and spleen cells fused with SP2/0-Ag14, or P3/x63-Ag8-653 myeloma cells. CB-EGF.1 was selected due to its high recognition of rhEGF in indirect ELISA, with the antigen coated to the plates. CB-EGF.2 was chosen after the demonstration that this MAb did not substantially interfere with CB-EGF.1 for the binding of radiolabelled rhEGF, when the latter antibody was adsorbed to plastic surfaces. CB-EGF.1 is specific for human EGF, and has an affinity constant in the order of 10 to the minus 9 M. CB-EGF.2 recognizes both human and mouse EGF.

Production and Purification of MAbs

BALB/c mice, primed with 0.5 ml of mineral oil, were injected i.p. with 3 million hybridoma cells. Ascites was collected by repetitive puncture, clarified

by centrifugation, and delipidized with chloroform (v/v) before purification. After dialysis against phosphate buffered saline (PBS), the ascites was diluted with 1.5 M glycine, 3 M NaCl buffer, pH 8.9, applied to a Protein A Sepharose CL-4B matrix (Pharmacia), and eluted as recommended by Ostlund (1986). The fractions were dialyzed against PBS, and tested for activity using an indirect ELISA (Gavilondo *et al.*, 1988) with the antigen coated to the plates. All protein determinations were carried out as described by Lowry *et al.* (1951), and the purity of the final antibody preparations was always more than 90%, as estimated by sodium dodecyl sulphate polyacrilamide gel electrophoresis (Laemli, 1970).

Radiolabelling of rhEGF

RhEGF was labelled with 125-Iodine (Amersham) following essentially the chloramine-T method (Hunter and Greenwood, 1962).

Peroxidase Conjugation of MAbs

Purified CB-EGF.1 was conjugated with horseradish peroxidase (HRP, Sigma, Fraction VI), using the periodate method, as suggested by Nakane (1979).

Solid-Phase Radioimmunoassay (SP-RIA)

Polyestirene (PE) strips (Dynatech) were coated with 5-30 μg of the purified CB-EGF.1 MAb, per ml of carbonate-bicarbonate buffer, pH 9.6, for different times at 37°C, and the remaining sites blocked with 2% delipidized milk in coating buffer, for two hours at 37°C. One hundred μl of the samples to be tested were mixed 1:1 with 100,000 cpm of radiolabelled rhEGF, and incubated for different periods of time (1-8 hours), at temperatures ranging between 4 and 37°C. After extensive washing with 0.05% Tween-20 in destiled water, the wells were individualized, and the radioactivity measured in a LKB Clinigamma counter. MAbs against Apo B lipoproteins (Sorell *et al.*, 1986) were adsorbed to the solid phase as negative controls. Controls for "total binding" (no "cold" samples), and "total radioactivity" were also included.

Enzyme Linked Immunosorbent Assay (ELISA)

Solutions of 5-30 μg of purified CB-EGF.2, per ml of carbonate-bicarbonate buffer, were adsorbed for different times at 37°C to Dynatech PE EIA plates (100 μl/well). The plates were blocked with 1% bovine seroalbumin (BSA, Sigma) in PBS for two hours at 37°C, and two variants were evaluated for the estimation of EGF in the samples:

(a) one hundred μl of sample were added to three replica wells and incubated 3 hours at 37°C. After extensive washing, the plates were incubated with 1:1000–1:2000 dilutions of HRP-CB-EGF.1, for 1 hour at 37°C.

(b) the sample and diluted conjugate were mixed for a total volume of 100 μl, added immediately to the plates, and incubated 1 hour at 37°C.

After these procedures, the plates were washed, and the reaction revealed with 0.04% ortho-phenylene diamine (Sigma), and hydrogen peroxide in citrate buffer, and evaluated at 492 nm in a Titertek Multiskan.

Samples

Highly purified standard preparations, with known quantity of rhEGF, were supplied by the Pilot Plant/Production Division of our Center. This unit also provided us with the supernatants from the fermentation process of recombinant and non-recombinant yeast, as well as with material coming from different steps of the purification process of rhEGF. For the measurement of EGF in the SP-RIA and ELISA systems, the samples were always prediluted 1:50–1:2000 with PBS-BSA (0.9–0.5% for each system, respectively).

RESULTS AND DISCUSSION

The obtention of mouse MAbs against EGF can be seen as a potentially difficult task, mainly based on the prediction that human EGF could be of low immunogenicity in mice, both because its structural homology with the murine molecule, and low molecular weight. Nevertheless, it has been shown that mouse MAbs against human EGF can be readily obtained, both using *in vivo*, and *in vitro* immunizations (Harper *et al.*, 1987; Freyre *et al.*, 1989; Gavilondo *et al.*, 1988; Nishikawa *et al.*, 1987; Ikuta *et al.*, 1985; Hissey *et al.*, 1985; Akai *et al.*, 1986). Such MAbs have been largely employed for the immunopurification of the molecule (Harper *et al.*, 1987; Hissey *et al.*, 1985; Akai *et al.*, 1986). The construction of radio and enzyme immunoassays have mainly depended on rabbit polyclonal antibodies (Connolly and Rose, 1988;

Pesonen *et al.*, 1987; Hirata *et al.*, 1980, 1982; Joh *et al.*, 1986; Byny *et al.*, 1972; Dailey *et al.*, 1978; Oka and Orth, 1983; Hayashi *et al.*, 1985; Tsutsumi *et al.*, 1988; Hayashi *et al.*, 1985; Kurobe *et al.*, 1986; Abe *et al.*, 1987), and MAbs for EGF have been seldomly employed, possibly because, being EGF a small molecule with few defined epitopes (Cooke *et al.*, 1987), the obtention of non-competitive, or non-interfering MAbs could be envisaged as a difficult task.

Being our most immediate goal the development of routine immunoassays to be employed during the production process of rhEGF in our Center, we took advantage of the existence of several MAbs recognizing this antigen that had been generated in our group (Freyre *et al.*, 1989). The construction of a radioimmunoassay that would employ one of such MAbs was seen as a first step, and for such work we selected CB-EGF.1, a MAb which had been shown to compete successfully with a hyperimmune rabbit antiserum in liquid phase RIA (Freyre *et al.*, 1989). CB-EGF.1 was coated to PE wells and evaluated as capture antibody with radioiodinated and "cold" rhEGF.

Twenty μg of MAb per ml of coating buffer, for 3 hours at 37°C, ensured a maximum radioactivity binding, without further increase in efficiency after variation of coating times. In subsequent experiments, the optimal incubation temperature and time for the samples of "cold" and radioiodinated rhEGF were determined as 37°C, and three hours. A typical standard curve is shown in Figure 1, indicating that the assay has a lower sensitivity level of 2 ng/ml, with an upper limit of 300 ng/ml.

The precision of the SP-RIA was assessed with a standard preparation of

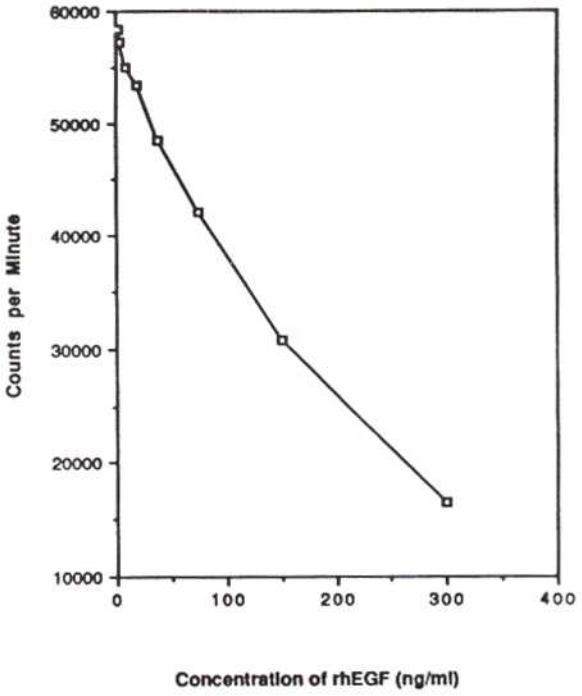

FIG. 1. Standard curve for the quantitation of recombinant human EGF, using the solid-phase RIA. Total radioactivity: 97,834 cpm; total binding: 28,681 cpm; negative control: 208 cpm.

rhEGF, in three independent experiments, in which each dilution was performed in quadruplicate. Under these conditions, the intra and inter-assay variabilities ranges between 3.7 and 11.4%, and 5.4 and 10.7%, respectively (Table 1).

Table 1
PRECISION OF THE SOLID-PHASE RADIOIMMUNOASSAY, AS DETERMINED WITH THE RhEGF STANDARD PREPARATIONS

EGF Concentration (ng/ml)	Intra-assay Coefficient of Variation (%) (n=4)	Inter-assay Coefficient of Variation (%) (n=3)
300	11.4	10.7
150 (a)	10.0	10.5
75	9.1	8.9
37.5	9.0	9.4
19	6.1	7.5
9.5	3.7	8.0
4.2	5.1	5.4
2.1	5.1	6.2

Note: *n* = number of replicas tested in the assay, or number of independent assays; *(a)* value correspondent approximately to the half-maximal radioactivity value.

This SP-RIA was immediately evaluated for the monitoring of the purification process of rhEGF; first, known amounts of rhEGF were added to the supernatants of non-recombinant yeast culture, so as to determine the possible influence of media and metabolic products on the measurements. An excellent correspondence between the actual and predicted quantities was noted (Table 2). Finally, three independent assays for the quantitation of 5 samples from the different steps of the purification process of rhEGF from recombinant yeast supernatants were performed with the SP-RIA; the reproducibility, as seen in Table 3, was always below 10%.

Table 2
EFFECT OF NON-RECOMBINANT YEAST SUPERNATANT IN THE QUANTITATION OF RhEGF CONCENTRATIONS BY THE SOLID-PHASE RADIOIMMUNOASSAY

Standard rhEGF Concentration	Estimated Concentrations (a) after the Assay	
10	8.7 ± 0.4;	8.6 ± 0.3
50	49.6 ± 1.2;	51.0 ± 1.1
200	198.1 ± 6.8;	198.3 ± 5.2

Note: All quantitations expressed in ng/ml; (a) = two independent assays were performed; values expressed as mean and standard deviation of tests in quadruplicate; the values were calculated by extrapolation from a simultaneous standard curve experiment.

Table 3
INTER-ASSAY REPRODUCIBILITY FOR THE SOLID PHASE RADIO-IMMUNOASSAY, WHEN QUANTITATING RhEGF FROM SAMPLES COMING FROM THE PURIFICATION PROCESS

Process Sample No.	Estimated RhEGF Concentration (a)	Coefficient of Variation (%)
1	8.5 ± 0.3	2.3
2	25.6 ± 1.2	2.5
3	67.0 ± 2.3	5.2
4	173.5 ± 9.9	8.0
5	255.7 ± 22.5	9.8

Note: (a) = as determined by extrapolation from a simultaneous standard curve; values expressed in μg/ml, as mean and standard deviation of three independent assays.

For the subsequent development of "sandwich"-type ELISA, alternative to the SP-RIA, we chose CB-EGF.1 as second antibody, taking into account both its specificity for rhEGF and the sensitivity shown in the SP-RIA. CB-EGF.2, a MAb that identifies both human and mouse EGF, and that does not substantially interfere with the binding of radiolabelled EGF by CB-EGF.1, was selected as "capture" antibody.

The best results for the adsorption of CB-EGF.2 to the PE plates were seen at a concentration of 20 μg/ml, with 3 hours and 37°C as other basic conditions for such step. Figure 2 shows that when the rhEGF sample is incubated for 3 hours at 37°C, followed by the HRP-conjugated CB-EGF.1 for 1 hour at room temperature, the sensitivity of the assay reaches 6 ng/ml, with an upper range value of 125 ng/ml. If the sample and conjugate are mixed and applied to the active solid phase for 1 hour at 37°C, the sensitivity approaches 400 pg/ml, with an upper range value of approximately 15 ng/ml (Figure 3).

An explanation for such differences is still mainly speculatory; if CB-EGF.1 determines the sensitivity of the system, then it is possible that the incubation of antigen and CB-EGF.2 reduces the sensitivity due to some steric hindrance for CB-EGF.1, if the sites recognized by the antibodies are close in the molecular structure. When the antigen is placed under "equal" reaction conditions for both antibodies, as is the case of the simultaneous incubation, the recognition of rhEGF by CB-EGF.1 could be optimal, rendering a better overall sensitivity for the assay. The absence of effective competition of these two antibodies in the SP-RIA does not rule out this possibility, as CB-EGF.2 is in this case adsorbed to the solid phase, and the effect is overall a quantitative one.

The sequential ELISA was tested for repeatibility and reproducibility, experiments that were performed with other five samples coming from the purification process of rhEGF; additionally, these same samples were tested simultaneously by the SP-RIA, and the results compared. Table 4 indicates that the ELISA is highly repeatible and reproducible. Figure 4 shows that both systems correlate well in the quantitation of rhEGF.

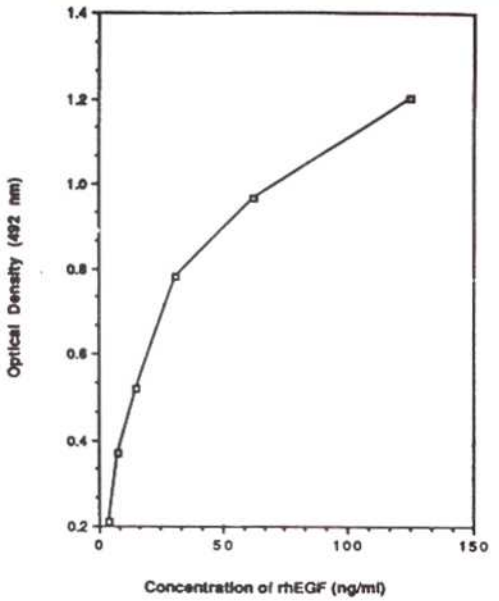

FIG. 2. Standard curve for the quantitation of recombinant human EGF, using the sequential ELISA variant.

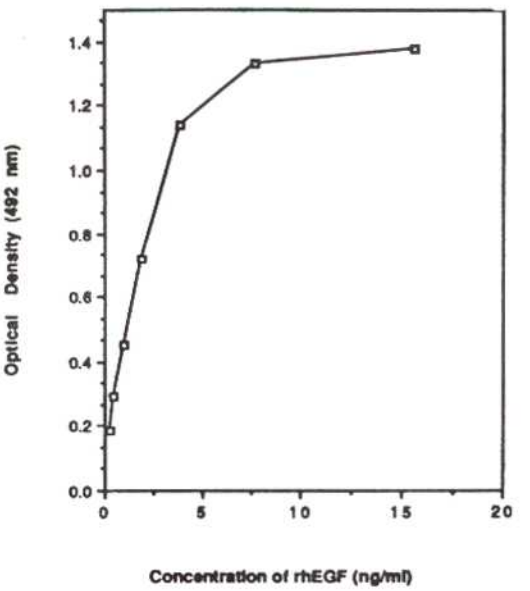

FIG. 3. Standard curve for the quantitation of recombinant human EGF, using the ELISA system involving the simultaneous incubation of samples and conjugated second antibody

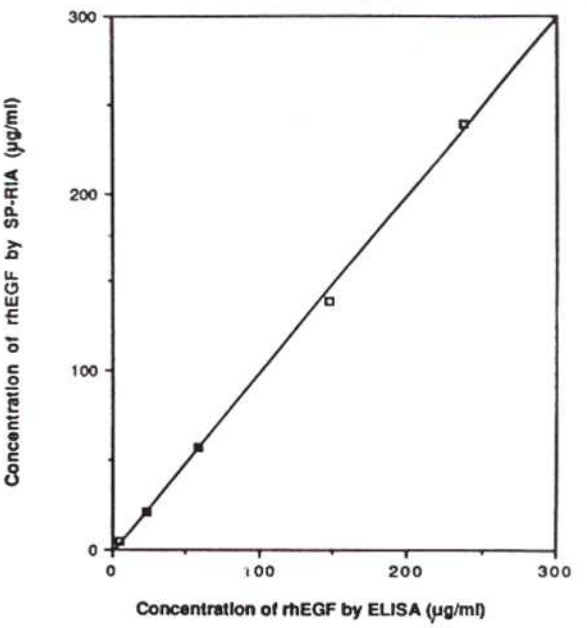

FIG. 4. Correlation between the solid-phase RIA and the sequential ELISA variant, in the quantitation of rhEGF samples derived from the purification process of the molecule. Correlation coefficient: $r = 0.99$; regression line: $y = x + 1.86$.

Table 4

PRECISION OF THE "SEQUENTIAL" ELISA, AS DETERMINED WITH SAMPLES COMING FROM THE PROCESS OF PURIFICATION OF RhEGF

Process Sample No.	Intra-assay Repeatibility (n = 5)	C.V. (%)	Inter-assay Reproducibility (n = 4)	C.V. (%)
1	4.5 ± 0.545	12.0	5.0 ± 0.1	1.9
2	21.4 ± 0.821	3.8	23.4 ± 1.41	6.0
3	57.1 ± 0.346	0.6	58.5 ± 2.96	5.0
4	143.3 ± 0.687	0.5	148.2 ± 7.45	5.0
5	241.2 ± 1.439	0.6	239.3 ± 19.30	8.0

Note: Values for intra- and inter-assay repeatibility and reproducibility expressed in μg/ml, as mean and standard deviation; n = number of replicas tested in the assay, or number of independent assays; C.V. = coefficient of variation.

These results have led us to the progressive substitution of the SP-RIA by the non-radioactive "sequential ELISA" in the routine determination of rhEGF in our Center. Also, using both ELISA variants, we have tested the presence of EGF in saliva and urine; the values have ranged from 0 to 3 ng/ml, and 17 to 55 ng/ml, respectively (data not shown in detail). These values agree, in general, with the concentration ranges reported by other authors (Connolly and Rose, 1988; Pesonen *et al.*, 1987; Hirata *et al.*, 1980, 1982; Joh *et al.*, 1986).

Due to the general interest that such systems could have for preclinical and clinical pharmacology, the assays are now under more extensive evaluation for such purposes.

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