[Chem. Pharm. Bull. 34(4)1721—1727(1986)]

Isolation of Epidermal Growth Factor with Monoclonal Antibody

KUNIHISA AKAI,* MASAAKI GOTO and MASATSUGU UEDA

Research Institute of Life Science, Snow Brand Milk Products Co., Ltd., 519 Shimo-ishibashi, Ishibashi-machi, Shimotsuga-gun, Tochigi 329-05, Japan

(Received August 21, 1985)

Three kinds of monoclonal antibodies directed to human epidermal growth factor (hEGF) were obtained. One of them did not react with mouse epidermal growth factor (mEGF) but the others showed reactivity with mEGF. Antibody having binding ability to both hEGF and mEGF was suitable for the purification of EGFs. Human EGF was isolated from normal adult human urine in a high yield by means of a simple purification process using an immunoadsorbent column of monoclonal antibody. Mouse EGF was also purified from mouse submaxillary gland using the same immunoadsorbent column and a Sephadex G-50 column. Both of the purified EGFs gave a single band with an apparent molecular weight of 6000 on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The amino acid sequences were consistent with those reported previously.

Keywords——monoclonal antibody to hEGF; human EGF; mouse EGF; immunoadsorbent column; EGF purification

Introduction

Epidermal growth factor (EGF) is a polypeptide hormone composed of 53 amino acids and is a potent stimulator of the growth of various cell types in culture as well as epithelium *in vivo*.[1] EGFs were isolated from mouse submaxillary gland[2] and human urine,[3] and human EGF (hEGF) was considered to be identical to human β-urogastron.[4] The primary structures of mouse EGF (mEGF)[5] and hEGF[4] have been determined and the two molecules are quite homologous. Recently, heterogeneity of EGF in a species was reported,[6-8] and moreover, EGF is considered to be derived from a large precursor protein.[9-13]

The effective purification of EGF is required for biological testing and for studying the molecular properties of EGF. Usage of monoclonal antibody for the purification is advantageous.[14,15] However, high specificity for the antigen is sometimes a disadvantage for purifying a protein that shows molecular heterogeneity. Therefore, an antibody having a broader epitope and/or recognizing a larger part of the antigen, is desirable in some purification processes. A monoclonal antibody to hEGF has already been reported[16] but its epitope was specific to hEGF and it did not react with mEGF. To overcome this problem, we tried to obtain monoclonal antibody having a broader epitope that would be suitable for the purification of various EGFs.

In this report, we describe the establishment of hybridoma cell lines producing the antibody to hEGF and demonstrate the effective purification of hEGF and mEGF with a monoclonal antibody.

Materials and Methods

Materials——Materials were purchased from the indicated sources: Bio-Gel P-10, Bio-Rex 70, silver-staining kit, protein assay kit and Affi-Gel 10 (Bio-Rad, Richmond, Calif.); DE-52 cellulose and CM-52 cellulose (Whatman, Clifton, N.J.); Radioactive compounds (Amersham International plc, Buckinghamshire, England and New England

Nuclear, Mass.); antibiotics (Meiji Seika Co., Ltd., Tokyo); disposable culture plastic ware (Nunc, Denmark); flexible microtiter plates (Dynatech, Alexandria, Va.); Freund's complete adjuvant (Difco, Detroit); IODO-GEN (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril, Pierce Chemical Co., Rockford, Ill.); polyethylene glycol (BDH Chemical, Poole, England); RPMI 1640 and Eagle's MEM (Gibco, N.Y.); fetal calf serum (M.A. Bioproducts, Walkersville, Md.); biotinylated anti-mouse immunoglobulin G (IgG), avidine and biotinylated horseradish peroxidase (Vector Labo, Burlingame, Calif.); Mono Ab-ID kit for mouse monoclonal antibody (Zymed Labo, Calif.); mEGF (Toyobo Co., Ltd., Osaka); human embryonic lung cell WI-38, mouse myeloma cell P3-NSI/1-Ag4-1 (Dainippon Seiyaku Labo, Osaka).

Radioreceptor Assay for EGF——The amount of EGF was measured by the radioreceptor assay method based on competitive binding of EGF to the placental cell membrane in the presence of ^{125}I-labeled mEGF (Amersham, IM. 124). The amount of hEGF was estimated based on the assumption that hEGF and mEGF bind equally to the plasma membrane of human placental cell membranes. The placental cell membranes were purified from term human placentas obtained immediately after spontaneous delivery according to the method of Hirata and Orth.[6] Radioreceptor assay (RRA) was performed in 12 × 80 mm polypropylene tubes at 25 °C. Sample or standard (100 μl) and 300 μl of RRA standard diluent (50 mM Tris–HCl, pH 7.4, and 2.5 mg/ml bovine serum albumin (BSA)) and 100 μl of suspended cell membrane (250 μg protein/ml) were incubated for 10 min and 100 μl of ^{125}I-mEGF (3×10^4 cpm, 200 μCi/μg) was added to each tube. After a 40 min incubation, the assay tube was centrifuged at $6000 \times g$ at 4 °C for 10 min. The supernatant was removed by aspiration and the pellet was washed by suspending it in 1 ml of cold RRA standard diluent. After the second centrifugation, the radioactivity in the pellet was counted with an auto-gamma counter. Specific binding was determined as the difference between the radioactivity bound in the assay tube and that in the control tube containing excess unlabeled mEGF.

***In Vitro* Bioassay of EGF**——*In vitro* bioassay of EGF was performed by measuring the stimulatory effect of EGF on incorporation of ^{3}H-thymidine into DNA in cultured human embryonic lung cells (WI-38) according to the methods described by Klagsbrun *et al.*[17,18]

Purification of Antigen hEGF from Human Urine——Antigen hEGF was purified from 50 l of adult human urine according to the conventional methods, involving 5 purification steps; adsorption on Bio-Rex 70, Bio-Gel P-10 gel filtration, Sephadex G-50 gel filtration, passage through DE-52 cellulose, and CM-52 cellulose ion-exchange chromatography.[3,19] By this 5-step purification, hEGF was purified about 280-fold with 20% recovery. This partially purified hEGF having about 15% purity was used as the antigen.

For the characterization of monoclonal antibody, hEGF was further purified by DE-52 cellulose chromatography based on the method of Savage Jr. and Happer.[19] The product was homogeneous on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

SDS Polyacrylamide Gel Electrophoresis——SDS polyacrylamide gel electrophoresis was performed by the method of Laemmli[20] and silver staining was done according to the manufacturer's (Bio-Rad) directions.

Protein Determination——The protein was determined by Coomassie brilliant blue binding assay according to the method of Bradford.[21] The reagents were purchased from Bio-Rad as a protein assay kit. A standard curve was made with BSA.

Amino Acid Sequence——The amino acid sequences of purified hEGF and mEGF were analyzed with an Applied Biosystems 470A protein sequencer, a gas-phase sequencer, and PTH amino acid was analyzed by high-performance liquid chromatography (HPLC) with reversed-phase silica-based supports (Spectra-Physics Model 8100, Senshukagaku SEQ-4 column).[22,23]

Immunization——Eight week-old female BALB/C mice were immunized intraperitoneally with 200 μg of partially purified hEGF (30 μg EGF/200 μg protein) emulsified 1 : 1 in Freund's complete adjuvant in a total volume of 0.5 ml, twice with an interval of 2 weeks. Two weeks later, they were boosted intraperitoneally with 0.5 ml, containing 100 μg of the antigen in phosphate-buffered saline (PBS) without adjuvant. At 1 week after the second immunization, samples of sera (100 μl) were obtained by cardiac puncture. The sera gave precipitation lines in the Ouchterlony double-immunodiffusion with hEGF. At 3 d after the booster, the spleen cells were collected for cell fusion.

Cell Fusion and Selection of Hybridoma Clones——Spleen cell suspension from mice was prepared in RPMI 1640, containing 2 mmol/l glutamine, 1 mmol/l pyruvate, penicillin (50 U/ml), streptomycin (50 μg/ml), and 15% (v/v) fetal calf serum (this is designated as medium I). Cells (2×10^9) from three spleens were fused with 7×10^8 mouse myeloma cells (P3-NSI/1-Ag4-1 cells) by the procedure described by Oi and Herzenberg,[24] using polyethylene glycol 1500. The fusion mixture in HT medium (medium I plus 100 μmol/l hypoxanthine and 15 μmol/l thymidine) was seeded in 96-well microtiter culture plates (0.1 ml of suspension containing 2×10^6 cells/well). Cells in the selective HAT medium (medium I plus 100 μmol/l hypoxanthine, 0.4 μmol/l aminopterine and 15 μmol/l thymidine) were cultured according to the procedures and schedules reported. After 3 weeks of culture, 384 wells demonstrating cell growth were tested for production of antibodies by a solid-phase antibody-binding assay and based on the inhibitory effects on binding of radio-labeled EGF to the receptor. Both hEGF and mEGF were used as the antigen. Fourteen antibody-positive cells were transferred into 1 ml cultures in 24-well tissue culture plates and switched to HT medium by removing half of the medium and replacing it with fresh medium every 3 d for 3 weeks. All positive cultures were

expanded in T flasks (50 ml), and HT medium was switched to medium I in a similar manner. Rescreening of the supernatants with the binding assay showed that 9 cultures produced antibodies consistently. Five of the cultures produced antibodies that could bind strongly to hEGF, one produced antibody binding strongly to mEGF and the other three cultures produced antibodies binding equally to hEGF and mEGF. The hybridomas of those 9 cultures were cloned by limiting dilution in microtiter plates.[24] Cloned hybridomas were tested for EGF antibody production in a similar manner and antibody production was confirmed.

Solid-Phase Antibody-Binding Assay——A solid-phase enzyme immunoassay used for the detection of antibody in hybridoma supernatants was performed on flexible round-bottomed 96-well microtiter plates using biotinylated anti-mouse IgG, avidin and biotinylated horseradish peroxidase, according to the manufacturer's (Funakoshi) directions, except for the detection of the peroxidase activity based on the appearance of fluorescence due to oxidized tyramine.

Preparation and Purification of Antibodies from Ascitic Fluid——BALB/C (8 to 10 weeks old) mice received intraperitoneal injections of 0.5 ml of pristane at 10 and 3 d before 0.5 ml (10^7 cells) of hybridoma cells was given intraperitoneally. Ten to 15 d after cell injection, 3 to 5 ml of ascitic fluid was obtained from each mouse. Antibodies from ascitic fluid were purified according to the method of Staehelin *et al.*[14]

Preparation of Immunoadsorbents——The immunoadsorbent column was prepared by fixation of the antibody on Affi-Gel 10 according to the procedure reported by Staehelin *et al.*[14]

Radiolabeling of hEGF——Labeling of purified hEGF with iodine 125 was done in Eppendorf microtubes (1.5 ml) by the solid-phase method with the IODO-GEN reagent according to the method of Garcia *et al.*[25]

Results

Characterization of Monoclonal Antibody

For selecting a suitable antibody for the purification of EGF, fundamental properties of the antibodies were investigated. Three kinds of hybridoma clone which produce monoclonal antibodies for hEGF were established. The subclass of all those antibodies was $\gamma_1\kappa$. The association constants to hEGF of the three kinds of antibody were 1.4×10^{10} to 1.06×10^{11} l/mol (Table I). The binding affinities to hEGF and mEGF were also investigated. As shown in Fig. 1, antibody produced by clone 8410 has high affinity for hEGF and also has the ability to bind to mEGF. Antibody produced by clone 9111 bound to hEGF but not to mEGF. Antibody from clone 2710 showed similar binding affinity to both hEGF and mEGF. These

TABLE I. Subclass and Binding Affinity of Monoclonal Antibodies

Clone	Subclass[a]	K value (l/mol)
2710	$\gamma_1\kappa$	1.43×10^{10}
8410	$\gamma_1\kappa$	1.06×10^{11}
9111	$\gamma_1\kappa$	5.10×10^{10}

a) Determined with mono Ab-ID kit according to the manufacturer's directions.

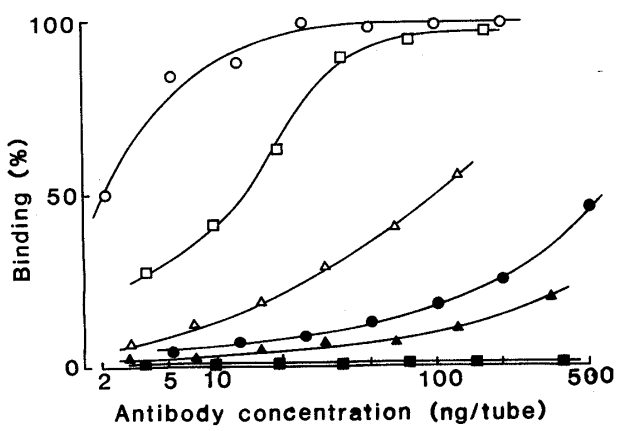

Fig. 1. Binding Curve for Monoclonal Antibodies to hEGF and mEGF

Monoclonal antibody solutions (100 μl) were incubated with radiolabeled EGF (^{125}I-hEGF; 27 μCi/μg, 3×10^4 cpm/100 μl, ^{125}I-mEGF; 100 μCi/μg, 10^4 cpm/100 μl) in the standard diluent (50 mM phosphate buffer, pH 7.4, 77 mM NaCl, 5 mg/ml BSA, 0.1 mg/ml merthiolate) and carrier mouse serum at 4 °C for 3 d and the bound radioactivity was precipitated with rabbit antimouse immunoglobulin (100 μl). In this experiment, hEGF was isolated as a single band on SDS–PAGE based on the methods of Cohen and Carpenter[3] and Savage, Jr. and Happer.[19]

Binding curves are for 2710 to hEGF (△) and mEGF (▲), 8410 to hEGF (○) and mEGF (●), and 9111 to hEGF (□) and mEGF (■).

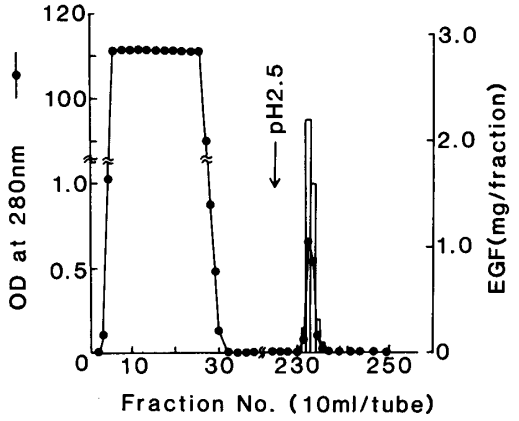

Fig. 2. Purification of hEGF from Human Urine on an Immunoadsorbent Column

Chromatography was carried out as described in the text. EGF activity was measured by the radioreceptor assay method.

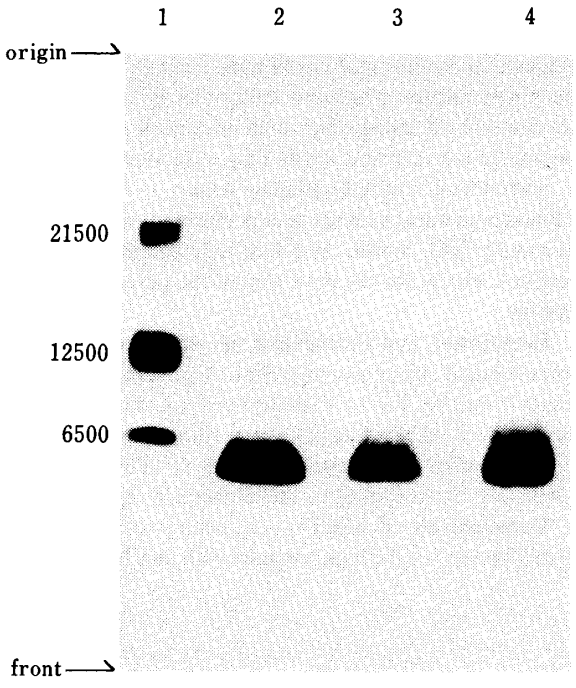

Fig. 3. SDS Polyacrylamide Gel Electrophoresis of Purified hEGF and mEGF

Polyacrylamide gel electrophoresis was carried out in 18% slab gel. Each lane contained 1—3 μg of sample: lane 1, M_r standard; lane 2, purified hEGF; lane 3, purified mEGF; lane 4, commercial mEGF.

TABLE II. Purification of hEGF from Human Urine

Step	Protein (mg)	EGF activity (mg)	Specific activity (mg/mg protein)	Purification	Yield (%)
Urine concentrate	7500	4	0.00053	1	100
Immunoadsorbent column	2.1	2.2	1.05	1980	55

results were consistent with the results obtained by the solid-phase antibody-binding assay. Antibodies produced by clones 8410 and 2710 showed binding ability to both hEGF and mEGF and were considered to be suitable for the purification. As antibody from clone 8410 has high binding affinity to both EGFs, antibody produced by clone 8410 was used for the following purifications.

Purification of hEGF from Urine

The lyophilized powder of urinary proteins was prepared from 100 l of adult human urine by a method similar to that described in Materials and Methods. Lyophilized powder (7.5 g) containing 4 mg of hEGF was dissolved in 100 ml of PBS and the solution was dialyzed overnight against PBS at 4°C, then applied to a column (1.7 × 9 cm, bed volume 20 ml) in which hEGF-directed monoclonal antibody fixed on Affi-Gel 10 had been packed. The column was extensively washed with 1.0 l of PBS, 500 ml of 10 mM Na–Pi buffer pH 7.4 and 0.15 M NaCl, and 500 ml of 0.15 M NaCl. It was then eluted by reverse flow of the pH 2.5 buffer (0.2 M acetate pH 2.5, and 0.15 M NaCl). As shown in Fig. 2, purification on the immunoad-

TABLE III. Purification of mEGF from Mouse Submaxillary Gland Extract

Step	Protein (mg)	EGF activity (mg)	Specific activity (mg/mg protein)	Purification	Yield (%)
Submaxillary gland	450	8.7	0.019	1	100
Immunoadsorbent column	8.8	4.5	0.51	27	52
Sephadex G-50	2.3	2.2	0.96	51	25

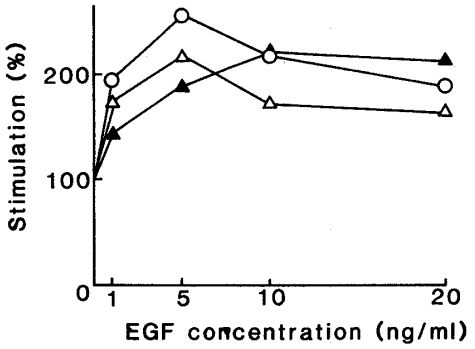

Fig. 4. Stimulation of DNA Synthesis in WI-38 Cell

Confluent monolayers of quiescent human fibroblast WI-38 cells were incubated with purified hEGF (○), purified mEGF (△) or commercial mEGF (▲) and ^{3}H-thymidine (4 μCi/ml). After 48 h the incorporation of ^{3}H-thymidine in the cells was measured.

sorbent column was tremendously effective. Most of the protein in the urine emerged in the flow-through fractions without being adsorbed, and hEGF was eluted sharply by the pH 2.5 buffer. About 2000 fold purification was achieved by this single step and 55% of the activity was recovered (Table II). The eluent containing hEGF was dialyzed against 10 mM ammonium acetate buffer (pH 8.0) and lyophilized. Sometimes immunoadsorptive contaminations of high molecular weight were recovered. These impurities were removed by gel filtration on Sephadex G-50.

Human EGF purified on the immunoadsorbent column gave a single band with an apparent molecular weight of 6000 on SDS polyacrylamide gel electrophoresis (Fig. 3). The amino acid sequence was also determined with the solid-phase peptide sequencer and was consistent with that reported previously.[4]

Purification of mEGF from Mouse Submaxillary Gland

The immunoadsorbent column with antibody produced by clone 8410 could also be used for purification of mEGF. Approximately 9.5 g submaxillary glands prepared from 40 ICR male mice (8 weeks old) was homogenized with 37 ml of 50 mM acetate in a Polytron and centrifuged at 23000 × *g* for 15 min. Thirty mililiter of supernatant obtained was acidified to pH 1.0 by the addition of conc. HCl and centrifuged again. The crude extract containing 8.9 mg of mEGF was obtained and lyophilized. The lyophilized powder was solubilized in 30 ml of PBS and dialyzed against PBS overnight. After centrifugation, the supernatant was applied to the immunoadsorbent column and mEGF was eluted in a manner similar to that used for hEGF. The eluent containing 8.8 mg protein (4.5 mg mEGF) was dialyzed and lyophilized. The lyophilized powder was dissolved in 3 ml of 10 mM ammonium acetate buffer (pH 8.0) and applied to a Sephadex G-50 column previously equilibrated with the same buffer, to remove the high molecular weight immunoadsorptive contaminants, and mEGF was isolated in homogenous form (Table III).

Purified mEGF gave a single band with an apparent molecular weight of 6000 on SDS polyacrylamide gel electrophoresis (Fig. 3), and the amino acid sequence was consistent with that of mEGF reported previously.[5]

Biological Activity of Purified EGF

Biological activities of purified hEGF and mEGF were investigated by measuring the stimulatory effect on incorporation of ^{3}H-thymidine into deoxyribonucleic acid (DNA) in cultured human embryonic lung cell line WI-38. Figure 4 shows that DNA synthesis was stimulated more than 100% by the addition of EGF to the culture medium at the concentration of 1 to 5 ng/ml.

Discussion

Application of antibody to affinity chromatography is very advantageous, and monoclonal antibody offers both high specificity and semipermanent availability. However, high specificity to the antigen is sometimes a disadvantage for purifying a protein having molecular heterogeneity. Therefore, an antibody having a broader epitope and/or recognizing a larger part of the antigen is considered to be preferable in some cases for purification processes. As the primary structures of hEGF and mEGF are quite homologous (about 60% homology),[4,5] antibody reacting with both EGFs was expected to recognize a large part of the antigen and to be suitable for purification of both of them.

For the establishment of a hybridoma cell line which produces monoclonal antibody against a specified antigen semipermanently, mouse myeloma cell line is available. Although mEGF is available as a commercial product, it was considered to be inappropriate to use mEGF for the immunization of mice. Human EGF was easily isolated from human urine in partially purified form by the conventional method.[3,20] Therefore, hEGF was used for immunization.

Three kinds of antibody to hEGF were obtained. Two of them reacted with mEGF whereas the other did not (Fig. 1). Antibody produced by clone 8410 that reacted with both EGFs was selected, and both EGFs were purified very effectively (Fig. 2, Tables II and III). In the case of mEGF, the eluent contained high molecular weight immunoadsorptive contaminants, and they were removed by gel filtration. Purified mEGF showed a single band on SDS polyacrylamide gel electrophoresis (Fig. 3), and also showed a single peak on the reversed-phase HPLC analysis. Commercial mouse EGF also showed a single peak on HPLC. However, Burgess *et al.* reported that mEGF was separated into two forms by HPLC.[7] As the conditions used for HPLC analysis were not identical, the reasons for the different results are not clear. On the other hand, hEGF purified from urine with the immunoadsorbent column was analyzed with SDS polyacrylamide gel electrophoresis and showed a single band, but it was separated into more than eight peaks on reversed-phase HPLC analysis and all of them showed binding activity to the receptor *in vitro*. Further, the number of peaks separable on HPLC was decreased by carboxymethylation of cysteine residues of the molecule (data not shown). These results suggest three possibilities. First, the molecular weight heterogeneity resulted from small deletions of terminal amino acid residues. Second, the molecular heterogeneity is based on differences of the primary structure of the EGF molecule. Third, conformational variations exist, caused by disulfide bonding in the molecule. Further investigation of the primary structure and conformation of the molecule should clear up these problems.

It would be very interesting to examine the structures of hEGF forms separated by HPLC and to look for differences in their biological activities. The effective purification of hEGF with monoclonal antibody described in this report has obvious advantages for obtaining suitable samples.

Acknowledgement The authors wish to thank Dr. Akihiko Murakami for technical assistance and valuable suggestion.

References

1) G. Carpenter and S. Cohen, *Annu. Rev. Biochem.*, **48**, 193 (1979).
2) S. Cohen, *J. Biol. Chem.*, **237**, 1555 (1962).
3) S. Cohen and G. Carpenter, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1317 (1975).
4) H. Gregory, *Nature* (London), **257**, 325 (1975).
5) C. R. Savage, Jr., T. Inagami and S. Cohen, *J. Biol. Chem.*, **247**, 7612 (1972).
6) Y. Hirata and D. N. Orth, *J. Clin. Endocrinol. Metab.*, **48**, 673 (1979).
7) A. W. Burgess, J. Knesel, L. G. Sparrow, N. A. Nicola and E. C. Nice, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 5753 (1982).
8) P. S. Olsen, E. Nexo, S. S. Poulsen, H. F. Hansen and P. Kirkegaard, *Regulatory Peptides*, **10**, 37 (1984).
9) A. F. Server and E. M. Shooter, *J. Biol. Chem.*, **251**, 165 (1976).
10) J. Scott, M. Urdea, M. Quroga, R. Sanchez-Pescador, N. Fong, M. Selby, W. J. Rutter and G. I. Bell, *Science*, **221**, 236 (1983).
11) A. Gray, T. J. Dull and A. Ullrich, *Nature* (London), **303**, 722 (1983).
12) R. F. Doolittle, D. F. Feng and M. S. Johnson, *Nature* (London), **307**, 558 (1984).
13) B. U. Zabel, R. L. Eddy, P. A. Lalley, J. Scott, G. I. Bell and T. B. Shows, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 469 (1985).
14) T. Staehelin, D. S. Hobbs, H. Kung, C.-Y. Lai and S. Pestka, *J. Biol. Chem.*, **256**, 9750 (1981).
15) S. Yanagawa, K. Hirade, H. Ohnota, R. Sasaki, H. Chiba, M. Ueda and M. Goto, *J. Biol. Chem.*, **259**, 2707 (1984).
16) D. M. Moriarity, R. A. Harper, B. B. Knowles and C. R. Savage, Jr., *Hybridoma*, **2**, 321 (1983).
17) M. Klagsbrun, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 5057 (1978).
18) M. Klagsbrun, R. Langer, R. Levenson, S. Smith and C. Lillehei, *Exp. Cell Res.*, **105**, 99 (1977).
19) C. R. Savage, Jr. and R. Happer, *Anal. Biochem.*, **111**, 195 (1981).
20) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
21) M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
22) M. W. Hunkapiller, R. M. Hewick, W. J. Dreyer and L. E. Hood, "Methods in Enzymology," Vol. 91, ed. by C. H. W. Hirs and S. N. Timasheff, Academic Press, New York, 1983, pp. 399—413.
23) M. W. Hunkapiller and L. E. Hood, "Methods in Enzymology," Vol. 91, ed. by C. H. W. Hirs and S. N. Timasheff, Academic Press, New York, 1983, pp. 486—493.
24) V. T. Oi and L. A. Herzenberg, "Selected Methods in Cellular Immunology," ed. by B. B. Mishell and S. M. Shiji, 1979, pp. 351—372.
25) J. F. Garcia, J. Sherwood and E. Goldwasser, *Blood Cells*, **5**, 405 (1979).