The Dynamics of Ligand-Receptor Interactions

Parallel cytometric and fluorimetric analyses of the interaction of a fluoresceinated N-formyl hexapeptide (N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein, Nle = norleucine) with its receptors on human neutrophils are presented. The cytometric analyses take advantage of the ability of the fluorescence flow cytometer to discriminate free and receptor-bound ligand in a homogeneous real-time assay. The spectrofluorometric analysis relies on a high affinity antibody to fluorescein to discriminate free and bound ligand. We find that the number of receptors for formyl peptides on the surface of a resting cell is 53,000 ± 13,000 (K_d ~ 0.6 ± 0.2 nM). We use commercially available cytometric standards to calibrate the cytometer and we obtain similar values for the number of receptors.

The temperature dependence of the kinetics of ligand-receptor interactions have been examined. The association rate constant varies from 3 × 10^8 M⁻¹ min⁻¹ at 4 °C to 10^9 M⁻¹ min⁻¹ at 37 °C (ΔH ~ 8 kcal/mol). While ligand internalization is blocked at 4 °C, at 37 °C internalization proceeds at an initial rate of ~24% of the occupied receptors/min following a latency period of ~20–30 s. Intermediate rates and longer latency periods are found at 15 and 25 °C. Dissociation of the ligand is heterogeneous and depends both on the length of time of association and the temperature. After short periods of association, the ligand dissociates with t½ = ~1–5 min. After longer periods (30 min at 15 °C or 100 min at 4 °C), while the ligand-receptor complex remains on the cell surface, t½ increases to >30 min. It appears that the ligand-receptor complex undergoes an alteration in affinity, with a time course at elevated temperatures, which parallels or lags behind the time course of the participation of the occupied receptors in cell activation.

Cell are activated by the binding of specific ligand molecules to cell surface receptors. The interaction between the ligand and the receptor is characterized by association and dissociation rate constants and by processing of the ligand-receptor complex which may involve internalization recycling and ligand degradation steps. All of these processes may contribute to the means by which the ligand-receptor interaction ultimately generates the signals necessary for cellular responses.

The analysis of ligand-receptor interaction is conventionally performed with radioligands. While radioligands provide the sensitivity necessary to detect small numbers of receptors (<1000/cell), their use suffers from several general types of limitations. First, the temporal resolution of the association or dissociation measurements depends upon the time required for the separation of free from bound ligand. The separation step often involves a centrifugation or filtration which usually requires at least several seconds. Second, the resolution between intracellular and extracellular (but surface bound) ligand depends upon subcellular fractionation. This may be both time consuming and subject to questions concerning the distribution and/or heterogeneity of the markers used to characterize the subcellular fractions.

The goal of our research is to describe the mechanism of the activation of human neutrophils. Neutrophils (or polymorphonuclear leukocytes), the most numerous of the human white blood cells, participate in the acute phase of the inflammatory response and contribute both to host defense and to tissue injury which occurs in inflammatory diseases. These cells are activated by a number of different ligands (C₅a, leukotrienes, acetyl glycoether phosphorylcholine, N-formyl peptides, immune complexes, etc.) for which specific individual receptors have been identified. The cells begin to respond within seconds after the administration of the ligand and within time intervals which precede the equilibrium interaction between the ligand and receptor (see references in Ref. 2). For these reasons and those cited above we have sought to develop homogeneous real-time analyses of ligand-receptor interactions using fluorimetric and flow cytometric results.

In this report, we validate and describe the results of new homogeneous assays for the analysis of ligand-receptor interactions. Parallel analyses by spectrofluorometry and flow cytometry of the kinetics and temperature dependence of association, dissociation, and internalization of a fluoresceinated N-formyl peptide, FLPEP, are presented. These new fluorimetric procedures are compared to radioligand procedures in terms of 1) their temporal resolution, 2) their rapidity and ease of measurement, 3) their potential utility in other ligand-receptor systems, and 4) their intrinsic limitations. In

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† Recipient of United States Public Health Service Grant AI 19032 in support of this work. Supported by a Grant-In-Aid and an Established Investigatorship from the American Heart Association.

‡ Recipient of United States Public Health Service Grant AI 17354 in support of this work.

(Received for publication, September 23, 1983)
a subsequent publication we will use these techniques to analyze the dependence of cellular responses and intracellular levels of potential signaling species (calcium and cAMP) on the interaction of formyl peptides with their receptor.

**MATERIALS AND METHODS**

Neutrophils—Neutrophils were obtained from fresh human blood by the method of Henson and Oades (3) and prepared for these studies as described previously.

**Reagents**—The high affinity antibody to fluorescein was prepared and characterized essentially as described (4). FLPEP was prepared by reacting the parent hexapeptide, 20 mM (Bachem Fine Chemicals, Torrance, CA) with fluorescein isothiocyanate, 20 mM (Molecular Probes, Inc., Eugene, OR) in dry dimethyl sulfoxide (Fiske Chemical Co., Rockford, IL). The product was purified by silicic acid thin layer chromatography using preparative layered plates (Analtech) and eluted with chloroform/methanol/acetonic acid, 3:1:0 (RF = 0.5) as suggested by J. E. Niedel, Dept. of Hematology, Duke University Medical Center. The peptide band was scraped from the plate and extracted with dimethyl sulfoxide. The purity (>90%) of the product was verified by analytical thin layer chromatography in the same solvent system (RF 0.3) and by high pressure liquid chromatography (Perkin-Elmer) using a 3-inch C18 reverse phase column and eluting with an 85% to 75% acetic acid with 0.25% triethylammonium phosphate, pH 3.2. The purity was quantitated by absorbance at 280 nm by the Neineh soft laser densitometer (Biomed Instrument, Chicago, IL) and on high pressure liquid chromatography by absorbance at 280 and 490 nm. The concentration of the peptide was determined by absorbance as described previously (4). The product was stored frozen as 10 μl aliquots in dry dimethyl sulfoxide to avoid repetitive freezing and thawing.

It is noteworthy that the labeled peptide is very difficult to purify away from a component which migrates with RF 0.4 in the analytical system. Puriﬁed labeled peptide is obtained by preparative thin layer chromatography in the same solvent system described above with absorbance at 254 nm. The absence of the parent peptide was further veriﬁed by the ability of antibody to fluorescein to inhibit neutrophil activation by the labeled peptide (4, 5). Preparations were discarded when their activity on a molar basis, in the interaction of neutrophils with antibody and extracellular ligand using the pH can be accomplished within 20 s. However, the loss of ﬂuorescence of FLPEP in the intracellular (lysosomal or receptosomal) compartment after more than 3 min of FLPEP binding and internalization of 37 °C, probably related to the scission of the cell suspension, precludes accurate analysis of internalization after this period (7).

Flow Cytometric Assays of Ligand Binding and Internalization—Flow cytometric analyses of ligand-receptor interactions were performed on a Becton-Dickinson FACS IV (Sunnyvale, CA), equipped with a Spectra-Physics 2-watt argon ion laser (Mountain View, CA). The spectroscopic conditions for all these measurements have been optimized since their original description (4, 7). The fluorescence is excited at 490 nm through 8-nm slits and emission is set at 520 nm with 8-nm slits. Stray light is ﬁltered with a 3-cavity interference ﬁlter centered at 490 nm (Corion, Worcester, MA) in the excitation path and a 3–70 glass ﬁlter (Corning, Corning, NY) in the emission path. Fluorescence signals are averaged for 1 s, and reagents are added to the stirred cuvettes through the top of the sample compartment using Hamilton syringes (Hamilton, Elko, NV) ﬁtted with PE-50 polyethylene tubing (Clay Adams, Parsippany, NJ).

The extent of receptor-bound FLPEP is assayed with the high affinity antibody to fluorescein (20 μM, Kᵣ = 10⁻⁹ M⁻¹). As indicated previously this antibody has the following properties. 1) It is rapidly (tᵣₑₛ = 1 ± 2 5 s) and efﬁciently bound and dissociated (100%) the fluorescence of the free fluoresceinated peptide (4), and 2) the receptor-bound ligand is inaccessible to quenching by the antibody until it dissociates from the receptor (6). The residual FLPEP fluorescence following antibody addition is proportional to the amount of ligand bound to the receptor (see results) the fluorescence of the ligand is unaffected by its association with the receptor. As FLPEP dissociates from the receptor it is rapidly quenched by antibody so that under the appropriate experimental conditions, the dissociation is revealed by the time dependence of the fluorescence losses following antibody addition.

The kinetic analysis of receptor-bound FLPEP is analyzed under similar conditions as binding with the use of an additional step.

Following the examination of FLPEP binding to the receptor, the pH of the cell suspension is adjusted from pH 7.4 (1.5 mM phosphate buffer) to pH 4.0 by the addition of 15 μl of 0.33 N HCl. As discussed previously, this pH change quenches the fluorescence of the extracellular but receptor-bound FLPEP instantaneously but only slowly quenches the fluorescence of intracellular fluorescein or internalized ligand (6). In these experiments, the extent of neutrophil ligand, using antibody and extracellular ligand using the pH can be accomplished within 20 s. However, the loss of fluorescence of FLPEP in the intracellular (lysosomal or receptosomal) compartment after more than 3 min of FLPEP binding and internalization of 37 °C, probably related to the scission of the cell suspension, precludes accurate analysis of internalization after this period (7).

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The analyses of internalization using the flow cytometer is accomplished in a parallel manner to that described above for the spectrofluorometer. In this case the external ligand is quenched instantaneously while intracellular ligand is quenched very slowly. The discrimination between the two compartments is based upon an analysis of the relative proportions of the components of the quenching signal (8). We have verified that exposure of neutrophils to pH 3.0 for 1 min neither releases the cytoplasmic marker lactate dehydrogenase, the granule marker β-glucuronidase, nor alters the scattered light profile of the cells in the cytometric analysis. Taken together, these results indicate that the cells are not disrupted by the pH treatment overnight (see frame requirement under "Reagents and methods") and that this assay is a valid one for use in the analysis of internalization.

Theoretical Calculations of the Kinetics of Ligand Binding—Calculations were performed on the HP 9825 using an iterative calculation
scheme (9). The accuracy of the kinetic calculation was verified in two ways. First, the equilibrium values of the ligand-receptor complex (based on values of $K_a$, $[L]$, $[R]$) in the kinetic calculation were found to be identical to equilibrium values calculated according to

$$K_a = [LR]/(L[R]).$$

(2)

Second, the rate calculations (dependent upon $k_{on}$ and $k_{off}$ (the on and off rate constants, respectively) were validated. In order to do so, curves of the ligand binding versus time were calculated for different concentrations of ligand. These data were replotted in the form described by Schonbrun and Tashjian (10) as log ($I - [LR(t)]/[LR-(eq)]$) versus $t$. In this case $[LR(t)]$ refers to the calculated value of the concentration of the ligand-receptor complex as a function of time and $[LR(eq)]$ refers to the equilibrium value at the same ligand concentration. These plots are linear. From the half-times, the apparent on rate constants are calculated as

$$k_{on(eq)} = 0.693/t_{1/2}.$$

(3)

When $k_{on(eq)}$ is plotted versus $[L]$, the slope is $k_{on}$ and the intercept is $k_{off}$. The $k_{on}$ and $k_{off}$ values obtained after calculation of the kinetic curves and reduction of the data match the original values input into the calculation.

**RESULTS**

Equilibrium Binding of FLPEP to PMN—The fluorescence flow cytometric profiles of neutrophils exposed to FLPEP at 4 °C at equilibrium are shown in Fig. 1A. In typical experiments, the mean fluorescence channel number (M) of unlabelled cells is ~20 and arises from cellular autofluorescence. When the cells are exposed to a near saturating concentration of FLPEP (~5 nM) in the presence of excess unlabelled peptide, M increases only about 10 channels. Since the laser beam in the cytomter is focused to a spot somewhat larger than the cell itself, the beam illuminates a volume which includes the cell and to a lesser extent the surrounding medium. This "nonspecific" increase in M may result, in part, from the fluorescence of ligand in the solution surrounding the cells.

When cells are equilibrated with increasing concentrations of FLPEP, the cellular fluorescence increases monotonically. At concentrations above 5 nM, M typically reaches a value 3–5 times greater than the cells alone. In Fig. 2A, a typical binding curve derived from such cytometric data is plotted. Note that the background fluorescence, measured as the increase in $M$ in the presence of blocking peptide is linear with increasing concentration of FLPEP and represents a small fraction of the increase in $M$ due to the binding of FLPEP. The "specific binding," $\Delta M$, is the difference between the "total" and background or nonspecific binding.

The specific binding at 4 °C is related to the fractional occupancy of the receptors as shown by Equation 1 (see under "Materials and Methods"). The fractional occupancy versus FLPEP concentration is plotted in Fig. 2B and compared to a theoretical curve for $K_d = 0.4$ nM. The binding curves appear to be adequately fit by a single binding constant. Since FLPEP is in excess in these cytometric analyses (see receptor number below), $K_d$ can also be estimated directly from the FLPEP concentration at which half-maximal binding occurs. In similar experiments with cells from more than 10 human donors we have observed that $K_d$ always falls between 0.4 and 1.0 nM with an average value of $0.60 \pm 0.29$ (13 determinations).

The Number of N-Formyl Peptide Receptors on Resting Neutrophils—We previously described a method, requiring no external calibration, for estimating receptor number by cytometry (6). This method relies on the fact that the fractional receptor occupancy, for a given FLPEP concentration, depends upon the cell concentration since $K_d$ and the concentrations of receptor and FLPEP are all similar in magnitude. We estimated 50,000–75,000 receptors/cell. However, since these measurements required large numbers of cells and many samples, this method is unsatisfactory for routine analysis.

Recently, we have used two more convenient methods to analyze the number of receptors. The first method has been made possible by the introduction of two calibration standards. These standards consist of "Fluorotrol GF" (thymocyte nuclei) stained with 0, 50,000, or ~220,000 equivalents of fluorescein isothiocyanate per particle obtained from Ortho Diagnostics, Westwood, MA) and "BDRC Green Beads" (polystyrene beads of ~5-μm diameter conjugated with different numbers of fluorescein equivalents and obtained from Becton-Dickinson FACS Systems, Sunnyvale, CA). In Fig. 1 the fluorescence histogram of a typical preparation of cells ex-
posed to saturating FLPEP is compared to Fluorotrol GF. In these experiments the instrument sensitivity was adjusted so that the brightest Fluorotrol GF subpopulation yields a histogram which is nearly full scale (M = 200-210 channels). As shown in Table I, these values reflect a sensitivity of ~1100 equivalents of fluorescein isothiocyanate/channel.

In parallel experiments (histograms not shown), BDRC green beads were shown to yield calibration values of ~700 fluoresceins/channel (Table I). Neutrophils saturated with FLPEP and analyzed with the same instrument settings exhibited specific binding, \( A_M \), corresponding to 80-100 channels. Using the green beads as standards, 80-100 channels represent 56,000-70,000 equivalents of fluorescein isothiocyanate; based on Fluorotrol GF, 80-100 channels represents ~90,000-110,000 equivalents of fluorescein isothiocyanate. Since there is a single fluorescein per ligand, these numbers are estimates of the number of receptors per cell. Direct spectrofluorometric analyses of FLPEP binding (Table I), which are described in more detail below, yield values comparable to the standards.

The spectrofluorometric analysis of FLPEP binding to the receptors is depicted in Fig. 3. The analysis relies on the use of a high affinity antibody to fluorescein to distinguish free and bound FLPEP as described under "Materials and Methods." As shown in Fig. 3A, the FLPEP fluorescence in a cell suspension at equilibrium at 15°C is not affected by its association with the receptor, i.e., in the presence or absence of a 1000-fold excess of unlabeled peptide. Since the antibody binds to and quenches the fluorescence only of free ligand (see Fig. 8), the residual fluorescence following the addition of antibody is proportional to the concentration of receptor-associated FLPEP.

The binding curve obtained from a spectrofluorometric assay is shown in Fig. 3B. The data are compared to a fit for \( K_o = 0.5 \text{ nM} \) and 34,000 receptors/cell for this particular donor. In these experiments which use 10\(^4\) cells/ml (~1 nM receptor), FLPEP is no longer in excess and \( K_o \) cannot be estimated simply from half-maximal binding. The average number of sites based on equilibrium measurements at 15°C is 53,000 ± 13,000 (15 donors). In Fig. 3C, cytometric data from the same experiment is shown. The equivalence of the results of the two methods is evident.

The Kinetics and Temperature Dependence of Ligand-Receptor Association — The kinetics of binding of FLPEP to its receptor has been examined both by flow cytometric and spectrofluorometric methods. Since the cytometric procedure permits continuous observation of the same cell suspension throughout the course of binding and requires much smaller quantities of cells, it is more suited to detailed studies.

The analysis of the kinetics of FLPEP binding to neutrophils by cytometry is provided in Fig. 4. When neutrophils are exposed to a moderate concentration of FLPEP (1 nM), the cellular fluorescence increases rapidly and reaches an apparent plateau within a few minutes at 37°C (Fig. 4A). The dose dependence of the kinetics of binding replotted as fractional receptor occupancy versus time is shown in Fig. 4B. The solid curves are calculated (see under "Materials and Methods") using the rate constants \( k_{on} = 10^9 \text{ M}^{-1} \text{ min}^{-1} \) and \( k_{off} = 0.25 \text{ min}^{-1} \). \( k_{off} \) thus calculated actually reflects a variety of processes including conversion of receptor affinity and ligand internalization (see under "Discussion"). These data have also been analyzed according to the method of Schonbrun and Tashjian (10) as shown in Fig. 4, C and D. While comparable results are obtained there is a systematic variation in the results which arise from the relative weighting of the individual data points in these two methods (see under "Discussion").

In all, the kinetics of FLPEP binding to neutrophils has been examined on 13 separate occasions and at four different temperatures. The results of these experiments are shown as an Arrhenius plot in Fig. 4E. \( k_{on} \) is observed to be moderately temperature dependent (\( \Delta H \approx 8 \text{ kcal/mol} \)) and increases roughly 3-fold between 4 and 37°C.

Spectrofluorometric measurements of the binding of FLPEP to its receptor involve the use of the high affinity antibody to fluorescein as illustrated in Fig. 5A. In parallel experiments where the cytometric and fluorimetric procedures are compared, essentially identical rate constants are obtained (Fig. 5B). Because the fluorimetric procedure involves separate samples for each time point and requires 10-fold more...
time of exposure and the temperature (Figs. 7 and 8). Even at 4 °C the ligand becomes largely nondissociable after 1 to 2 h of incubation while the ligand-receptor complex is located on the plasma membrane (Fig. 7). This slowly dissociating or "high affinity" complex is formed more rapidly at 15 °C (i.e., within 30 min). At higher temperatures, the rapid internalization of the ligand obscures the analysis of the formation of the slowly dissociating species. Semi-log plots of the time course of ligand dissociation, following different periods of binding at 4, 15, 25, and 37 °C are shown in Fig. 8. Under all conditions the dissociation is heterogeneous.

We have compared the dissociation observed in the presence of nonfluorescent peptide or antibody to fluorescein under limiting conditions where the ligand-receptor complex has been shown to be on the cell surface, after short times at 4 or 37 °C (where ligand is dissociable) and after 100 min at 4 °C (where ligand is only slowly dissociable). We always observe an identical rate of dissociation following the addition of either antibody to fluorescein or excess nonfluorescent ligand. If FLPEP which was bound to receptor were accessible to binding and quenching by the antibody, we should have observed a more rapid reduction in the fluorescence following antibody addition than that observed when dissociation followed the addition of cold ligand. The equivalence of these dissociation rates demonstrates the inaccessibility of receptor-bound FLPEP to quenching by the antibody.

This result validates the use of antibody to discriminate free and receptor-bound FLPEP in the spectrofluorometric assays of ligand binding described above (Figs. 3A and 5A). Furthermore it permits the use of antibody in evaluation of FLPEP dissociation in a continuous spectrofluorometric assay. This arises because FLPEP in solution is quenched by antibody with a half-time of ~1 s under our experimental conditions. Thus, when antibody is added to a cell suspension containing free and bound FLPEP, the free is rapidly quenched (see Fig. 3) while the bound can only be quenched after it dissociates from the receptor. By monitoring the residual fluorescence of FLPEP following antibody addition, the rate of FLPEP dissociation can be determined.

FLPEP dissociability has been examined in this manner at 15 (Fig. 9) and 37 °C (not shown). A loss of dissociability of FLPEP at 15 °C occurs with a half-time of ~10 min, prior to internalization, while the complex of FLPEP and the receptor is effectively trapped on the cell surface. The dissociability in the cytometric and fluorometric assays (Figs. 8, lower left, and 9) are identical.

At 37 °C there appear to be two components of dissociation. A rapid component appears to decrease in amplitude within seconds following exposure of the cells to FLPEP. After 2 min of binding the extent of dissociability is also reduced because of rapid internalization of FLPEP.

**DISCUSSION**

The binding, dissociation, and internalization of FLPEP at its receptor on human neutrophils are all processes which occur rapidly at 37 °C. Cellular responses to these ligandreceptor interactions are initiated within seconds following the addition of ligand (2). In this report we have applied cytometric and spectrofluorometric methods which are suited to the analysis of ligand-receptor dynamics on this time scale.

By a combination of three independent methods we have analyzed the number of receptors on resting cells (at 4 or 15 °C). These methods involve the use of: 1) particles with known numbers of fluoresceins in a cytometric assay; 2) antibody to fluorescein to discriminate free and bound FLPEP in a spectrofluorometric assay; and 3) a cytometric assay...
described previously which examines the variation of receptor occupancy as a function of cell concentration (6). Taken together, these methods yield a value of 53,000 ± 13,000 receptors on a resting neutrophil.4 These numbers are comparable to those obtained in a variety of studies using radioligands (see Ref. 2). Of these three methods, the first is the most convenient, being rapid and requiring the smallest number of cells. Due to the differences in the "brightness" of the two commercial standards, there remains some uncertainty in the absolute instrumental calibration. Of primary significance, however, is the utility of the calibration standard as a tool to standardize the instrumental sensitivity of the cyto-

cmeter so that cells from the same or different donors may be compared on different days. The affinity of FLPEP for its receptors has been analyzed at equilibrium by the spectrofluorimetric and cytometric methods. Both of these methods assume that the intensity of fluorescence of FLPEP is not influenced by the binding event. This assumption is validated in Figs. 3 and 5. We observe that the binding curve is reasonably well described by a single dissociation constant $K_D = 0.6 ± 0.2 \text{nM}$.

Our present results concerning the kinetics of N-formyl peptide-receptor interaction extend and confirm the earlier observations of Zigmond and Sullivan (11, 12) and Niedel and co-workers (13, 14). Specifically we have now quantitatively evaluated the temperature dependence of association and the temperature dependence of the initial rate of internalization. We have begun to dissect the elements which influence ligand dissociation. The kinetics of FLPEP association has been analyzed as a function of FLPEP concentration and temperature. The association rate constant varies roughly 3-fold over a temperature range from 4 to 37 °C. $k_{on}$ at 37 °C equals $10^9$ M$^{-1}$ min$^{-1}$, a value roughly 1 to 2% of the diffusion limit predicted for a molecule this size (15). When we consider the fact that the solid angle for entry of the ligand into the receptor binding pocket is likely to be diminished to a few per cent of $4\pi$ radians, $k_{on}$ is of comparable magnitude to the diffusion limit. There are differences in the results of the kinetic analysis of association based on the two methods we have used. The graphical analysis of Schonbrun and Tashjian (Fig. 4, C and D) (10) relies on an estimate of the half-time of binding and tends to weight early points in the binding curve particularly if the association is not strictly exponential. Our analysis fits the entire binding curve (Fig. 4B) but is very sensitive to the actual occupancy at "equilibrium." As seen in Fig. 5B, rela-

![Fig. 4. Analysis of FLPEP association kinetics by cytometry. A, time dependence of the histograms of cellular fluorescence following the addition of 1 nM FLPEP at 37 °C. The histogram noted "blocked" was obtained at equilibrium in the presence of 1 μM nonfluorescent peptide. B, the fractional receptor occupancy for different FLPEP concentrations is plotted versus time. C, calculated according to Ref. 9 using the rate constants $k_{on} = 10^9$ M$^{-1}$ min$^{-1}$ and $k_{off} = 0.25$/min. C, the data are plotted as log[1 - B(t)/B(max)] versus time for the different FLPEP concentrations. B(t)/B(max) refers to the fractional occupancy at time t compared to the maximal occupancy for the same concentration of FLPEP. D, the data of C are replotted according to Equation 3 as $K_{on}$ versus $K_{on}$. E, Arrhenius plot of association rate constants. The data are plotted as In $k_{on}$ versus 1/K. Four and five determinations were made at 4 and 37 °C, respectively. Duplicate determinations are shown for 15 and 25 °C. The average values are $k_{on} = 10.7 ± 2.8 \times 10^9$ M$^{-1}$ min$^{-1}$ with $k_{off} = 0.60 ± 0.31$ min$^{-1}$ at 37 °C and $k_{on} = 2.8 ± 0.4 \times 10^9$ min$^{-1}$ at 4 °C. The line drawn is the least squares fit to the data, calculated for the mean values determined at each temperature and equally weighted. The slope is $-4124.41$ and the intercept is 94.247. Various weighting schemes approximating the number of determinations yield similar lines.](https://www.jbc.org/)
processing which could include up-regulation at 37°C. Under most conditions the dissociation is resisted by the extent of internalization and by the loss of fluorescence of intracellular FLPEP. We are investigating the use of NRCl to inhibit the acidification of the endosomal vesicles (see Fig. 5A) for the first few minutes at 37°C and for much longer times at lower temperatures. However, it precludes an analysis of any longer term aspects of receptor dissociability. Our method tends to yield smaller estimates (see references in Refs. 16 and 20).

The extent of dissociability and the rate of dissociation of FLPEP are dependent upon the length of time of binding and the temperature. Under most conditions the dissociation is heterogeneous. At 15°C, where internalization of FLPEP is slow, we detect a rapid alteration in the dissociability of FLPEP with a conversion in the dissociation rate from koff about 0.4/min to <0.02/min. The half-time for conversion is about 10 min. At elevated temperatures, analysis of the dissociation is obscured by rapid internalization of FLPEP, but preliminary evidence is consistent with a much more rapid alteration in affinity. Even at 4°C there is a time-dependent loss of dissociability. These observations have been confirmed by independent measurements of the subcellular processing of the receptor. The high-affinity slow dissociating complex apparently arises as the complex of receptor and FLPEP associated with the cytoskeleton in a transient high molecular weight species (16).

The heterogeneity in dissociability may have as many as three components or more. There is a fraction of sites, ~10–20% after short periods of binding, whose dissociation is observed within the first 15 s of antibody or addition of unlabeled peptide. This component diminishes with time. It has been suggested that these sites arise from a subpopulation of cells which bind and internalize peptide differently (17). The bulk of the dissociation occurs with a halftime of ~3–5 min. This latter population appears to convert to the very slowly dissociating form (t1/2 > 30 min). Whether these sites are all interconvertible is not known.

Moreover, in demonstrating a conversion of the ligand-receptor complex to a slow dissociating state we have verified an observation which appears common to several receptor systems (see references in Refs. 16 and 20). A theoretical
FIG. 8. The dissociability of FLPEP after addition of 1 μM nonfluorescent peptide (○, △, □, PEP) or 20 nM antibody to fluorescein (●, ▲, Anti-FL) is plotted versus the length of time of binding: upper left, 4 °C; upper right, 37 °C; lower left, 15 °C; lower right, 25 °C. Data were obtained as in Fig. 7. Representative error bars (5 or 4 determinations) are provided in the lower left portion.

FIG. 9. The dissociability of FLPEP at 15 °C as a function of the length of time of binding in the spectrofluorometric assay. A suspension of cells (4.5 ml, 10⁷/ml) was exposed to 1 nM FLPEP at 15 °C. After 3, 10, or 30 min, a 1.5-ml aliquot was placed in a cuvette and antibody to fluorescein was added. The data are plotted as the FLPEP fluorescence versus time following addition of antibody. Duplicate samples were analyzed. The "blocked" sample was preincubated with 1 μM nonfluorescent peptide. Data are scaled in terms of FLPEP fluorescence. Broad lines are drawn to indicate the time course of the "average" dissociation. These lines represent averages of the duplicate determinations followed by three consecutive point-by-point smoothings of the data.

The analysis of these phenomena will require an analysis of the reversibility of the conversion and its occupancy dependence, the role of diffusion of the ligand away from the membrane, and clustering of the receptor in the membrane (21, 22) on the dissociation rates. Depending upon the exact conditions used in equilibrium measurements, heterogeneous or homogeneous formyl peptide binding (11, 12, 18, 19) is observed.

Features and Limitations of the Cytometric Assay of Ligand- Receptor Dynamics—The cytometric assay arises from the fact that at low ligand concentrations fluorescence flow cytometry intrinsically discriminates between the fluorescence which is concentrated on a cell surface and the fluorescence which is diffuse in the volume surrounding the cell. Thus, we observe that 50,000 FLPEP molecules associated with the cell surface contributes roughly 10 times more intensity to the profile of cellular fluorescence (100 channels) than 5 nM FLPEP in the surrounding medium (10 channels).

For a system of Kd = 30 nM and 50,000 receptors, with saturation at ~100 nM free ligand, the receptor-bound ligand and the free ligand would contribute roughly equally to the fluorescence profiles. Thus for cytometry to be applicable to systems of still lower affinity in a homogeneous assay, a larger number of receptors per cell would be required. There is no upper limit for the affinity for the system which can be measured, and receptor occupancy as low as a few thousand per cell is readily detected as a shift in the fluorescence profile.

Perhaps the most important feature of the cytometric assay is that the homogeneous assay conditions permit real-time and essentially continuous observation of ligand-receptor dynamics in a single sample of a cell suspension. With as few as 10⁶ cells in a milliliter, the time course of ligand binding and dissociation can be examined. We discuss elsewhere how these general procedures can be utilized in the analysis of ligand-receptor-competitor kinetics, even if the competitor is not labeled, is rapidly dissociable, or is of low affinity (9).

Whereas we have chosen to analyze binding kinetics using separate profiles of fluorescence obtained within narrow time windows at the desired time intervals, it is also possible to examine the time course of binding continuously using the "list mode" data acquisition option which is available in some cytometric systems. The major barriers to kinetic analysis are the delay time between the sample and the point of analysis and the fact that in many systems the pressurized sample chamber is not readily accessible for real-time addition of reagents (i.e. labeled or unlabeled ligand, etc). The first problem primarily affects the time of the first data acquisition (~5 s in our system). The second problem affects conditions under which the sample is to be altered in order to observe internalization or dissociation. We previously described an instrumental modification which permits on-line sample alteration (8), and we are presently developing a sample chamber with improved accessibility and mixing.

Features and Limitations of the Spectroscopic Assay of Ligand Binding Using the High Affinity Antibody to Fluorescein—Several aspects of this assay have been described elsewhere (4, 7). The basic feature of this system is the ability of antibody to fluorescein to discriminate free and receptor-bound ligand. It is noteworthy that the fluorimetric analysis which uses antibody and the cytometric analysis, which does not, yield identical results in four types of measurements: 1) equilibrium binding (Figs. 2 and 3); 2) association kinetics (Figs. 4 and 5); 3) dissociability (Figs. 8 and 9); and 4) internalization (Ref. 8). The use of antibody in the binding and quenching of free FLPEP is an extension of the work of Levison et al. and Watt et al. in which antibody is used to quench fluorescein (23, 24). The inaccessibility of the fluorescein of the receptor-associated FLPEP to antibody probably arises from steric hindrance at the receptor. While it is not possible to generalize at this point about systems in which antifluorophore antibodies will be applicable, we know already that it can be used with the activated complement component with C5a which is fluorescent at its NH₂-terminal threonine. We know, moreover, that the general principles are applicable not merely to fluorescein, but to eosin, erythrosin, and tetramethylrhodamine. Antibodies which quench these chromophores have also been obtained.

At this time we suggest the utility of these high affinity antibodies in ligand-receptor systems where homogeneous and univalent ligand labeling is possible. From a spectroscopic

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5. L. A. Sklar and D. E. Chenoweth, unpublished results.
6. L. A. Sklar, unpublished result.
point of view, there are several limitations to the generalization of these procedures. These depend on the concentration of receptor and the $K_d$. Due to the contributions of cellular autofluorescence and light scattering, a practical detection limit for bound ligand is $\sim 0.1 \text{ nM}$ fluorescent. However, in order to resolve free and bound ligand, a significant fraction of the ligand must be bound to the receptor. In order to meet these conditions $K_d$, [L], and [R] must all be similar in magnitude. Since $10^7$ cells/ml bearing $60,000$ receptors provide about $1 \text{ nM}$ receptor, ligand binding can be measured for $K_d$ in the nanomolar range and lower. Only if higher receptor concentrations can be achieved (i.e., if there are more receptors per cell or higher cell concentrations can be used) can lower affinity systems be examined. At higher ligand concentrations, direct spectral methods (i.e., fluorescence polarization) for discriminating free and bound ligand also become available.

The major advantage of the antibody method is that it permits continuous observation of ligand dissociation with a time resolution of seconds. This enables us to observe the reduction in the dissociation rate of the ligand. Even though both association and internalization can also be measured in real-time, each time point requires $\sim 10^7$ cells, and the measurements are not continuous. However, the time resolution in all of these measurements is excellent ($\sim 1 \text{s}$) and dependent only on the time required from the antibody to quench the fluorescence of the free ligand.

The most important aspect of the antibody technique for us lies in its utility to generate a stimulus “pulse” (4, 5). In such a pulse, cells are exposed to FLPEP over a desired time interval (occupying a defined fraction of receptors). By examining the details of the cellular responses elicited by this pulse, we believe it is possible to gain insight into the pathway and mechanism of cell activation. This issue will be the topic of a subsequent publication. In it we relate the contributions of the kinetics of the various aspects of ligand-receptor interaction to cellular activation.

Acknowledgments—We would like to thank Robert Hoffmann, Ortho Diagnostics, Westwood, MA for providing the Fluorotrol GF.

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