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# DNP-Phycobiliproteins, Fluorescent Antigens to Study Dynamic Properties of Antigen-IgE-Receptor Complexes on RBL-2H3 Rat Mast Cells[1]

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**In RBL-2H3 rat mucosal mast cells, the crosslinking of cell-surface IgE-receptor complexes by multivalent antigens initiates a sequence of responses leading to degranulation. We have developed a family of dinitrophenol (DNP)-conjugated fluorescent antigens to study dynamic membrane events associated with these responses. Lysyl groups on the phycobiliproteins, B-phycoerythrin and C-phycocyanin, were labelled with DNP, yielding fluorescent conjugates that cause the release of [$^3$H]serotonin from anti-DNP-IgE-primed RBL-2H3 cells. The binding of these antigens to IgE-receptor complexes was observed by fluorescence microscopy and quantified by flow cytometry. Incubation with 1 μg/ml $DNP_{42}$-B-phycoerythrin stimulates maximum degranulation from IgE-saturated cells. Under these conditions, approximately $26 \times 10^3$ molecules of $DNP_{42}$-B-phycoerythrin are bound per cell at equilibrium. The rate and extent of antigen binding and of antigen-stimulated mediator release decrease in parallel as the concentration and DNP:protein ratio of the fluorescent conjugates is reduced. Secretion stops immediately when the nonfluorescent monovalent antigen, DNP-lysine, is added to degranulating cell suspensions. DNP-lysine also displaces surface-bound antigen when added during the first minutes after multivalent antigen. However, the ability of DNP-lysine to displace surface-bound $DNP_{42}$-B-phycoerythrin from IgE-receptor complexes decreases progressively with time. Treatment with dihydrocytochalasin B and several analogs that prevent antigen-stimulated F-actin assembly enhances secretion and delays the transition of antigen to its DNP-lysine-resistant form. Cytochalasin treatment also permits the long-range movement of antigen into surface caps. Based on these data, we propose that secretion is triggered by the act of IgE-receptor crosslinking or by a short-lived excited state of the crosslinked antigen-IgE-receptor complex. We propose further that antigen-stimulated F-actin assembly contributes to the transition of antigen-IgE-receptor complexes to a DNP-lysine-resistant form that does not trigger secretion. Two possible mechanisms for the transition to DNP-lysine resistance are discussed.**

**Key terms: Transmembrane signalling, ligand-receptor binding kinetics, phycobiliproteins, mast cells, IgE receptors**

The IgE-dependent release of histamine, serotonin, and other inflammatory mediators from mast cells and basophils is the triggering event in a variety of acute allergic, asthmatic, and inflammatory conditions. Although the events leading to release are still imperfectly understood, the development of new tools and techniques has contributed to recent progress. In particular, Siraganian and colleagues (2,26) have characterized a family of rat basophilic leukemia cell lines, including RBL-2H3; Katz et al. (15) have demonstrated the homology of these lines with rat mucosal mast cells; Metzger and colleagues have isolated and characterized the RBL-2H3 receptor for IgE (22); and Liu et al. (16) have generated stable hybridomas secreting mouse monoclonal antidinitrophenol (DNP) IgE. The crosslinking of anti-DNP-

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IgE-receptor complexes on RBL-2H3 cells by multivalent DNP-conjugated proteins is the first of a cascade of intra- and transmembrane events, including the stimulation of inositol phospholipid turnover and the mobilization of $Ca^{2+}$ (3,4), that lead to mediator release.

We have developed a family of DNP-conjugated fluorescent proteins for dynamic studies of antigen-IgE-receptor interaction in RBL-2H3 cells. The fluorescent moieties are provided by two phycobiliproteins (10,20), B-phycoerythrin (B-PhE) and C-phycocyanin (C-PhC). The binding of DNP-B-phycoerythrin (DNP-B-PhE) to membrane IgE-receptor complexes has been observed by fluorescence microscopy and measured by flow cytometry. Our data provide new information about the role of dynamic membrane events in signal transduction in RBL-2H3 cells.

## MATERIALS AND METHODS

### Cells

The RBL-2H3 cell line (2) was a generous gift of Dr. C. Fewtrell, Cornell University. The cells were grown on bacteriological plates in Dulbecco's Modified Eagle's Medium with 15% fetal calf serum (DMEM-FCS). RBL-2H3s are readily detached from these plates by gentle pipetting or scraping.

### Reagents

Affinity-column-purified monoclonal mouse anti-DNP-IgE (16) was a generous gift of Dr. H. Metzger, National Institutes of Health. B-PhE from *Porphidium cruentum* and C-PhC from *Synechococcus 6301* were isolated and conjugated with DNP by Dr. Rosaria Haugland, Molecular Probes, Inc. Junction City, OR (11), and are now commercially available from Molecular Probes. Their protein concentrations were determined according to Bradford (6). Their DNP concentrations were determined from the absorption at 480 nm, using DNP-lysine as standard. The DNP substitution levels were calculated assuming that B-PhE is principally in the hexameric form ($[\alpha\beta_6]\gamma$, mol. wt. 240,000) and C-PhC is principally in the monomeric form ($\alpha\beta$; mol. wt. 36,700) when dissolved in Hanks' medium at low protein concentration (9,20). Making these assumptions, the calculated DNP:B-PhE ratios of the conjugates ranged from 20:1 to 45:1 and the calculated DNP:C-PhC ratios ranged from 4:1 to 16:1. DNP substitution did not alter the absorption and emission spectra of the phycobiliproteins. Dr. Haugland also prepared a series of DNP-bovine serum albumin (DNP-BSA) conjugates with DNP:BSA ratios ranging from 30:1 to 50:1; 5-(1,2-[$^3$H]N)-hydroxytryptamine binoxalate ([$^3$H]serotonin; 24.1 Ci/mmol) was from New England Nuclear; phenylglyoxal was from Aldrich Chemical Company; and cytochalasins B and D and dihydrocytochalasin B were from Sigma Chemical Company.

### Mediator Release

Mediator release was assessed from the loss of [$^3$H]serotonin by modification of the procedures of Taurog et al. (32). Cells were incubated for 2 h with 2 $\mu$Ci/ml [$^3$H]serotonin and 3$\mu$g/ml anti-DNP-IgE in DMEM-FCS. They were washed twice with modified Hanks' Medium (5), resuspended to $2 \times 10^6$ cells/ml in Hanks' Medium at 37°C, and 0.2-ml portions were transferred into 1.5-ml plastic tubes containing either 0.02 ml of 10 mM Hepes buffer, pH 7.4, or appropriate concentrations of DNP-proteins in Hepes buffer. After incubation for specified times at 37°C, the reaction was terminated by addition of 0.5 ml of ice-cold Dulbecco's phosphate-buffered saline (PBS) and immediate 3-min centrifugation in an Eppendorf minicentrifuge; [$^3$H]serotonin in 0.5-ml portions of the supernatant was determined by liquid scintillation counting in 4 ml Aquasol. A background value was determined by cold dilution and centrifugation of a portion of cells without the addition of cross-linker or incubation at 37°C. Total serotonin content was determined from the supernatant radioactivity when 0.5% Triton-X-100 was added to the cells. Each assay was performed in duplicate or triplicate.

### Fluorescence Microscopy

IgE-primed cells (0.5 ml per incubation; $2 \times 10^6$ cells/ml) were incubated in 1.5-ml plastic tubes at 37°C in Hanks'-BSA containing DNP-B-PhE. At specified times they were centrifuged (3 s in an Eppendorf minicentrifuge), resuspended for 10 min in 0.5 ml 2% paraformaldehyde in PBS, and then washed and mounted in 50% glycerol on glass slides. The distribution of DNP-B-PhE bound to IgE-receptor complexes was observed by epillumination using the rhodamine filter combination of a Zeiss Photomicroscope III. Fluorescence was recorded by photography on Kodak Tri-X-Pan film.

### Flow Cytometry

The binding of DNP conjugates of B-PhE ($\lambda$ $max_{ex}$ = 545 nm; $\lambda$ $max_{ex}$ = 575 nm) and C-PhC ($\lambda$ $max_{ex}$ = 620 nm; $\lambda$ $max_{ex}$ = 650 nm) to cell-surface IgE-receptor complexes was quantified by flow cytometry. The analyses were performed at the National Flow Cytometry Resource, Life Sciences Division, Los Alamos National Laboratories, using Sorter I, a three-laser flow cytometer, and Sorter II, a two-laser flow cytometer. Sorter I has an argon ion laser that can excite DNP-B-PhE at 514 nm and a krypton laser that can excite DNP-B-PhE at 530 nm and DNP-C-PhC at 570 or 630 nm. Sorter II can excite only B-PhE (argon but no krypton laser). Its beams can be pulsed by using an electro-optic modulator (see below). Both instruments permit measurements of fluorescence binding kinetics over time in live cells (17). Both can collect multiple additional parameters including Coulter volume, axial light loss, 90° scatter, 2° or forward scatter, and time (29).

In most experiments, DNP-protein was added to 2 ml of RBL-2H3 (around $0.5 \times 10^6$ cells/ml) at 37°C. Simultaneously, an adjustable time ramp generator (30) was activated. The cells were placed in a 37°C sample holder and data collection was begun within 20–30 s of ligand addition. Multiparameter list mode data were acquired

by using the Los Alamos LACEL data acquisition system (12) interfaced to a PDP 11/23 computer. The multiparameter data were subsequently analyzed by using programs developed for the LACEL system (25).

Two principal experiments were performed. The flow cytometer was used to assess the relative rates of binding and displacement of DNP-B-PhE as a function of antigen concentration and of DNP substitution. For total binding, fluorescence intensity per cell was followed over time for 10 min or until no increase in fluorescence mean channel number was seen in 1–2 min, indicating that equilibrium had been reached. Antigen displacement was followed by addition of DNP-lysine (usually 10$\mu$M) and measurement for 5 min.

The flow cytometer was also used to determine the number of DNP-B-PhE molecules bound to IgE-receptor complexes. This was possible by application of a new calibration method (Martin et al., in preparation) in which the fluorescence intensities of cell-equivalent volumes of DNP-B-PhE solutions are measured in the sample stream–laser beam intersection and compared with the fluorescence intensities of DNP-B-PhE-labelled cells. To establish this calibration curve, the solution dye concentration, sample stream diameter, focused laser spot size, and the sample velocity must all be known. Fluorescence pulses were obtained from the steadily flowing solution sample stream by pulse-modulating the laser beam with an electro-optic modulator (Coherent Radiation, Palo Alto, CA, model 20). The temporal shape of the modulated laser pulses was adjusted to mimic the fluorescence pulses normally generated by labelled RBL-2H3 cells. The sample stream diameter was determined by weighing the sample tube before and after running the sample for a measured amount of time, and from the sample flow velocity. The sample flow velocity was determined by measuring the transit time for fluorescent microspheres passing between two focused laser beams, whose separation was also measured. The diameter of the laser spot was estimated from measurements of the unfocused beam diameter and the focusing lens that was used. A calibration curve of measured fluorescence intensity against number of fluorochrome molecules in the cell equivalent volume was constructed from these data. After obtaining this curve, IgE-treated cells in Hanks'-BSA containing at least 0.1 $\mu$g/ml DNP-B-PhE were introduced and fluorescence intensity per cell was recorded at equilibrium by using the same optics, laser power levels, and electronic gain settings. These data were corrected for background fluorescence, obtained by measurements of the fluorescence of cells incubated with the same concentration of DNP-B-PhE but without IgE pretreatment. The average number of bound fluorochrome molecules per cell was finally read from the calibration curve.

## RESULTS

We reported before (23) that 1 $\mu$g/ml DNP-BSA and 1 $\mu$g/ml $DNP_{42}$-B-PhE (corresponding to 13 nM $DNP_{34}$-BSA and 3.8 nM $DNP_{42}$-B-PhE) stimulate the release of between 25 and 40% of the total [$^3$H]serotonin content of RBL-2H3 cells. The kinetics of release were identical for the two antigens. These data are extended in Table 1. It is shown that maximal [$^3$H]serotonin release is maintained over a broad protein concentration range (50 ng/ml–1 $\mu$g/ml) when B-PhE is conjugated with greater than 30 DNP molecules per hexamer and 0.01 $\mu$l/ml $DNP_{42}$-B-PhE stimulates around 50% of maximal release. In contrast, phycobiliproteins substituted with 20–30 DNP molecules per hexamer are less effective degranulating agents, and conjugates with fewer than 20 DNP bound per B-PhE do not stimulate IgE-dependent mediator release. Similarly, maximum serotonin

Table 1
*Antigenic Activity of the DNP-Phycobiliproteins*[a]

| Antigen | 1.0 $\mu$g/ml | 0.5 $\mu$g/ml | 0.1 $\mu$g/ml | 0.05 $\mu$g/ml | 0.01 $\mu$g/ml |
|---|---|---|---|---|---|
| $DNP_{34}$-BSA | 100 | 102 | 97 | 104 | 98 |
| $DNP_{19}$-B-PhE | 0 | 0 | 0 | 0 | — |
| $DNP_{21}$-B-PhE | 2 | 0 | 0 | 0 | — |
| $DNP_{22}$-B-PhE | 5 | 7 | 1 | 0 | — |
| $DNP_{24}$-B-PhE | 83 | 77 | 36 | 21 | — |
| $DNP_{27}$-B-PhE | 93 | 97 | 104 | 83 | — |
| $DNP_{31}$-B-PhE | 101 | 113 | 111 | 97 | — |
| $DNP_{42}$-B-PhE | 98 | 101 | 117 | 106 | 58 |
| $DNP_4$-C-PhC | 18 | — | — | 12 | — |
| $DNP_6$-C-PhC | 98 | — | — | 97 | — |
| $DNP_8$-C-PhC | 105 | — | — | 102 | — |

[a][$^3$H]-Serotonin released by DNP-phycobiliproteins (expressed as % of serotonin released by 1 $\mu$g/ml $DNP_{34}$-BSA in 20 min). [$^3$H] serotonin-loaded cells (2 $\mu$Ci/ml; 12–15 h) were primed with anti-DNP-IgE (3 $\mu$g/ml) for 1–2 h and then incubated with antigen at the concentration indicated for 20 min. Results are the average of at least three determinations of serotonin release caused by each concentration of DNP-phycobiliproteins compared to 1 $\mu$g/ml $DNP_{34}$-BSA. The data are corrected for the spontaneous release that occurs in the absence of antigen (approximately 0.5% per minute). The net % serotonin released by 1 $\mu$g/ml $DNP_{34}$-BSA varied between experiments from 25 to 40%. This variability is a function of cell passage number.

release is obtained when C-PhC is conjugated with at least six DNP molecules per monomer and little or no release occurs at lower DNP substitution levels. Neither DNP-BSA nor DNP-phycobiliproteins stimulate serotonin release in the absence of IgE or in the absence of extracellular $Ca^{2+}$. Furthermore, neither B-PhE nor C-PhC without DNP substitution stimulates serotonin release (data not shown).

Secretion caused by multivalent antigen is rapidly and completely inhibited by the addition of an excess of competing, monovalent antigen. Figure 1 shows that DNP-lysine (10 $\mu$M) blocks [$^3$H]serotonin release caused by 0.5 $\mu$g/ml (1.9 nM) $DNP_{42}$-B-PhE when added at any point in the secretory process.

When cells are incubated with antigen plus 5 $\mu$g/ml dihydrocytochalasin B, which inhibits antigen-stimulated F-actin assembly (JC. Seagrave, unpublished), both the rate and extent of mediator release are increased (Fig. 2). The same effect is observed when RBL-2H3 cells are exposed to cytochalasin B and to cytochalasin D (not shown) and when primary basophils are exposed to cytochalasin B (8). This effect is optimal when antigen and drug are present over the entire experimental period. However, the cytochalasins also stimulate release when added up to 10 min after the antigen (Fig. 2).

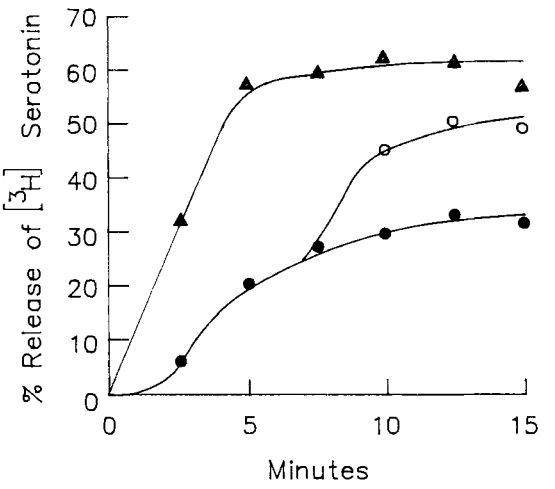


FIG. 2. The effect of dihydrocytochalasin B on antigen-dependent [$^3$H]serotonin release. IgE-primed cells were incubated with $DNP_{42}$-B-PhE (0.1 $\mu$g/ml) in the presence or absence of 5 $\mu$g/ml dihydrocytochalasin B. Cells incubated with Hanks' medium alone (solid circles) released around 30% of their total [$^3$H]serotonin. Both the rate and extent of mediator release were increased when dihydrocytochalasin B was present throughout the experiment (triangles). A smaller but still significant enhancement was observed when the drug was added 7 min after addition of antigen (open circles). These results are corrected for antigen-indepependent [$^3$H]serotonin release (that was not altered by dihydrocytochalasin B).

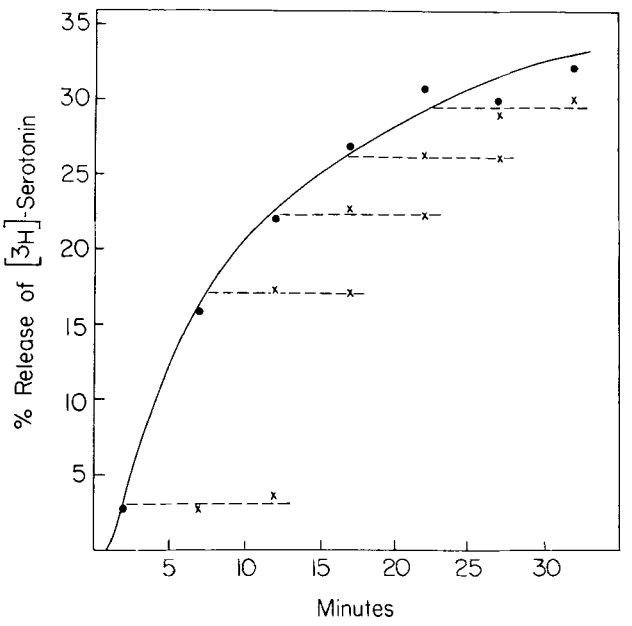


FIG. 1. The effect of DNP-lysine on antigen-dependent [$^3$H]serotonin release. RBL-2H3 cells were incubated for 2 h in DMEM-FCS with anti-DNP-IgE and [$^3$H]serotonin. The cells were washed into Hanks' medium and 1.9 nM $DNP_{42}$-B-PhE was added. The solid line shows the time course of antigen-stimulated [$^3$H]serotonin release measured over 30 min. The dashed lines show that DNP-lysine (10 $\mu$m) causes the rapid and complete inhibition of [$^3$H]serotonin release when added at any time during the process of secretion. Curve fitting was by the method of least squares. Results are corrected for antigen-independent [$^3$H]serotonin release (approx. 0.5% of the total [$^3$H]serotonin content of the cells per minute).

## The Kinetics of DNP-B-PhE Binding

Figure 3 shows the time course of binding of various concentrations of $DNP_{42}$-B-PhE to IgE-saturated cells. High concentrations of DNP-B-PhE (1 $\mu$g/ml) reach equilibrium binding in the 20–30 s before data collection is begun. At lower antigen concentrations both the rate and extent of $DNP_{42}$-B-PhE binding are reduced. There is no measurable binding of $DNP_{42}$-B-PhE to surface receptors at concentrations of 0.01 $\mu$g/ml and lower.

The effect of DNP conjugation on the rate and extent of DNP-B-PhE binding is shown in Figure 4. Conjugates with greater than 30 DNP per B-PhE bind rapidly and reach similar equilibrium binding values. Both the rate and extent of binding decline rapidly at lower levels of DNP conjugation and there is no measurable binding of conjugates with less than 20 DNP per B-PhE molecule. The binding curves are essentially unaltered when cells are incubated in $Ca^{2+}$-free medium (not shown); in medium containing 0.75 mM phenylglyoxal, an arginine-reactive reagent (31) that inhibits endocytosis (21; see below); and in medium containing 5 $\mu$l/ml dihydrocytochalasin B. Binding curves for phenylglyoxal and dihydrocytochalasin B-treated cells are illustrated in Figure 5.

## The Number of DNP-B-PhE Molecules Bound per Cell

The maximum number of $DNP_{42}$-B-PhE molecules bound per cell at equilibrium was estimated from multiple measurements made in two separate experiments.

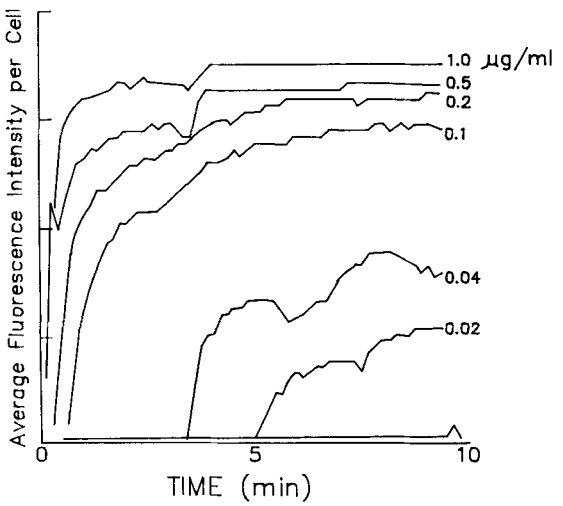


FIG. 3. The concentration dependence of $DNP_{42}$-B-PhE binding to RBL-2H3 cells. $DNP_{42}$-B-PhE at the indicated concentrations (in μg/ml) was added to 2 ml of IgE-primed cells ($1 \times 10^6$/ml), mixed, and inserted into the 37°C sample holder of Sorter I. Measurements of cellular fluorescence were recorded and analyzed by programs developed at the Los Alamos National Laboratory. Cellular fluorescence is reported as arbitrary units on a logarithmic scale.

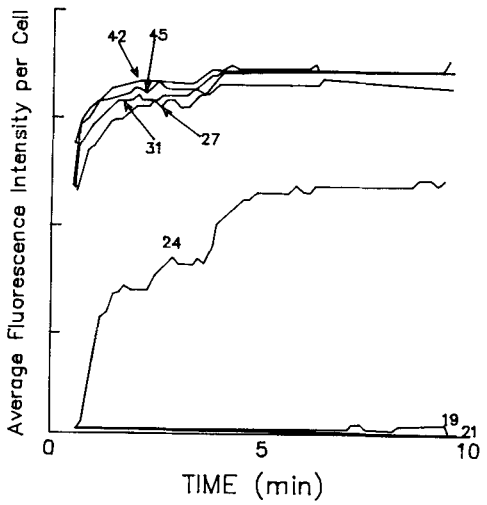


FIG. 4. Dependence of B-PhE binding on DNP substitution. Phycoerythrins conjugated with the indicated numbers of DNP's per molecule were added to IgE-primed cells at 1 μg/ml. Fluorescence was recorded as described in the legend to Figure 3. Cellular fluorescence is reported as arbitrary units on a logarithmic scale.

The cell equivalent volumes for the two sets of solution calibration experiments were 6.5 and 5.1 pl. It was calculated that an average of 25,700 ± 2,000 molecules of $DNP_{42}$-B-PhE were bound per cell.

### The Distribution of DNP-B-PhE

The initial binding of antigen is followed by predictable changes in the distribution of antigen-IgE-receptor complexes. This is illustrated in the series of fluorescence micrographs of $DNP_{42}$-B-PhE-labelled RBL-2H3 cells in Figure 6. $DNP_{42}$-B-PhE is distributed uniformly on IgE-primed RBL-2H3 cells that were labelled with antigen at 4°C (Fig. 6A). IgE-receptor complexes thus

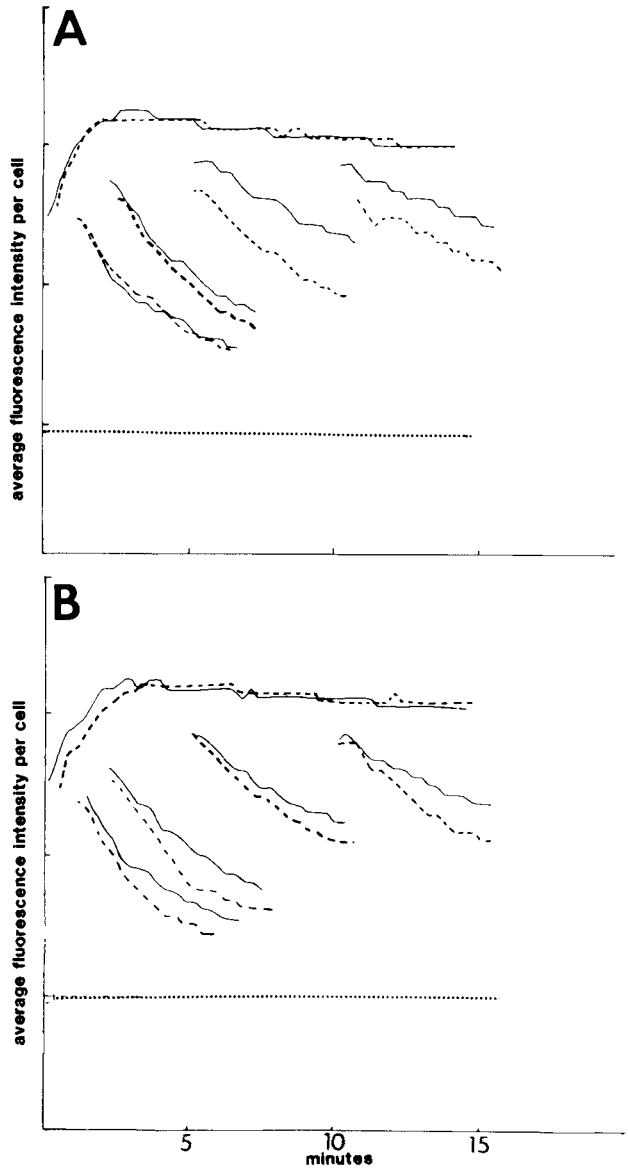


FIG. 5. The binding of $DNP_{42}$-B-PhE to IgE-receptor complexes and its displacement by DNP-lysine. In panel A, IgE-primed RBL-2H3 cells were incubated with $DNP_{42}$-B-phycoerythrin (1 nM; 0.25 μg/ml) in Hanks' medium alone (solid lines) or in Hanks' plus 5 μg/ml dihydrocytochalasin B (dashed lines). In panel B, incubation was in Hanks' with phenylglyoxal (0.75 mM; solid lines) and in Hanks' with phenylglyoxal plus dihydrocytochalasin B (dashed lines). Binding was followed continuously at 37°C for 15 min toi give the upper curves. The lower curves were obtained by incubating separate samples for 1, 2, 5, or 10 min with $DNP_{42}$-B-PhE before addition of 10 μM DNP-lysine. Cellular fluorescence is given in arbitrary units on a logarithmic scale. Neither dihydrocytochalasin B nor phenylglyoxal alters the rate or extent of antigen binding. However, antigen bound to cytochalasin-treated cells remains more readily displaceable by DNP-lysine. The dotted lines represent the fluorescence of cells that were incubated with 1 nM $DNP_{42}$-B-PhE without prior exposure to IgE.

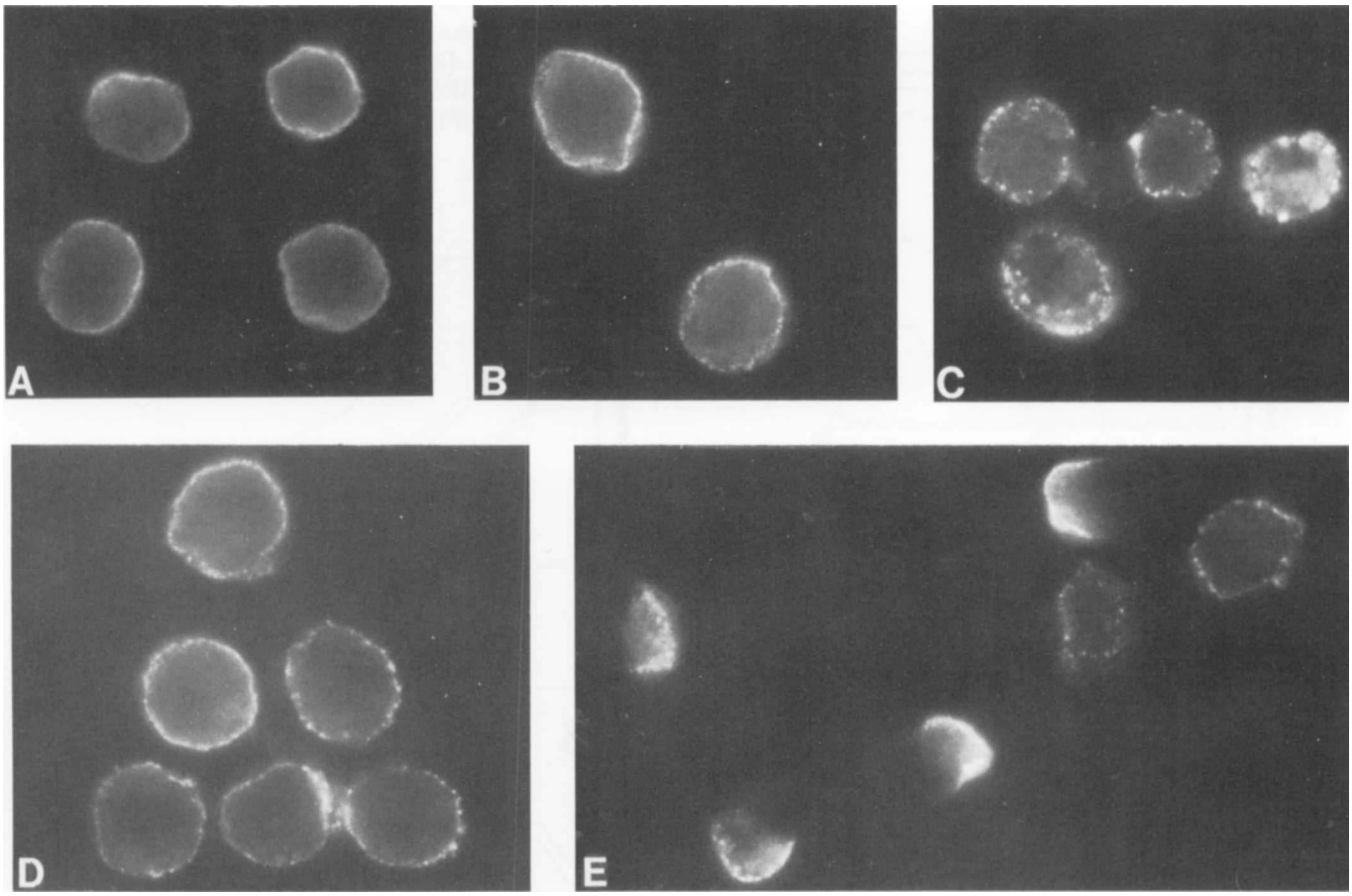


FIG. 6. The distribution of $DNP_{42}$-B-PhE on RBL-2H3 cells. IgE-primed RBL-2H3 cells were incubated under various conditions with $DNP_{42}$-B-PhE (1 μg/ml), fixed, and examined by fluorescence microscopy. Antigen binds uniformly to IgE-primed cells that were incubated for 10 min at 4°C (A). Cells incubated with antigen at 37°C develop surface clusters after 5-min incubation (B) which are internalized after 10 min (C). Cells incubated at 37°C in the presence of 0.75 mM phenylglyoxal form surface clusters that are not internalized (D). Treatment with dihydrocytochalasin B (5 μg/ml, 10 min) promotes antigen capping on about 30% of cells (E).

appear to have an inherently uniform distribution on the cell surface. In contrast, antigen is redistributed into clusters when cells are warmed in the presence of $DNP_{42}$-B-PhE (Fig. 6B). Beyond 10 min of incubation, the internalization of antigen is apparent (Fig. 6C). Antigen can still cluster but it is not internalized in phenylglyoxal-treated cells (Fig. 6D). Antigen-stimulated capping of receptors does not normally occur on RBL-2H3 cells. However, endocytosis is reduced and around 30% of cells are capped following incubation with antigen plus 5 μg/ml dihydrocytochalasin B (Fig. 6E).

### The Kinetics of Antigen Displacement

Figure 5 shows one of a series of five replicate experiments that measured the kinetics of $DNP_{42}$-B-PhE displacement by the monovalent, competing antigen, DNP-lysine. Multivalent antigen was allowed to bind for various times; then DNP-lysine was added to the sample chamber of the flow cytometer and the displacement of antigen was followed. DNP-phycoerythrin that bound during brief (0.5–2 min) incubation at 37°C was rapidly and almost completely displaced by a 10,000-fold molar excess of DNP-lysine. With longer incubation times, there was always a progressive decrease in the slope of the displacement curve. There was also a progressive increase in the amount of antigen that was not displaced by DNP-lysine during a 5–10-min incubation (Fig. 5A, solid lines). The same pattern of progressively slower and less complete displacement of bound antigen with increasing incubation time was seen in cells treated with phenylglyoxal, the reagent shown above to inhibit the endocytosis of bound antigen (Fig. 5B, solid lines). Thus the transition to poorly displaceable binding cannot be explained solely by antigen internalization.

Most importantly, antigen bound to cytochalasin-treated cells was always more readily displaceable by DNP-lysine than antigen bound to control cells. This greater rate of displacement in cytochalasin-treated cells

was observed in the presence or absence of phenylglyoxal (Fig. 5A, B, dashed lines). Thus, the inhibition of endocytosis by cytochalasins cannot explain the altered time course of antigen displacement.

## DISCUSSION

We have developed a family of DNP-conjugated fluorescent proteins to study the role of dynamic cell-surface properties in the control of mediator release from anti-DNP-IgE-primed RBL-2H3 cells. Two phycobiliproteins, B-PhE and C-PhC, were selected for conjugation with DNP. These fluorescent proteins are well suited for biological studies (10,20). They can be detected with great sensitivity because of their high extinction coefficients, their high fluorescence quantum yields, and their large Stokes shift. Furthermore, they emit in the orange-red spectral region, where cellular autofluorescence is usually low; they are insensitive to most quenching agents and conditions; and their fluorescence spectra are relatively unaltered by changes in pH (at least within the range of 6–8) and by conjugation with proteins and other biologically interesting molecules. Finally, they are fairly stable and highly water-soluble.

The fluorescence studies reported here are focused on DNP-B-PhE. This conjugate is easily detected with conventionally equipped fluorescence microscopes and flow cytometers because its spectral properties overlap those of rhodamine. In contrast, DNP-C-PhC detection requires the use of long-wavelength excitation and emission filters (for fluorescence microscopy) and access to a krypton laser and red-sensitive photomultipliers (for flow cytometry). We have developed this conjugate because DNP-B-PhE and DNP-C-PhC constitute a natural pair of reagents for future analyses of antigen clustering by the method of fluorescence resonance energy transfer.

We report that DNP-B-PhE conjugates containing approximately 30 DNP:B-PhE hexamer and DNP-C-PhC conjugates with approximately 6 DNP:C-PhC monomer cause the time, protein concentration, and $Ca^{2+}$-dependent release of [$^3$H]serotonin from IgE-primed RBL-2H3 cells. Conjugates with lower DNP:phycobiliprotein ratios do not stimulate mediator release. Since the B-PhE molecules are approximately six times larger than the C-PhC molecules, the surface distributions of DNP at the effective levels of substitution are presumably similar. Previous investigators have shown that secretion can be triggered by the crosslinking of as few as three IgE receptors (7). We suppose that our reagents require a higher minimal level of DNP substitution because some DNP residues are inaccessible to surface-bound IgE or are inappropriately spaced to crosslink receptors effectively (14).

The kinetics of DNP-B-PhE binding to IgE-receptor complexes are similar to the kinetics of DNP-B-PhE-induced mediator release. There is no detectable binding to cells that were not previously labelled with IgE. Antigens that cause optimal serotonin release bind rapidly to cell-surface IgE-receptor complexes and equilibrium is reached in minutes. At antigen concentrations that cause slower and less complete mediator release, DNP-B-PhE also binds more slowly and reaches lower equilibrium binding values. Similarly, antigens whose DNP:protein ratios are suboptimal for mediator release show reduced binding to IgE-primed cells.

However, there are some differences between the binding and release responses. First, concentrations of $DNP_{42}$-B-PhE below the level of fluorescence detection (0.01 $\mu$g/ml) still cause some release of [$^3$H]serotonin. That is, the biochemical assay maintains a greater sensitivity than the flow cytometric assay. In addition, the time course of antigen binding is unaltered by treatments that can prevent mediator release (incubation in $Ca^{2+}$-free medium) or can enhance mediator release (incubation with cytochalasins). Thus, antigen binding is necessary but not sufficient for mediator release.

The number of antigen molecules bound at equilibrium was calculated by using a new method (Martin et al., in preparation) to convert relative fluorescence intensity measurements to numbers of dye molecules. The method involves establishing a calibration curve by using solutions of DNP-B-PhE at known concentrations. Solution fluorescence measurements are made by pulse activating the sample stream with an optical pulse shape that corresponds to the average pulse shape generated by a cell. Using cells saturated with IgE, we calculated that an average of 25,700 molecules of $DNP_{42}$-B-PhE are bound per cell at equilibrium and under conditions that permit maximum degranulation. RBL-2H3 cells have about 300,000 IgE-receptors per cell (19). Thus a single $DNP_{42}$-B-PhE molecule may bind up to 12 bivalent IgE-receptor complexes.

The initial binding of antigen is followed by changes in antigen distribution. As expected from previous studies (18,23) crosslinked DNP-B-phycoerythrin-IgE-receptor complexes were redistributed at 37°C into surface clusters that were internalized. The endocytosis of DNP-B-phycoerythrin was specifically inhibited by phenylglyoxal, an arginine-reactive compound (31) that inhibits both phagocytosis and pinocytosis in macrophages and neutrophils (21). No long-range movement of antigen-IgE-receptor complexes into surface caps was observed on either untreated or phenylglyoxal-treated cells. However, up to 30% of dihydrocytochalasin-B-treated cells developed surface caps during brief (5–10 min) incubation with DNP-B-phycoerythrin. We reported elsewhere (23) that IgE-receptor crosslinking increases F-actin assembly and F-actin-membrane interaction. One of us (JC. Seagrave) has also found that cytochalasin treatment prevents the antigen-induced polymerization of F-actin. From these data, we speculate that antigen-stimulated F-actin-membrane interaction may be responsible for the topographical restriction of antigen-IgE-receptor complexes on RBL-2H3 cells.

Previous investigators have established that the crosslinking of IgE-receptor complexes by antigen is the key event that initiates secretion in RBL-2H3 cells. Our experiments with DNP-lysine indicate more specifically that secretion depends on the act of crosslinking and/or

or the formation of short-lived triggering-competent complexes. The monovalent antigen, DNP-lysine, was added to IgE-primed cells at various times after $DNP_{42}$-B-PhE and either the kinetics of [$^3$H]serotonin release or the kinetics of $DNP_{42}$-B-PhE displacement from the cell surface was followed. It was found that DNP-lysine always causes the immediate and complete inhibition of [$^3$H]serotonin release. DNP-lysine added shortly after $DNP_{42}$-B-PhE also displaces the majority of surface-bound multivalent antigen. However, with increasing incubation time, antigen becomes progressively more resistant to displacement by monovalent ligand. This conversion of antigen-IgE-receptor complexes to a DNP-lysine-resistant form that does not trigger secretion occurs whether or not the cells are treated with phenylglyoxal to inhibit endocytosis, indicating that the effect cannot be explained solely on the basis of antigen internalization. Further experiments indicated a role for antigen-stimulated F-actin assembly (23) in this transition to DNP-lysine-resistant and biologically inactive complexes. It was found that the time-course of antigen binding is unaltered by cytochalasin B and its analogs. However, treatment with cytochalasins delays the shift from readily displaceable to poorly displaceable antigen and it simultaneously enhances the rate and extent of secretion.

Two hypotheses to explain these data are being investigated. It is possible that the interaction of F-actin with IgE-receptor complexes on antigen-stimulated cells causes a conformational change in the complexes that increases their affinity for antigen and decreases their ability to trigger secretion. According to this proposal, cytochalasin B inhibits antigen-stimulated F-actin assembly, and this in turn inhibits the postulated increase in binding affinity of IgE-receptor for antigen. This inhibition may extend the lifetime of the DNP-lysine-sensitive excited state of antigen-IgE-receptor complexes. In addition, the continued higher rate of dissociation of antigen from IgE-receptor complexes may permit new crosslinking (triggering) events to occur over longer times than in control cells. Consistent with such a model, Jesaitis et al. (13) have already presented evidence for a cytochalasin-inhibited (presumably F-actin-dependent) increase in binding affinity of the formylpeptide receptor for its ligand that may terminate signal transduction in neutrophils.

Alternatively, F-actin-membrane interaction may anchor clusters of IgE-receptor complexes so that complexes that dissociate from antigen are more likely to reassociate with the same population of antigen molecules than to diffuse away. Such an effect would minimize the dissociation of multivalent antigen from the cell surface. By restricting receptor movement, anchoring may simultaneously limit the association of crosslinked IgE-receptor complexes with elements of the signal transduction pathway (G proteins, phospholipase C) whose activation is required to cause secretion. In this view, cytochalasin treatment enhances secretion by preventing the anchoring of IgE-receptors, thus prolonging the sensitivity of antigen to DNP-lysine displacement and simultaneously increasing the receptor-transducer interaction. The increased long-range mobility of antigen-IgE-receptor complexes, as indicated by the caps on cytochalasin-treated cells, is consistent with the hypothesis that F-actin-membrane interaction causes the anchoring of IgE-receptor complexes on RBL-2H3 cells. Recent evidence from Baird, Holowka, and colleagues that receptor crosslinking is followed by receptor immobilization and by a detergent-resistant interaction of receptors with cytoskeletal elements (18,24) is also consistent with the anchoring hypothesis.

Sklar and his colleagues have demonstrated the utility of the flow cytometer for kinetic analysis of ligand-receptor interaction in neutrophils (27,28). Our data establish the value of flow cytometry for analyses of dynamic membrane events associated with antigen-stimulated mediator release in the RBL-2H3 mast cell model. We anticipate that flow systems will become increasingly popular tools to study receptor-dependent functions of animal cells.

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