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EGF Cell Surface Receptor Quantitation on Ocular Cells by an Immunocytochemical Flow Cytometry Technique

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A method is presented for the rapid flow cytometric determination of epidermal growth factor (EGF) receptor densities on the surface of cultured ocular cells. The technique uses a biotinylated monoclonal antibody directed against the EGF receptor in conjunction with a streptavidin-bound fluorochrome and requires the specific fluorescence per cell to be measured as a function of ligand and receptor concentration. Because the measurement is noninvasive and restricted to cell surface-bound material, the cells can be kept in a physiologic environment, even at the moment of assay. Calculated receptor densities ranged from 5142/cell (infant human corneal endothelium) to 35,678/cell (infant human keratocytes) to $>5 \times 10^5$/cell for an A431 control cell line. Species and donor age differences were noted, as was transient receptor downregulation after EGF administration. Flow cytometry represents a valuable time saving procedure for large scale applications while providing the same level of sensitivity as standard radioimmunoassays. This technique is applicable to quantitation of other growth factor cell surface receptors and could greatly expand the use of flow cytometry in the research laboratory. Invest Ophthalmol Vis Sci 33:2053–2062, 1992

Epidermal growth factor (EGF) is a small (6045 D), well-characterized mitogenic polypeptide present within a wide variety of mammalian tissues and bodily fluids. EGF stimulates the proliferation and differentiation of a wide variety of ectodermal and mesodermal cells (eg, fibroblasts, glial cells, epithelial cells, endothelial cells, chondrocytes)[1,2] by binding to a specific cell surface receptor homogeneously distributed in the plasma membrane. Binding typically triggers, via a cascade effect, cellular oncogene expression and a number of biologic responses in the target cell, including increased glucose uptake and increased amino acid transport and growth. Because of its ability to accelerate wound healing, EGF has been suggested to have important clinical ophthalmic applications.[3–5]

The EGF receptor (EGF-R), the best understood growth factor receptor, consists of a 170 kD transmembrane glycoprotein with an extracellular binding domain and an intracellular tyrosine phosphorylation domain.[6] The receptor is found in many cells and tissues of mammalian and avian species and is postulated to play a significant role in growth control. Many agents, natural and pharmacologic, affect the affinity, number, or response of EGF-R, and these may have a bearing on the role of EGF in growth control. The oncogene v-erbB codes for a product homologous to a portion of the EGF receptor in which the EGF-binding domain has been deleted.

Historically, EGF-R have been readily detected and quantified by ^{125}I-EGF binding assays.[6,7] Although sensitive and relatively precise, these assays have several drawbacks. The procedures are tedious and time-consuming, and, because of radiation, can pose a health hazard. The useful lifetime of an assay kit is limited by the half-life of the isotope. In addition, cell suspensions contain diverse cell types (eg, dead cells, cells in various phases of the cell cycle, and cellular debris), and conventional isotope binding techniques are unable to discriminate between these cell subpopulations.[8]

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Flow cytometric (FCM) analysis of cellular receptor content is achieved by labeling the moiety of interest with a monoclonal antibody-fluorochrome conjugate. During FCM analysis, the bound fluorochrome in stained cells is excited at an appropriate wavelength, resulting in emission of light of a longer wavelength in all directions. The excitation wavelength and color of this emitted fluorescence are characteristic of each fluorochrome. If the dye is bound specifically to a receptor of interest, and the staining reaction is stoichiometric, the fluorescence intensity reflects, quantitatively, the cellular receptor content.

Although extremely well suited for cell surface receptor detection,[9-12] flow cytometry is not entirely free of shortcomings. Quantitation of cell surface receptors with flow cytometric immunoassays becomes a problem when cellular immunofluorescence is only slightly more intense than cellular autofluorescence. Background fluorescence, consisting of cellular autofluorescence and nonspecific binding of immunoreagents, is often the limiting factor in the optimization of the signal-to-noise ratio in sensitive flow cytometric immunoassays.[13,14] Typically, the lower-limit detectability of cell surface fluorescein antibody conjugates is 3000–5000 molecules, and this sensitivity can be obtained only when low levels of cellular autofluorescence are present.[15,16]

The solution to this problem involves the use of a different fluorescent probe whose emission wavelength lies outside the region of cellular autofluorescence. Of those available, the phycobiliproteins (phycoerythrin, for example) are the best dyes for applications that require high sensitivity.[17] It has been reported that the fluorescence of a single molecule of B-phycoerythrin has been detected.[18,19] Femtomole (10^{-15} mole) quantities of phycoerythrin conjugates can be detected because of the high extinction coefficient ($\epsilon_M = 2.4 \times 10^6$ cm^{-1} M^{-1} for 2.4×10^5 daltons) and high fluorescence quantum yield ($Q > 0.8$) of the phycoerythrin moiety. These conjugates emit in the orange-red spectral region, where background fluorescence (cellular autofluorescence) is far less than at shorter wavelengths.

The high sensitivity of R-phycoerythrin fluorescence measurements in combination with the high affinity and stoichiometric interactions of biotin and streptavidin provide for very low nonspecific binding and accurate results while avoiding high levels of cellular autofluorescence. By using a biotinylated monoclonal antibody in conjunction with streptavidin-R-phycoerythrin, EGF cell surface receptors can be readily detected on living ocular cells, within a working range of essentially 775–525,000 receptors per cell.

Materials and Methods

Reagents

Biotinylated anti-EGF-R antibody (mouse monoclonal IgG_{2a} EGF-R AB-1, clone 528, cell surface domain epitope) was purchased from Oncogene Science, Inc. (Manhasset, NY). R-phycoerythrin-conjugated streptavidin was obtained from Molecular Probes, Inc. (Eugene, OR). The composition of this fluorochrome complex is such that streptavidin retains primarily one active binding site for reaction with biotin that's present with the monoclonal antibody. Fluorescence quantitation was performed using a Quantum PE microbead kit (Flow Cytometry Standards Corp., Research Triangle Park, NC) with molecules of equivalent soluble fluorochrome (MESF) content ranging from 10^4 to 5×10^5. Fluorescence to protein (F/P) ratios were derived from control runs using Simply Cellular Microbeads (Flow Cytometry Standards Corp.).

Bovine serum albumin (Cohn Fraction V), sodium azide, heparin sulfate, gentamicin, and Fungizone were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's minimum essential medium (D-MEM; high glucose, 320-1965AJ), Coon's modified Ham's F-12 medium, Hank's balanced salt solution without phenol red, Dulbecco's phosphate buffered saline without phenol red (D-PBS), heat-inactivated fetal calf serum (FCS; 230-6170AJ), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). Eagle's minimum essential medium with Earle's balanced salt solution (E-MEM), D-PBS, essential and nonessential amino acids, minimum essential medium vitamin mixture, and gentamicin were obtained from Whittaker Bioproducts, Inc. (Walkersville, MD). NuSerum IV was obtained from Collaborative Research (Bedford, MA). Collagen-coated dextran microcarriers (Cytodex 3) were purchased from Pharmacia Fine Chemicals, AB (Uppsala, Sweden). Human recombinant EGF, produced by Creative Biomolecules (Hopkinton, MA) was the gift of Alcon Laboratories, Inc. (Fort Worth, TX).

Cell Culture

Human epidermoid carcinoma cells (A-431, Cell Repository Line 1555) and contact-inhibited NIH/3T3 Swiss mouse embryo fibroblasts (CRL 1658) were purchased from American Type Culture Collection (Rockville, MD). Rabbit corneal epithelium was harvested from New Zealand white rabbits using a urethane stripping method.[20] Rabbit corneal fibroblasts were a gift of Dr. B. Gebhardt, LSU Eye Center,

New Orleans, LA. Human trabecular meshwork cells were obtained from Alcon Laboratories, Inc. Human retinal pigment epithelial cells, derived from infant donors, were the gift of Dr. M. Neisman, LSU Eye Center, New Orleans, LA. Human corneal endothelial cells and stromal fibroblasts were derived from infant donor corneas (mean donor age, 2 mo) obtained from regional eye banks. Stromal fibroblasts were also harvested from adult donor tissue (mean donor age, 53 yr). Infant human corneal endothelium and rabbit corneal endothelium were isolated nonenzymatically with Cytodex 3 microcarriers as previously described.[21] A431 cells, NIH/3T3 cells, rabbit and human corneal fibroblasts, and human trabecular meshwork cells were maintained in 90% D-MEM with 4.5 g/l glucose and 10% FCS. Human retinal pigment epithelial cells were maintained in 95% Coon's modified Ham's F-12 medium supplemented with 5% FCS and 1% gentamicin/Fungizone. Infant human corneal endothelial cells were established and maintained in a supplemented version of E-MEM containing essential and nonessential amino acids, minimum essential medium vitamin mixture, L-glutamine (560 μg/ml), sodium bicarbonate, and 15% NuSerum IV. Human erythrocytes were obtained fresh from Tulane Medical Center Blood Bank, New Orleans, Louisiana. Procurement of rabbit ocular tissues adhered to the ARVO Resolution on the Use of Animals in Research.

Immunofluorescence Staining of EGF Cell Surface Receptors

Confluent early passage cells, cultured in plastic tissue culture flasks (Corning Scientific Products, Corning, NY), were harvested by trypsinization, pelleted at $400 \times g$, washed two times with ice cold phenol red-free Hank's balanced salt solution, and suspended in D-PBS (pH 7.4) containing 0.1% (weight/volume) sodium azide (to prevent receptor internalization) at a concentration of 5×10^5 cells per 100 μl. Cell viability was assessed by trypan blue exclusion. Two-and-a-half microliters of biotinylated anti-EGF-R antibody (1 μg/μl) were added to 100 μl cell aliquots, and the suspensions were incubated for 20 min at 4°C. After incubation, the cells were washed twice with D-PBS containing 0.1% sodium azide and 2% (w/v) bovine serum albumin and incubated in the presence of two units (2 μl) of streptavidin-R-phycoerythrin for 20 min at 4°C. After incubation, the cells were washed three times with D-PBS supplemented with 2% bovine serum albumin and 0.1% sodium azide. After the final wash, the cells were maintained in supplemented D-PBS at 4°C in light-proof containers until analyzed by flow cytometry. To avoid potential blocking of EGF receptors by any EGF present in FCS, the culture medium was replaced with serum-free D-MEM 12 or 24 hr before the addition of the primary antibody. This step could be omitted, however, without a significant loss of sensitivity, because the concentration of EGF in inactivated FCS is very low. Human corneal endothelial cells, however, were switched to NuSerum-free medium 24 hr prior to EGF receptor quantitation because NuSerum IV is supplemented with 5 ng/ml EGF.

Flow Cytometric Analysis of Stained Cells

Flow cytometry was performed with an EPICS Profile 1 (Coulter Electronics, Inc., Hialeah, FL) equipped with a 15 mW air-cooled argon ion laser and a 250 μm^2 flow chamber. Fluorescence excitation was accomplished at 488 nm using a dichroic laser blocker. Optimal signal-to-noise ratio for collection of the R-phycoerythrin fluorescence was obtained using a 575 nm band-pass filter with a 20 nm pass band (3802082; Coulter Electronics). Sheath fluid consisted of a nonfluorescing isotonic saline solution (Isoton; Coulter Electronics). R-phycoerythrin fluorescence emissions were collected using a four decade logarithmic amplifier. Sample runs were collected in single and dual (forward scatter versus side scatter) parameter histograms using a 256 channel scale. Cell debris and undispersed cell aggregates were excluded from analysis by selective gating. In each experiment, two negative controls were performed without the biotinylated monoclonal antibody, permitting the definition of the threshold used to discriminate between receptor positive cells and receptor negative cells (monoparametric analysis). A shift in the mean channel fluorescence of greater than 4 channels compared to control cells stained with streptavidin-R-phycoerythrin only was considered a positive result. Triplicate runs were performed for all cell types tested, with a minimum of 5000 cells analyzed for each sample. Laser power and photomultiplier tube gain were kept constant for sample and control runs.

Determination of EGF Cell Surface Receptor Density

The number of EGF cell surface receptors was determined by analytical flow cytometry in conjunction with fluorescent R-phycoerythrin microbead standards.[22,23] Cells were incubated in the presence of biotinylated anti-EGF-R antibody, washed, and stained to saturation with streptavidin-R-phycoerythrin. The resulting cellular fluorescence intensity was extrapolated onto a standard fluorescence calibration curve derived from an R-phycoerythrin quantitative fluorescence standards kit (Quantum PE; Flow Cytometry

Standards Corp.). These microbead standards consist of R-phycoerythrin molecules covalently bound to alkyl spacer groups on the exterior of 5.8 μm hydrophobic polymeric beads. The calibration curve was generated by plotting the given number of equivalent soluble R-phycoerythrin molecules per bead (range 10^4 to 5×10^5) versus the log of its mean fluorescence intensity. Use of the microbeads permitted the direct conversion of fluorescence intensity of the labeled cells to the number of MESF.[22,23] The fluorescence threshold (sensitivity) was defined as the minimum number of molecules of R-phycoerythrin detectable above the noise level of the flow cytometer and was derived from the analysis of dye-free microbeads. Results were normalized with the F/P ratio determined empirically for the anti-EGF-R antibody using Simply Cellular Microbeads.[23–25]

Relative EGF receptor sites per cell, which represents the stoichiometric binding of the streptavidin-R-phycoerythrin to the receptor-bound biotinylated anti-EGF-R antibody, were calculated by dividing the number of R-phycoerythrin molecules bound per cell by the F/P ratio of the biotinylated antibody/streptavidin-R-phycoerythrin conjugate. For each experiment, the flow cytometer was calibrated with an R-phycoerythrin reference microbead standards kit (FCSC Fluorescence Reference Standards, 899; Flow Cytometry Standards Corp.).[26]

The mean number of EGF receptor binding sites per cell was directly determined by initially quantitating the average fluorescence intensity of the labeled cells in MESF, converting the MESF value into the number of bound antibodies and dividing by the effective F/P ratio. Conversion from MESF to the number of antibodies bound per cell involved the determination of fluorescence intensity/antibody ratios in contrast to the number of fluorochromes per antibody.

EGF Receptor Regulation

Secondary cultures of recently confluent infant human corneal fibroblasts, derived from the same donor, were rinsed twice with H-BSS and fed either D-MEM/10% FCS or D-MEM/10% FCS supplemented with 100 μmol/l atropine sulfate. Twenty-four hours later, cell samples were analyzed for baseline EGF receptor densities. Lyophilized human recombinant EGF (>99% pure, as determined by high performance liquid chromatography) was reconstituted with D-PBS (pH 7.0) and added to D-MEM/10% FCS (with and without atropine), yielding a final hEGF concentration of 100 ng/ml. The culture medium was again changed and the cells incubated in the presence of hEGF for 2 and 12 hr. After incubation, the cells were analyzed by flow cytometry for EGF receptor density.

Results

Seven ocular cell types, derived from rabbit and human tissue, and four control cell lines (human and murine) were analyzed by flow cytometry for the presence of EGF cell surface receptors. The quantities of detectable EGF receptors are reported in Table 1. Calculated mean receptor densities ranged from a low of 800 per cell (transformed NIH/3T3 mouse fibroblast) to in excess of 5×10^5 (A431, human epidermoid carcinoma). The lower limit of receptor detection (fluorescence threshold) was derived from analysis of dye-free microbeads and calculated to be approximately 775 EGF receptors per cell. Human erythrocytes, which are known not to possess EGF receptors, demonstrated no primary or secondary staining.

The streptavidin-R-phycoerythrin fluorochrome produces a pronounced yellow-orange stain in positive cells, with minimal background (high signal-to-noise ratio). Flow cytometric evaluation of unstained cells at the standard fluorescein (525 nm) and R-phycoerythrin (575 nm) emission maxima wavelengths revealed significantly less interfering autofluorescence at the R-phycoerythrin wavelength (Figs. 1a–d). As a result, background controls for autofluorescence did not exceed 3% of a given population.

Table 1. Quantitation of EGF surface receptors of ocular cells by flow cytometry

Cell type	*Mean EGF receptors per cell*
Rabbit corneal epithelium*	5503 ± 470
Rabbit keratocytes	8962 ± 322
Rabbit corneal endothelium	15,074 ± 269
Human keratocytes†	35,678 ± 2120
Human keratocytes‡	>85,000
Human keratocytes§	9210 ± 427
Human corneal endothelium†	5142 ± 2412
Human corneal endothelium‖	>30,000
Human retinal pigment epithelium	72,934 ± 1292
Human trabecular meshwork fibroblasts	40,488 ± 2200
Control cell lines:	
A431 (Human epidermoid carcinoma)	$>5 \times 10^5$
NIH/3T3 (Mouse embryo fibroblast)¶	2200 ± 302
NIH/3T3**	800 ± 291††
Erythrocytes (Human)	0

* Ex vivo; harvested mechanically.
† Infant donors.
‡ Infant donor; approx. 30% of the total number of infant donors yielded keratocytes possessing EGF receptor densities of this level.
§ Adult donors.
‖ Infant donor, passage 7, preconfluent; senescent.
¶ Contact-inhibited cellular proliferation.
** Noncontact inhibited; transformed cell line.
†† Lower limit of detection—775 receptors/cell.

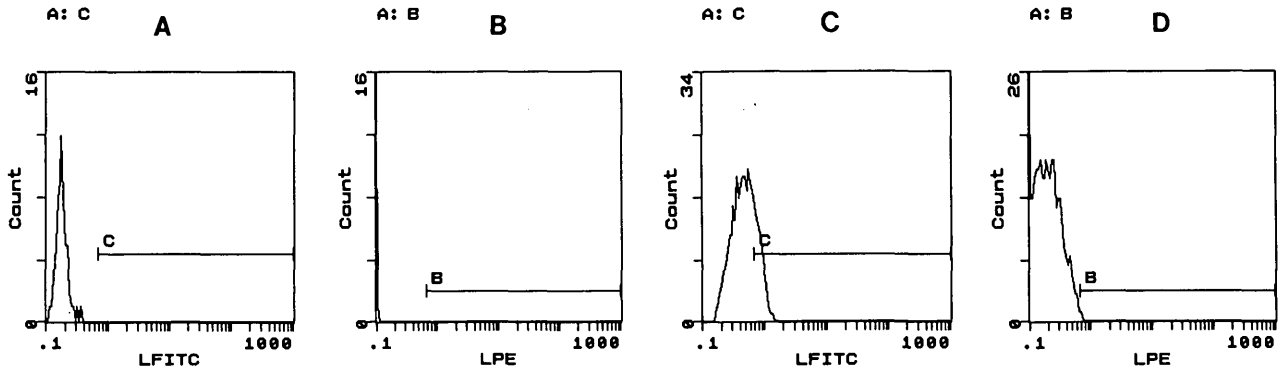

Fig. 1. (A) Plot of log fluorescence versus cell count for unstained NIH 3T3 cells evaluated at the fluorescein maximum emission wavelength (525 nm ± 15 nm pass band). An increase of 15 channels represents a doubling of the fluorescence signal. The inherent autofluorescence of the cultured cells is obvious. (B) Plot of log fluorescence versus cell count for unstained NIH 3T3 cells evaluated at the R-phycoerythrin maximum emission wavelength (578 nm ± 15 nm pass band). The autofluorescence of the cultured cells is significantly less at this wavelength than at 525 nm. (C) Plot of log fluorescence versus cell count for unstained infant human corneal fibroblasts evaluated at the fluorescein maximum emission wavelength (525 nm ± 15 nm pass band). By comparison with (A), it can be seen that the inherent autofluorescence of cultured human corneal fibroblasts is higher than that of NIH/3T3 fibroblasts. (D) Plot of log fluorescence versus cell count for unstained infant human corneal fibroblasts evaluated at the R-phycoerythrin maximum emission wavelength (578 nm ± 15 nm pass band). The autofluorescence of the cultured cells is significantly less at this wavelength than at 525 nm.

Mean EGF-R densities on the surface of live ocular cells were derived by comparison with standard R-phycoerythrin-labeled microbeads. A standard curve was obtained from the analysis of R-phycoerythrin Quantum microbeads, in which the number of equivalent soluble R-phycoerythrin molecules per bead (range 10^4 to 5×10^5) was plotted against the log of fluorescence intensity (Fig. 2). Fluorochrome-free beads delineated the lower limit of detection.[23]

Three separate control runs consisting of unstained cells (autofluorescence control), cells incubated with anti-EGF-R antibody only, and cells incubated with streptavidin-R-phycoerythrin only were performed on all cell types prior to receptor quantitation runs, which combined the biotinylated anti-EGF receptor antibody and the streptavidin-labeled fluorochrome (Fig. 3). Addition of recombinant hEGF to single cell suspensions for 30 min prior to the addition of the anti-EGF-R antibody resulted in significantly decreased (>90%) binding of the study antibody and consequently decreased cellular fluorescence. For all cell types tested, no increase in fluorescence was observed with the addition of biotinylated anti-EGF receptor antibody only. The streptavidin-R-phycoerythrin conjugate is particularly useful in that it has an inherent low nonspecific binding (as was evident from the third control run) and does not contain carbohydrate moieties, thus minimizing nonspecific binding. Results of EGF-R determinations for human corneal endothelial cells, rabbit and human corneal fibroblasts, and A431 human epidermoid carcinoma cells are presented (Figs. 3–6). Species and donor-age related differences in EGF-R densities were noted (Table 1).

Transient EGF receptor down regulation could be easily and rapidly monitored by flow cytometry. Con-

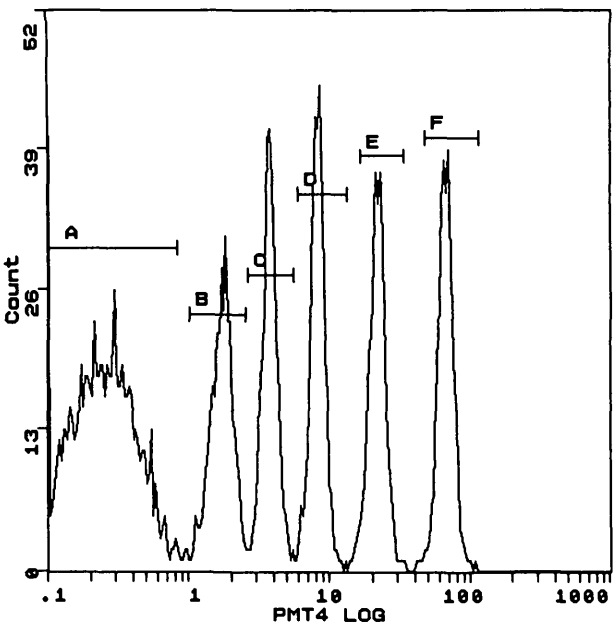

Fig. 2. Fluorescence calibration based on analysis of dye-loaded microbeads; plot of log mean fluorescence intensity versus number of R-phycoerythrin Quantum beads. The equivalent molecules of soluble phycoerythrin per bead ranged from 10^4 (peak B) to 5×10^5 (peak F); beads containing no dye are represented by peak A. The use of R-phycoerythrin microbead standards permits the direct conversion of fluorescence intensity of the labeled cells into the number of molecules of equivalent soluble fluorochrome (MESF).

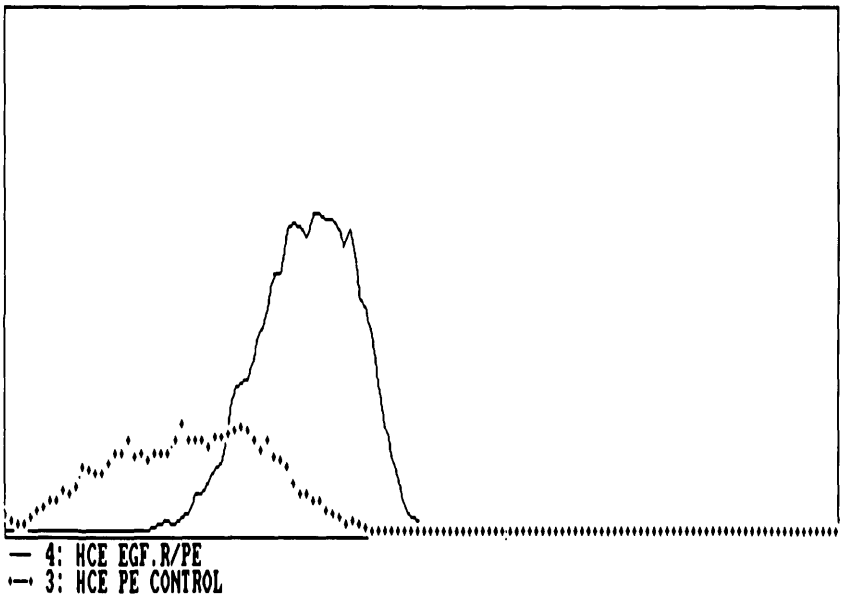

Fig. 3. Single parameter frequency distribution for a population of cultured infant human corneal endothelium incubated in the presence of biotinylated anti-EGF-R antibody and subsequently stained with streptavidin-R-phycoerythrin. The histogram clearly demonstrates the presence of EGF cell surface receptors (solid line); note the shift in mean peak fluorescence intensity away from the secondary control (dotted line), which represents a combination of predominately cellular autofluorescence in conjunction with minimal nonspecific binding of the streptavidin-labeled fluorochrome. Calculated mean EGF receptor density is 4978/cell. The X-axis illustrates fluorescence labelling intensity on a four-decade log scale; the Y-axis illustrates the number of cells at each intensity.

fluent cultures of infant human corneal fibroblasts were exposed for 2 or 12 hr to basal medium containing human recombinant EGF. A significant reduction (>80%) in EGF receptor density was noted at 2 hr after administration of 100 ng/ml hEGF. An almost complete recovery to predosing levels was observed at 12 hr. This recovery could be inhibited, however, by the use of 100 μmol/l atropine sulfate in the culture medium (Table 2).

Discussion

Cell membrane receptor quantitation by flow cytometry relies upon fluorescence and light scatter measurements of individual cells, and the power of the technique derives from the ability to make these measurements of each cell simultaneously and at very rapid speeds.

The early studies by Casperson et al,[27,28] employing the then infant science of quantitative cytophotometry, first demonstrated the potential for quantitative measurements of biochemical constituents in single cells. These studies demonstrated the potential for performing biochemical analysis on a cell-by-cell basis so that distinct subpopulations could be discriminated and identified. Kamentsky et al,[29] in the earliest report on flow cytometry, used ultraviolet absorption cytophotometry with light-scattering measurement to

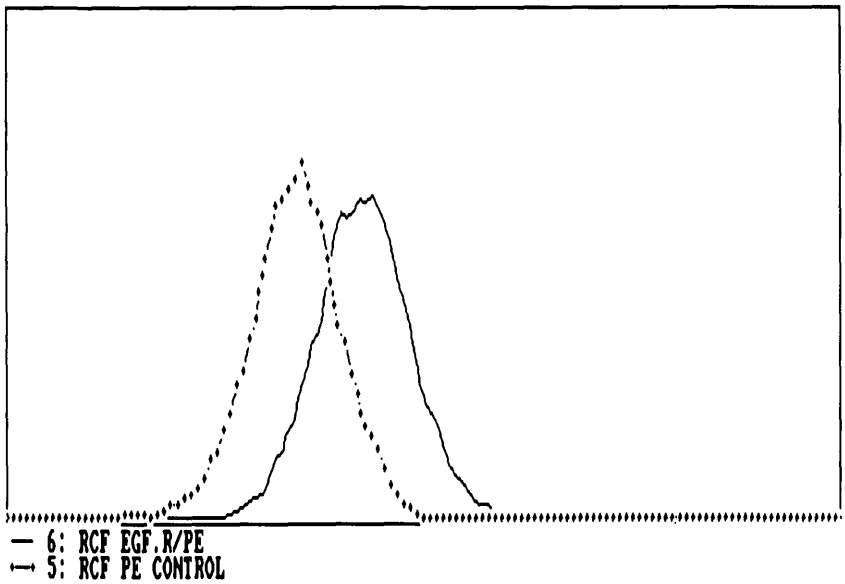

Fig. 4. Single parameter frequency distribution for a population of cultured rabbit corneal fibroblasts incubated in the presence of biotinylated anti-EGF-R antibody and subsequently stained with streptavidin-R-phycoerythrin. The histogram demonstrates the presence of EGF cell surface receptors (solid line); note the shift in mean peak fluorescence intensity away from the secondary control (dotted line). Calculated EGF receptor density is 9023/cell. The X-axis illustrates fluorescence labeling intensity on a four-decade log scale; the Y-axis illustrates the number of cells at each intensity.

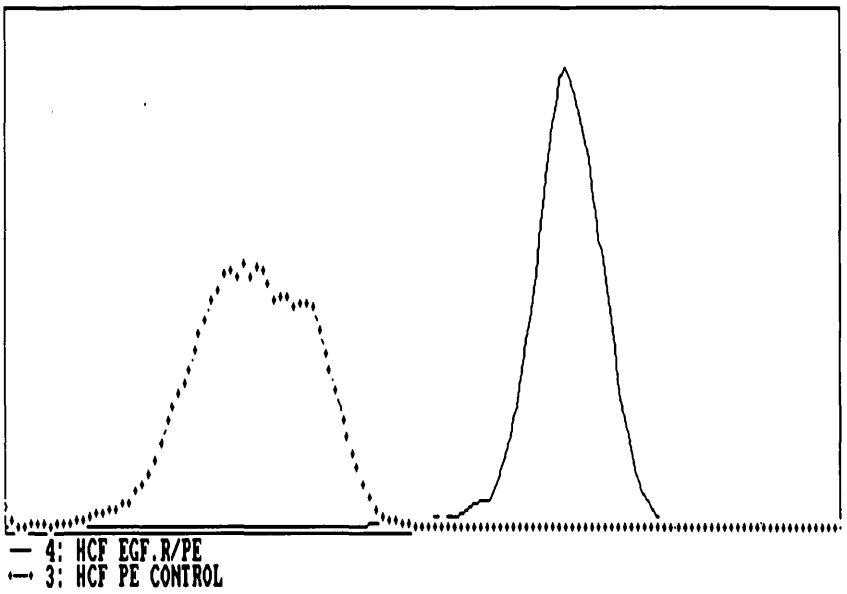

Fig. 5. Single parameter frequency distribution for a population of cultured infant human corneal fibroblasts incubated in the presence of biotinylated anti-EGF receptor antibody and subsequently stained with streptavidin-R-phycoerythrin. The histogram demonstrates the presence of EGF cell surface receptors (solid line); note the shift (increase) in mean peak fluorescence intensity away from the secondary control (dotted line). Calculated EGF receptor density is 87,119/cell. The X-axis illustrates labeling intensity on a four-decade log scale; the Y-axis illustrates the number of cells at each intensity.

quantify nucleic acid content and cell size. Quantitative cytochemical analysis using colorimetric procedures such as the Feulgen reaction allowed for microspectrophotometric assay of single cell DNA content.

Flow cytometry incorporates many of the principles established in the previous cytophotometric studies. In addition, flow cytometry provides ease, speed, and statistical accuracy of the measurements as well as other desirable features. For example, when cellular components are labeled stoichiometrically with fluorochromes, subsequent fluorescence measurements by flow cytometry are less sensitive than absorption cytophotometry to distributional errors caused by nonhomogeneous distribution of the materials within the cells. Furthermore, the loss of quantitative accuracy because of fluorescence fading does not appear to limit the precision of flow measurements, because the stained cells are exposed uniformly and only briefly (3 to 5 μsec) to a high intensity excitation source.

Establishing optimal specificity and staining stoichiometry is critical to receptor density measurements. Fluorometric measurements represent indirect assays. The flow instrument measures fluorescence, not the biochemical moiety directly. Therefore, the assay method relies on the accuracy of the fluorochrome labeling technique. To extract ligand-cell binding parameters from flow cytometric fluorescence data, it is necessary to convert the measured cellular fluores-

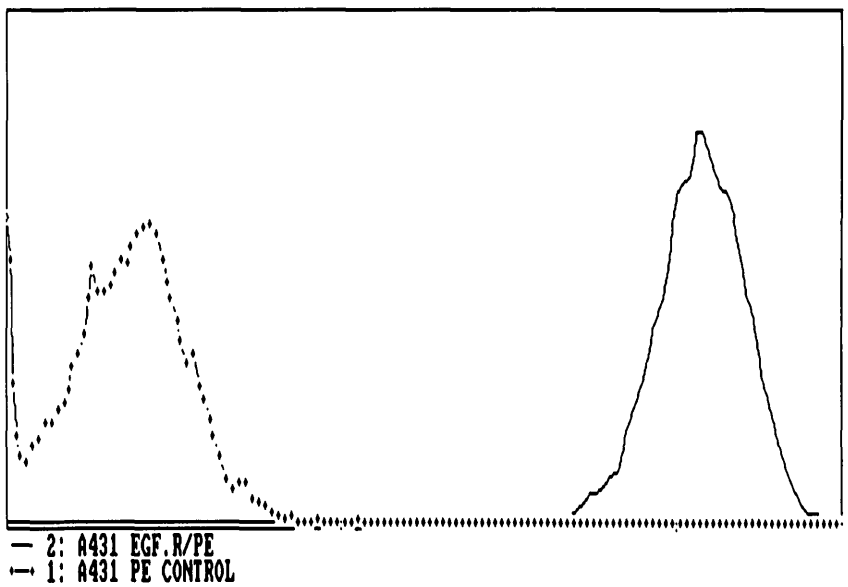

Fig. 6. Single parameter frequency distribution for a population of A431 human epidermoid carcinoma cells incubated in the presence of biotinylated anti-EGF receptor antibody and subsequently stained with streptavidin-R-phycoerythrin. Compare the shift in mean channel of the positive (solid line) to that of the previous figures: the calculated EGF receptor density is $>4.5 \times 10^5$ per cell. The X-axis illustrates labeling intensity on a four-decade log scale; the Y-axis illustrates the number of cells at each intensity.

Table 2. Human corneal fibroblast EGF receptor downregulation following administration of r-hEGF

	Mean EGF receptor density	
Time after dosing	*Basal medium*	*Basal medium + 100 μmol/l atropine*
0 hr	37,648	37,337
2 hr	6534	7778
12 hr	34,225	8090

cence to the actual number of bound molecules producing that fluorescence.[30]

Avidin-biotin based amplification and detection systems have proven exceptionally useful, particularly in immunohistochemistry, ELISA, immunoblotting, and DNA probe technology, where detection methods having the highest possible sensitivity are imperative.[31] When used in combination with monoclonal antibodies, the detection of very low levels of antigens (surface receptors) can be accomplished. Streptavidin, with a molecular weight of about 60,000 daltons, has a near-neutral isoelectric point and exhibits very low nonspecific binding to negatively charged surfaces,[32] including cells and nucleic acids, compared to avidin, which has a molecular weight of about 68,000 daltons and an isoelectric point of about 10.5. Streptavidin in its unmodified form is capable of binding four biotin ligands per mole with high affinity and low reversibility. When conjugated to the fluorochrome R-phycoerythrin, the number of potential binding sites is effectively reduced to one, with little affect on affinity. The high affinity of streptavidin for biotin (dissociation constant of approximately 10^{-15} M^{-1}) is exploited in our present study to provide a highly sensitive means of antigen detection and reduced nonspecific staining.

Autofluorescence from naturally occurring materials, generally pyrimidines and flavin nucleotides, is found in many cell types and contributes to unwanted fluorescence emissions in unstained cells.[33-35] When the levels of autofluorescence are far below that produced from cell staining, analytical problems are not encountered. Difficulties from cellular autofluorescence can arise, however, when the number of bound dye molecules is small, such as for antibodies against cell surface antigens or when the autofluorescence spectrum overlaps that of the labeling fluorochrome.[22,35,36] Under these conditions, resolution of the labeled fluorochrome and subsequent quantitative analysis may be impaired. Fluorescence analysis of unstained cells at the excitation wavelength, laser power, and electronic gain setting predetermined for the fluorochrome-labeled studies was used to detect and determine the degree of analytical distortion due to autofluorescence (Figs. 1a, b).

In the present study, a fluorochrome with emission spectral properties different from the cellular autofluorescence was successfully used to minimize background intrusion. R-phycoerythrin, isolated from the higher red macroalga Rhodymeniales (*Gastroclonium coulteri*), is superbly suited for use as a fluorescent label. The characteristics of R-phycoerythrin, with an extinction coefficient of 2.0×10^6 M^{-1} cm^{-1}[37]—some 24 times greater than that of the frequently used label fluorescein isothiocyanate—a large Stoke's shift (87 nm), and a high quantum efficiency of 0.85, permit the detection of low receptor numbers.[17,38] The R-phycoerythrin emissions overlap fluorescein emissions by only 10%, accounting for the significantly reduced autofluorescence at the R-phycoerythrin emission maximum (578 nm) compared to the fluorescein isothiocyanate emission maximum (525 nm; Figs. 1a–d).

The EGF receptor densities as determined in this present study represent total numbers of EGF receptors present. No attempt was made to study binding kinetics or differentiate between high and low affinity EGF receptors. These calculated densities correlate well with previously published radioimmunoassay studies of ocular cells and further support how the high affinity binding of a ligand to a relatively small number of sites (<100,000 per cell) can be measured by flow cytometry.[12] Schultz et al,[39] using ^{125}I-EGF binding studies, found EGF receptor densities for adult human keratocytes to be approximately 8500 per cell, compared to our calculated value of 9210. It is interesting to note that early passage keratocytes derived from infant donors displayed two larger and distinct densities: 35,678 receptors/cell for the majority of the donors and, for approximately 30% of the infant donor population, in excess of 85,000 receptors/cell. The significance of this remains unclear and no data exist that reveal the age at which EGF receptor numbers decrease to adult values. Published NIH/3T3 EGF receptor densities of 2300–2500 per cell compare favorably with our determination of 2200 per cell.[40] Stimulated in vitro transformation of these cells resulted in the loss of contact inhibition of cell proliferation and yielded cultures that routinely displayed fewer than 1000 EGF receptors per cell.

Although the molecular weight of R-phycoerythrin is large (240,000 D), Oi et al[41] demonstrated that this did not normally interfere with its usefulness. We, however, observed an upper limit of EGF-R detection using streptavidin-R-phycoerythrin for primary antibody labeling. Cultured A431 cells repeatedly demonstrated a mean EGF receptor density of approxi-

mately 525,000/cell when analyzed by FCM. However, previous studies employing radioimmunoassay routinely yielded EGF receptor densities in excess of 2×10^6.[40,42] We attribute this marked discrepancy to the size of the antibody-fluorochrome conjugate employed, resulting in stearic hindrance and occlusion of the high number of EGF receptors typically found on A431 cells.[17]

Flow cytometry has been used to elucidate physiologic mechanisms that modulate gene expression and, in addition to DNA metabolism, regulate and control cell proliferation. Many current studies include staining and measurement of other cellular constituents, such as protein and RNA simultaneously with DNA.[43-45] Cellular levels of such descriptors and others are known to be important indicators of cell cycle progression capacity. With the methods developed in this study for receptor density determinations, multiparametric studies may be performed and could include the investigations of cell cycle transverse rates and DNA replication in conjunction with growth factor cell surface density and responsiveness to growth factor concentrations.[46,47] In addition, the measurement of binding kinetics could be achieved in which histograms are recorded at selected time points for a sample continuously incubated with a selected antibody. In most cases, the techniques would involve minor modifications of pre-existing staining methods. These would allow the appropriate combination of the dyes and near-optimal quantitative analysis.

Flow cytometry represents a substantial time saving procedure for large scale applications while providing the same or greater level of sensitivity as a standard radioimmunoassay.[45] Harel-Bellan et al[48] recently demonstrated significantly higher sensitivity of flow cytometry over Scatchard analysis in the study of interleukin-2 binding sites. Flow cytometry allows for rapid receptor density determinations (hours versus days for radioimmunoassay) and permits parametric determination on a cell-by-cell basis, independent of total cell number, because fluorescence is measured at the single cell level. These studies confirm that flow cytometry is a useful, expeditious technique for detecting EGF cell surface receptors.

Key words: flow cytometry, immunofluorescence, epidermal growth factor, cell surface receptor, monoclonal antibody, R-phycoerythrin, streptavidin

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References

1. Carpenter G and Cohen S: Epidermal growth factor. Annu Rev Biochem 48:193, 1979.
2. Carpenter G: Epidermal growth factor. *In* Tissue Growth Factors, Baserga R, editor. Berlin, Springer Verlag, 1981, pp. 1-26.
3. Tripathi RC, Raja SC, and Tripathi BJ: Prospects for epidermal growth factor in the management of corneal disorders. Surv Ophthalmol 34:457, 1990.
4. Tripathi BJ, Kwait PS, and Tripathi RC: Corneal growth factors: A new generation of ophthalmic pharmaceuticals. Cornea 9:2, 1990.
5. Brazzell RK, Stern ME, Aquavella JV, Beuerman RW, and Baird L: Human recombinant epidermal growth factor in experimental corneal wound healing. Invest Ophthalmol Vis Sci 32:336, 1991.
6. Carpenter G: Receptors for epidermal growth factor and other polypeptide mitogens. Annu Rev Biochem 56:881, 1987.
7. Schlessinger J, Lax I, Yarden Y, Kanety H, and Liberman T: Monoclonal antibodies against the membrane receptor for epidermal growth factor: A versatile tool for structural and mechanistic studies. *In* Monoclonal Antibodies to Receptors: Probes for Receptor Structure and Function, Greaves MF, editor. London, Chapman and Hall, 1984, pp. 279-303.
8. Bohn B: Flow cytometry: A novel approach for the quantitative analysis of receptor-ligand interactions on surfaces of living cells. Mol Cell Endocrinol 20:1, 1980.
9. Sklar LA and Finney DA: Analysis of ligand-receptor interactions with the fluorescence activated cell sorter. Cytometry 3:161, 1982.
10. Watson JV and Walport MJ: Molecular calibration in flow cytometry with sub-attogram detection limit. J Immunol Methods 93:171, 1986.
11. Cohen JHM, Aubry JP, Jouvin MH, Wijdenes J, Bancherau J, Kazatchkine M, and Revillard JP: Enumeration of CR1 complement receptors on erythrocytes using a new method for detecting low density cell surface antigens by flow cytometry. J Immunol Methods 99:53, 1987.
12. Murphy RF: Ligand binding, endocytosis, and processing. *In* Flow Cytometry and Sorting, Melamed MR, Lindmo T, and Mendelsohn ML, editors. New York, Wiley-Liss, 1990, pp. 355-366.
13. Lim VL, Gumbert M, and Garovoy MR: A flow cytometric method for the detection of the development of antibody to Orthoclone OKT3. J Immunol Methods 121:197, 1989.
14. Corsetti JP, Sotirchos SV, Cox C, Cowles JW, Leary JF, and Blumburg N: Correction of cellular autofluorescence in flow cytometry by mathematical modeling of cellular fluorescence. Cytometry 9:539, 1988.
15. Loken MR and Herzenberg LA: Analysis of cell populations with a fluorescence activated cell sorter. Ann NY Acad Sci 254:163, 1975.
16. Jett JH, Keller RA, Martin JC, Nguyen DC, and Saunders GC: Ultrasensitive molecular-level flow cytometry. *In* Flow Cytometry and Sorting. New York, Wiley-Liss, Inc., 1990, pp. 381-396.
17. Kronick MN: The use of phycobiliproteins as fluorescent labels in immunoassay. J Immunol Methods 92:1, 1986.
18. Nguyen DC: Detection of single molecules of phycoerythrin in hydrodynamically focused flows by laser-induced fluorescence. Anal Chem 59:2158, 1987.

19. Peck K, Stryer L, Glaser AN, and Mathies RA: Single-molecule fluorescence detection: Autocorrelation criterion and experimental realization with phycoerythrin. Proc Natl Acad Sci USA 86:4087, 1989.
20. Steineman TL, Thompson HW, Clarke AF, Malter JS, Nixon J, and Beuerman RW: Wounding of the cornea increases the density of epidermal growth factor (EGF) receptors on corneal epithelial cells and the production of high molecular weight forms of EGF in lacrimal gland. Curr Eye Res (submitted for publication) 1992.
21. Lopez JG and Insler MS: Isolation and mass culture of neonatal human corneal endothelium using a nonenzymatic microcarrier isolation technique. Invest Ophthalmol Vis Sci 31(suppl):473, 1990.
22. Vogt RF, Cross GD, Henderson LO, and Phillips DL: Model system evaluating fluorescein-labeled microbeads as internal standards to calibrate fluorescence intensity of flow cytometers. Cytometry 10:294, 1989.
23. Flow Cytometry Standards Corp.: Fluorescent Microbead Standards (monograph). Flow Cytometry Standards Corp., Research Triangle Park, North Carolina, 1988.
24. Schwartz A, Fernández-Repollet E, Velilla M, and Ocasio B: Calibration of flow cytometers to directly measure numbers of antibody binding sites on cells. Cytometry (Suppl) 4:463B, 1990.
25. Fulwyler MJ and McHugh TM: Flow microsphere immunoassay for the quantitative and simultaneous detection of multiple soluble analytes. Methods Cell Biol 33:613, 1990.
26. Le Bouteiller P, Mishal Z, Lemonnier FA, and Kourilsky FM: Quantitation by flow cytofluorometry of HLA class I molecules at the surface of murine cells transformed by cloned HLA genes. J Immunol Methods 61:301, 1983.
27. Caspersson T and Santesson L: Studies on protein metabolism in the cells of epithelial tumors. Acta Radiol 46(suppl):1, 1942.
28. Caspersson T and Schultz J: Nucleic acid metabolism of the chromosomes in relation to gene reproduction. Nature 142:294, 1938.
29. Kamentsky LA, Melamed MR, and Derman H: Spectrophotometer: New instrument for ultrarapid cell analysis. Science 150:630, 1965.
30. Chatelier RC and Ashcroft RG: Calibration of flow cytometric fluorescence standards using the isoparametric analysis of ligand binding. Cytometry 8:632, 1987.
31. Wilchek M and Bayer EA: The avidin-biotin complex in immunology. Immunology Today 5:39, 1984.
32. Chaiet L and Wolf FJ: The properties of streptavidin, a biotin-binding protein produced by streptomycetes. Arch Biochem Biophys 106:1, 1964.
33. Corsetti JP, Sotirchos SV, Cox C, Cowles JW, Leary JF, and Blumburg N: Correction of cellular autofluorescence in flow cytometry by mathematical modeling of cellular fluorescence. Cytometry 9:539, 1988.
34. Steinkamp JA and Stewart CC: Dual-laser, differential fluorescence correction method for reducing cellular background autofluorescence. Cytometry 7:566, 1986.
35. Roederer M and Murphy RF: Cell-by-cell autofluorescence correction for low signal-to-noise systems: Application to EGF endocytosis by 3T3 fibroblasts. Cytometry 7:558, 1986.
36. Sisken JE: Fluorescent standards. *In* Methods in Cell Biology, Taylor DL and Wang Y, editors. New York, Academic Press, 1989, vol 30, pp. 35–70.
37. Kronick MN and Grossman PD: Immunoassay techniques with fluorescent phycobiliprotein conjugates. Clin Chem 29:1582, 1983.
38. Hemmila I: Fluoroimmunoassays and immunofluorometric assays. Clin Chem 31:359, 1985.
39. Schultz GS, Woost PG, and Eiferman RA: Modification of corneal wound healing by growth factors. *In* The Cornea: Transactions of the World Congress on the Cornea III, Cavanagh HD, editor. New York, Raven Press, Ltd., 1988, pp. 15–21.
40. Adamson ED and Rees AR: Epidermal growth factor receptors. Mol Cell Biochem 34:129, 1981.
41. Oi VT, Glazer AN, and Stryer L: Fluorescent phycobiliprotein conjugates for analyses of cells molecules. J Cell Biol 93:981, 1982.
42. Kawamoto T, Sato JD, Le A, Polikoff J, Sato GH, and Mendelsohn J: Growth stimulation of A431 cells by epidermal growth factor: Identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. Proc Natl Acad Sci USA 80:1337, 1983.
43. Crissman HA, Darzynkiewicz Z, Tobey RA, and Steinkamp JA: Correlated measurements of DNA, RNA and protein in individual cells by flow cytometry. Science 228:1321, 1985.
44. Crissman HA and Steinkamp JA: Cytochemical techniques for multivariate analysis of DNA and other cellular constituents. *In* Flow Cytometry and Sorting, Melamed MR, Lindmo T, and Mendelsohn ML, editors. New York, Wiley-Liss, 1990, pp. 227–247.
45. McHugh TM, Reid ME, Stites DP, Chase ES, and Casavant CH: Detection of the human erythrocyte surface antigen gerbich by flow cytometry using human antibodies and phycoerythrin for extreme immunofluorescence sensitivity. Vox Sang 53:231, 1987.
46. Bergsma DR, Lopez JG, Insler MS, and Malter JS: Flow cytometric DNA analysis of cultured human corneal endothelium. Invest Ophthalmol Vis Sci 32:(suppl)1178, 1991.
47. Lopez JG, Insler MS, and Malter JS: Flow cytometric discrimination of mitotic human ocular cells: Resolution of cell cycle phase with propidium iodide. Cytometry (Suppl) 5:120, 1991.
48. Harel-Bellan A, Krief P, Rimsky L, Farrar WL, and Mishal Z: Flow cytometry resonance energy transfer suggests an association between low-affinity interleukin 2 binding sites and HLA class I molecules. Biochem J 268:35, 1990.