ANTIGEN-INDUCED AGGREGATION AND MODULATION OF RECEPTORS ON HAPTEN-SPECIFIC B LYMPHOCYTES*

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Since the discovery of the high density of immunoglobulin (Ig) present at the surface of mouse B lymphocytes (1), the changes in the distribution of these Ig receptors after attachment of divalent antiglobulin have been extensively studied (2–4). The formation by metabolism-independent diffusion of receptor patches, and the subsequent metabolism-dependent emergence of a "cap" of aggregated receptors over one pole of the cell is well known. In contrast, there have been few studies of a parallel nature on the movement of B-lymphocyte receptors induced by specific antigens. This is because only a small minority of a random population of B lymphocytes react with a given antigen, and thus the investigator must search amongst many negative cells before finding suitable objects for study. It has been observed that polymeric antigens can, as expected, cap B-lymphocyte receptors (5–8); that immunogenic concentrations of antigen can be quickly eliminated (9) and can cause emergence of a denser receptor coat within about 6 h (5, 9); that tolerogenic antigens can cause failure of capping (5) or prolonged surface persistence of antigen (10); and that the totality of the surface Ig on a B lymphocyte can be brought into a cap by a given antigen (6, 8), suggesting homogeneity of the receptor population (6).

Recently, a new, simple technique for the fractionation of hapten-specific B lymphocytes has been developed in our laboratory (11, 12). It depends on the adherence of lymphocytes to a thin layer of hapten-gelatin at 4°C, recovery of bound cells by melting and centrifugation, and removal of the adherent antigen by collagenase treatment. The resultant population consists mainly of viable B cells, and is greatly enriched for cells capable of binding the relevant hapten (11, 12) or of responding to hapten-protein conjugates in vitro by antibody formation (12, 13). The present paper describes in quantitative terms the reactions of such hapten-fractionated cells with haptenated, fluorescent polymeric antigens under different circumstances. A variety of binding conditions, reaction times, and tissue culture periods are used, including concentrations of antigen chosen as being either immunogenic or tolerogenic in tissue culture (14). The enriched population was found to contain a mean of 37% of detectably antigen-binding cells, making observation no more tedious than in the case of antiglobulins and unfractionated spleen cells. The chief novel observations were failure of receptor reappearance after attachment and endocytosis of tolerogenic antigen, i.e.,

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apparently irreversible modulation; continued availability of randomly distributed, unoccupied receptors in cells undergoing immunogenesis; and surface persistence of traces of antigen for many hours despite much earlier clearance of the bulk of antigen.

Materials and Methods

Mice. Specific pathogen-free, 8-to 12-wk-old (or, in a few experiments, 8-day-old) CBA/CaH/WEHI mice were used.

Antigens. 2,4-dinitrophenylated polymerized flagellin (DNP-POL)[1] was prepared as described by Feldmann (15). 4-hydroxy-3-iodo-5-nitro-phenylacetylated POL (NIP-POL) and NIP-gelatin (NIP-GEL) were prepared according to Brownstone et al. (16).

Rabbit Anti-NIP Ig (Anti-NIP) Serum. Anti-NIP serum was prepared by immunizing a rabbit with NIP-conjugated keyhole limpet hemocyanin. Anti-NIP antibodies were purified from whole serum on a NIP-bovine serum albumin-Sepharose column (kindly supplied by J. W. Goding, The Walter and Eliza Hall Institute of Medical Research). Bound antibodies were eluted with 1 M propionic acid in normal saline (17) into an equal volume of 3 M Tris-HCl buffer, pH 8.55, and promptly dialyzed against phosphate-buffered saline (PBS).

Fluorescent Reagents. Fluorescein isothiocyanate (FITC) isomer I and tetramethylrhodamine isothicyanate (TRITC) crystalline isomer R were obtained from Baltimore Biological Laboratories, Cockeysville, Md. All conjugations were carried out at room temperature for 2 h in 0.05 M carbonate/bicarbonate buffer at pH 9.5. The amount of fluorochrome required was determined empirically and varied from 10 μg/mg protein to 50 μg/mg protein. The fluorochrome was dissolved in dimethylsulphoxide as described by Bergquist and Nilsson (18). Conjugated proteins were separated from the reaction mixture on a Biogel P6 column (Bio-Rad Laboratories, Richmond, Calif.), equilibrated with PBS or with 0.01 M phosphate buffer, pH 8.0, for the anti-NIP antibodies.

FITC-conjugated (Flu) anti-NIP antibodies were further fractionated on DEAE-Sephadex equilibrated with 0.01 M phosphate buffer, pH 8.0. Antibodies were eluted with a 0–1 M NaCl gradient in the phosphate buffer. The resulting conjugates were DNP_6-POL-Flu_1 (i.e., six DNP groups and one Flu group per flagellin monomer), NIP_4-POL-TRITC $conjugated_{0.3}$ (Rho), NIP_3-GEL-Rho_2, and anti-NIP-Flu_1. Fluorescein-conjugated goat antimouse μ-chain serum (anti-μ-Flu) was obtained from Meloy Laboratories, Springfield, Va. It was absorbed with an equal volume of CBA thymus cells before use.

Isolation of Hapten-Specific Cells. DNP- and NIP-specific cells were isolated by the method of Haas and Layton (11) as modified by Nossal and Pike (13). Briefly, red blood cells and damaged cells were removed from a spleen cell suspension which was then fractionated on DNP- or NIP-GEL-coated plastic Petri dishes. Approximately 0.06–0.09% of the input cells remained bound to the dishes after 15 min of fractionation. These were recovered by melting the gel at 37°C after the unbound cells had been washed off. The bound cells were treated with collagenase to remove bound hapten-GEL.

Labeling Procedures. Hapten-specific cells (10^4–10^5 per sample) were incubated at 4°C or 37°C with antigen in HEPES-buffered Eagle's minimal essential medium (HEM) containing 10% fetal calf serum (FCS) in 0.5 ml conical based, capped plastic tubes (Kayline, Adelaide, South Australia, MCP5505C). For incubation periods of 6 h or longer, the cells were suspended in microculture medium as described by Pike (19) and cultured in Terasaki Histocompatibility Plates (Costar Histoplate; Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Alexandria, Va.) in a 10% CO_2-in-air incubator. The cells (usually in 20 μl) were washed once through 0.4 ml FCS in a 0.5 ml tube. All centrifugations were for 15 s in a Beckman 152 Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). Manipulations of cells were done via Pasteur pipettes with finely drawn out tips.

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[1] *Abbreviations used in this paper:* anti-μ, goat antimouse μ-chain serum; anti-NIP, rabbit anti-NIP Ig; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Flu, FITC conjugated; GEL, gelatin; HEM, HEPES-buffered Eagle's minimal essential medium; HSF, horse spleen ferritin; NIP, 4-hydroxy-3-iodo-5-nitro-phenylacetyl; PBS, phosphate-buffered saline; POL, polymerized flagellin; Rho, TRITC conjugated; TRITC, tetramethylrhodamine isothiocyanate.

When a second label was used, the cells were fixed in 1% paraformaldehyde in PBS for 30 min at 4°C, washed through FCS, then labeled with anti-NIP-Flu (100 μg/ml) or anti-μ-Flu (diluted 1/10) for 30 min at 4°C. Cells were washed through FCS and examined at 1,000 magnification under both phase contrast and incident light fluorescence using a Zeiss III RS fluorescence attachment (Carl Zeiss, Inc., New York). Cells were scored as negative, ± (trace), + (moderate), and ++ or +++ (strong).

Results

Comparison of Labeling of Hapten-Fractionated and Unfractionated Spleen Cells. Unfractionated spleen cells and cells fractionated on NIP-GEL dishes, representing 0.06–0.09% of the original population, were held at 4°C for 30 min with various concentrations of NIP-POL-Rho, washed twice, and examined. The results are given in Table I and Fig. 1. It is evident that high antigen concentrations labeled a considerable proportion of unfractionated spleen cells, though, in the main, only lightly. Concentrations of 10 μg/ml or lower showed labeling of only a few unfractionated spleen cells. At every concentration tested, but particularly 10 μg/ml or less, the NIP-GEL-fractionated cells gave a much higher proportion of labeling.

In these and subsequent experiments, we made use of the fact that cells with adherent NIP-POL-Rho brought their label into a cap-like distribution very quickly as cells warmed on the microscope stage. This could be prevented by fixing the cells in paraformaldehyde immediately after staining at 4°C. However, unless otherwise stated, cells were examined unfixed. Of the unfractionated cells showing fluorescence, over 60% could be inhibited by POL, i.e., were probably POL-specific rather than NIP-specific. Amongst fractionated cells, the percentage labeling with 10 μg/ml NIP-POL-Rho could be doubled by a second cycle of NIP-GEL binding (data not shown). However, as this involved considerable yield losses, it was not instituted as a routine.

TABLE I

The Labeling of Fractionated Hapten-Specific Lymphocytes by NIP-POL-Rho

	Concentration of NIP-POL-Rho/ml	Percent of cells showing fluorescence			
		Strong	Moderate	Trace	Total
Unfractionated spleen cells*	1 mg	0	2.4	4.2	6.8
	100 μg	0.7	1.6	2.6	4.9
	10 μg	0.3	0	0.3	0.5
	1 μg	0.1	0.3	0.5	0.8
	0.1 μg	0	0	0	0
NIP-GEL-binding cells‡	1 mg	36.4	16.7	15.2	68.2
	100 μg	21.8	11.5	9.0	42.3
	10 μg	14.7	9.8	10.8	35.3
	1 μg	5.1	17.2	7.1	29.3
	0.1 μg	0	3.7	7.5	11.2

* Only 17% of moderately or strongly labeled cells in the two highest concentrations showed capping, vs. 80% in the lower ones.

‡ Of moderately or strongly labeled cells, 33% in the highest concentration and 68% in the other groups showed capping.

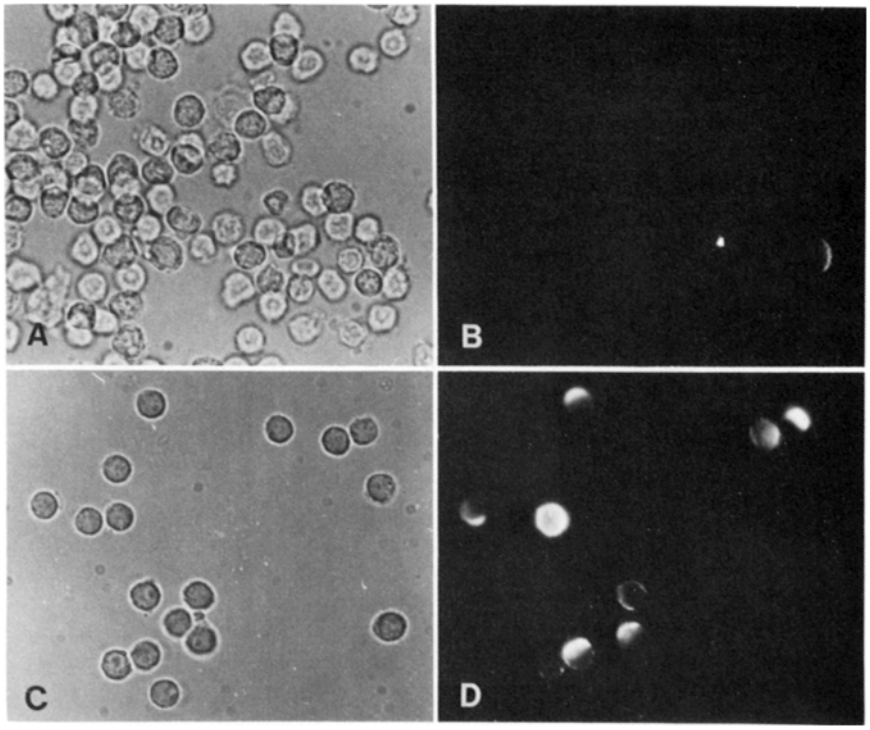

FIG. 1. A comparison of the ability of unfractionated cells (A and B) and NIP-GEL-fractionated cells (C and D) to bind NIP-POL-Rho (10 μg/ml). B and D show the number of fluorescent cells in fields A and C, respectively. × 660.

Reproducibility of the Fractionation and Labeling Procedures. Table II gives the results of 12 successive experiments in which 10 μg/ml of NIP-POL-Rho was used to label NIP-GEL-bound cells. The procedures are shown to be readily reproducible.

Labeling Percentage Stays Constant on Short-Term Tissue Culture. As most experiments to be reported involved short-term tissue culture of hapten-specific cells, it was desirable to show that such cells cultured for 24 h without antigen retained their Ig receptor coat. Table III shows that such cells showed no significant change in their capacity for NIP-POL-Rho binding.

Collagenase is Effective in Removing most Bound NIP-GEL. The fractionation procedure depends on the union of NIP-specific Ig receptors with NIP determinants on NIP-GEL. In order to subsequently study the interaction of antigen with NIP-specific lymphocytes, it is important to achieve removal of adherent NIP-GEL before commencing further studies. Accordingly, fractionated cells were studied before and after collagenase treatment for their capacity to bind purified rabbit anti-NIP antibody conjugated with fluorescein (anti-NIP-Flu). It was shown that 23% of noncollagenase-treated, fractionated cells stained with this reagent, but 0% did after collagenase treatment. When collagenase-treated cells were fixed with paraformaldehyde before staining with NIP-POL-Rho at 10 μg/ml, the fluorescence was in a ring distribution, with no capping. As one focussed up and down from the plane of the cell profile, a few fine patches

TABLE II
Repeatability of Enrichment for NIP-Binding Cells by NIP-GEL Fractionation

Exp. No.	Percent of cells showing fluorescence after 10 μg/ml NIP-POL-Rho			
	Strong	Moderate	Trace	Total
1	14.3	8.9	4.5	27.7
2	5.1	14.5	2.6	22.2
3	17.9	16.0	10.4	44.3
4	18.9	11.8	5.5	36.2
5	25.8	15.2	10.6	51.5
6	24.6	12.5	9.7	50.8
7	1.6	21.0	3.2	25.8
8	14.7	9.8	10.8	35.3
9	18.1	10.1	5.1	33.3
10	12.5	18.3	3.8	34.6
11	17.0	11.9	9.6	38.3
12	11.0	19.4	8.4	38.7
Mean ± SEM	15.1 ± 2.0	14.1 ± 1.1	7.0 ± 0.9	36.6 ± 2.6

TABLE III
The Persistence of Receptor Characteristics of Hapten-Specific Lymphocytes for 24 h in Tissue Culture

Duration of culture period	Percent of cells showing fluorescence after staining with 10 μg/ml NIP-POL-Rho			
	Strong	Moderate	Trace	Total
h				
0	12.5	18.3	3.8	34.6
24	18.6	12.4	1.6	32.6

were seen, but as this was observed to an equal extent on the few NIP-POL-Rho-binding cells from unfractionated spleens after fixation, it probably represents a limitation of the fixation technique. For all practical purposes, it appears that after collagenase treatment, the receptors on hapten-fractionated cells are randomly distributed.

The Specificity of Antigen-Binding by Hapten-GEL-Fractionated Lymphocytes. Spleen cell suspensions were fractionated on either NIP-GEL or DNP-GEL dishes and collagenase treated as usual. The specificity of their capacity to bind various antigens was tested. As Table IV shows, there was a slight one-way cross-reactivity between the two haptens, in that DNP-fractionated cells bound NIP-POL-Rho a little more frequently than vice versa. However, most of this labeling was light. NIP-GEL-fractionated cells showed little capacity to bind horse spleen ferritin (HSF)-fluorescein even at high concentration. HSF-Flu was kindly donated by Dr. D. De Luca, The Walter and Eliza Hall Institute of Research.

The nature of the binding was further investigated through binding various

TABLE IV
The Specificity of Antigen-Binding by Hapten-Fractionated Cells

Cells fractionated on:	Percent of cells showing fluorescence after:		
	10 μg/ml NIP-POL-Rho	10 μg/ml DNP-POL-Flu	500 μg/ml HSF-Flu
NIP-GEL	38.5	0.8	1.6*
DNP-GEL	4.6	26.0	ND‡

* Of the HSF-Flu-positive cells, 33% showed red fluorescence also when HSF-Flu and NIP-POL-Rho double staining was performed.
‡ ND, not done.

inhibitors onto the surface of NIP-specific cells before binding NIP-POL-Rho. The results (Table V) show that POL could not, but NIP-POL could completely, inhibit labeling, i.e., the cells were NIP specific as expected. Pretreatment with rabbit antimouse globulin serum caused 86% inhibition. While 86–94% of NIP- or DNP-GEL-binding cells regularly stain with polyvalent, fluorescein-labeled antimouse globulin reagents, there are consistently a few percent of Ig-negative cells amongst both the NIP-POL-Rho-binding and the NIP-POL-Rho-negative cells harvested from NIP-GEL dishes. These are the subject of current investigation.

Behavior of Cell Surface-Attached Antigen during Tissue Culture. In preliminary experiments, DNP- and NIP-specific cells were held with DNP-POL-Flu or NIP-POL-Rho for 5 min at 4°C, washed by micromanipulation, fixed, and examined. Under these circumstances, smooth rings of fluorescence were observed (Fig. 2 A). However, the antigens proved to be very powerful patching and capping reagents. If cells were intentionally held at 37°C for 5 min and not fixed, over half the labeled lymphocytes showed capping and the rest exhibited patched fluorescence (Fig. 2 B–D). Endocytosis was also observed within 10 min or less. This could be seen most clearly by focussing on the plane of the widest cell profile, when spots of label (presumably in pinocytic vacuoles) could be seen internal to the surface cap.

We were particularly interested in whether the early changes in receptor distribution were different with low concentrations of antigen, e.g. 0.3 μg/ml, such as those used to induce immunity, vs. high concentrations, e.g. 30 μg/ml, such as those used to induce B-cell paralysis or blockade (14). Our usual, optimally immunogenic concentration of 0.1 μg/ml (13, 19) gave fluorescence that was too faint to be readily followed for the requisite several hours, but 0.3 μg/ml of NIP-POL-Rho, mixed for 30 min at 37°C with NIP-fractionated cells, gave adequate labeling. Accordingly, cells were treated with this concentration or with 30 μg/ml, washed, a proportion examined immediately, and the rest cultured in the absence of further antigen. The results, an example of which is given in Table VI, showed that the overall sequence of events was essentially similar in the two situations, though, of course, the intensity of fluorescence was much lower in the 0.3-μg groups. There was initial rapid capping and evidence of endocytosis, followed by a progressive loss of label. In comparison with the

TABLE V

Inhibition of Binding of NIP-POL-Rho to NIP-Fractionated Cells by Various Agents

Inhibitor*	Percent of cells showing fluorescence after staining with NIP-POL-Rho at 10 μg/ml			
	Strong	Moderate	Trace	Total
None	18.1	10.1	5.1	33.3
POL, 1 mg/ml	23.5	10.8	6.9	41.2
NIP-POL, 1 mg/ml	0	0	0.4	0.4
RAMG‡, 1/5	1.0	2.4	1.4	4.8

* Cells were held first in the inhibitor for 30 min at 4°C and then in NIP-POL-Rho for 30 min at 4°C.

‡ A polyvalent rabbit antimouse globulin reagent, diluted 1:5.

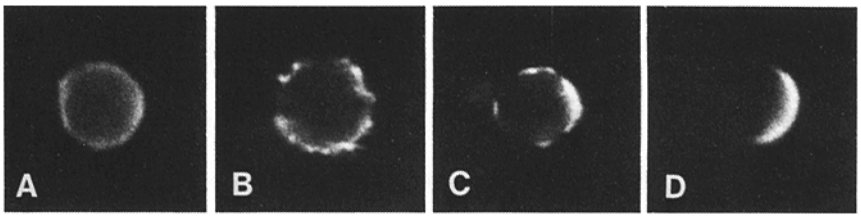

FIG. 2. An illustration of the sequence of events which rapidly occurred when NIP-specific cells, which had bound NIP-POL-Rho at 4°C, were warmed on the microscope stage: (A), a cell showing ring fluorescence; (B), a patched cell; (C), a patched cell beginning to cap; and (D), a capped cell. × 2,300

TABLE VI

The Persistence of NIP-POL-Rho on and in Hapten-Specific Lymphocytes after 30 min Preincubation at 37°C with Antigen and Further Culture without Antigen

Time of incubation without antigen	Concentration of NIP-POL-Rho during preincubation	Percent of cells showing fluorescence	Percent of fluorescent cells showing "caps"	Percent of fluorescent cells showing endocytosis*
h	*μg*			
0	0.3	27.1	68.2	54.5
	30	28.7	71.0	67.7
3	0.3	17.6	41.9	35.5
	30	20.8	48.1	29.6
24	0.3	3.8	20‡	20‡
	30	12.5	29.5	14.8

* As judged subjectively; see text and Table VII.

‡ Too few cells seen for accurate estimate.

rapid total loss of cell surface caps reported for cells labeled with antiglobulins, some capping persisted after attachment of NIP-POL-Rho. However, the picture at later time points was frequently a mixed one, with the caps lacking the clear linear definition of the earliest time points ("patched caps"). The results were consistent with some endocytosis but some residual surface adherence.

With only one marker to aid observation, it was usually difficult to be certain whether label was on the cell surface or inside the cell. Therefore, the estimates for cells showing endocytosis in Table VI are too subjective and likely to be an underestimate. Accordingly, we studied the fate of antigen by a double-label study. Cells were first incubated with NIP-POL-Rho for 30 min at 37°C, washed, then reincubated without antigen, then fixed with paraformaldehyde, and stained with anti-NIP-Flu. With this protocol, internalized antigen showed red fluorescence only, but residual cell surface antigen fluoresced under both red and green filter conditions. The results (Table VII) showed that, for as long as a reasonable number of fluorescent cells could be seen, over half of the NIP-binding cells retained some antigen in surface patches or caps. Though the proportion of labeled cells showing endocytosis was, as expected, higher with this objective aid, it was of interest that around 30% of antigen-binding cells showed no evidence of endocytosis. As these eventually lost their label (Table VI), this suggests that shedding occurs in addition to endocytosis and catabolism of attached antigen. The reason why most cells endocytosed their cell surface caps, whereas a minority did not, has not emerged.

To show that fractionated cells behave as expected with antiglobulins, a strong, well-characterized anti-μ-Flu at 1:10 dilution was attached to NIP-fractionated cells at 4°C, staining 93.8% of them. After washing, the cells were incubated at 37°C and examined at intervals. By 1 h, only 9.1% of cells and by 2 h only 6.7% of cells showed residues of their caps so clearly evident at 10 min. At this one concentration, antiglobulin was clearly shed more rapidly than NIP-POL-Rho.

Identity of Surface Receptors for Antigen on NIP-GEL-Fractionated Cells. Raff et al. (6) have already reported that a single antigen can cap all the detectable surface Ig on a given antigen-binding lymphocyte. The availability of the present enriched population of cells allowed us to check this finding with a much larger number of antigen-binding cells. Experiments were performed in which NIP-fractionated cells were first incubated for 15 min at 37°C in 30 μg/ml of NIP-POL-Rho, then washed, fixed in paraformaldehyde, and stained for 20 min at 4°C with a 1:10 dilution of anti-μ-Flu. This reagent was preferred to a polyvalent antiglobulin to avoid the possibility of staining loosely adsorbed IgG. The results are given in Table VIII. As usual, about two-thirds of the antigen-binding cells gave typical "caps," and of these 73.3% had aggregated all detectable surface Ig into the cap. 10% failed to stain with the anti-μ reagent, either because there was insufficient surface IgM on those cells, or conceivably because of steric hindrance by attached antigen. In only 16.7% of cases was there some green fluorescence remaining in a linear ring form in addition to the more intense green fluorescence over the cap.

A similar study was performed on DNP-fractionated cells with DNP-POL-Flu and a rhodaminated sheep antimouse globulin reagent. The DNP-POL-Flu was

TABLE VII
The Persistence of Cell Surface-Bound NIP-POL-Rho as Judged by Double Labeling with Anti-NIP-Flu‡*

Time of incubation without antigen	Percent of red fluorescent cells with residual surface antigen (green fluorescence)	Percent of red fluorescent cells with some internalized antigen
h		
0	76.0	72.0
2	66.7	73.3
4	72.2	61.1
6	53.3	66.7

* Cells were preincubated with 0.3 μg/ml of NIP-POL-Rho for 30 min at 37°C.
‡ After incubation without further antigen, cells were fixed in 1% paraformaldehyde and stained with a purified anti-NIP antibody that had been fluoresceinated.

TABLE VIII
*The Capacity of Specific Antigen to Cap all Detectable Ig Receptors on the Surface of Hapten-Specific B Lymphocytes**

Percent of cells showing red fluorescence:	35.0
Percent of red fluorescent cells showing a red "cap":	64.3
Percent of cells showing green fluorescence:	87.5
Percent of red caps showing identical green caps:	73.3
Percent of red caps showing residual green ring fluorescence:	16.7
Percent of red caps negative for green fluorescence:	10.0

* Cells were incubated for 15 min at 37°C in 30 μg/ml of NIP-POL-Rho, washed, fixed in 1% paraformaldehyde, stained for 20 min at 4°C with anti-μ-Flu, washed, and examined.

an even better capping reagent than NIP-POL-Rho. The study showed that, at high concentrations of DNP-POL-Flu, 89% of antigen-binding cells showed all the surface Ig in a cap, with a linear residuum in only 11% of cases (omitting the few cells not staining with anti-μ); at intermediate concentration, an Ig ring was left in 48% of cases, whereas at low concentrations of antigen, though the antigen still capped the receptors to which it attached, some linear ring Ig was left in 91% of cases. In other words, the question of whether all the Ig on the cell surface moves into a cap depends critically on the antigen concentration used to induce capping.

The Fate of Antigen when Continuously Present in Culture. So far we have discussed the fate of antigen presented to hapten-specific cells as a short pulse. In most situations of immunization in vitro, antigen is continuously available to the responding lymphocytes. Accordingly, we addressed this more complex question by incubating NIP-fractionated cells and NIP-POL-Rho together for various periods at putatively immunogenic and tolerogenic (14, 15) concentrations. Under these circumstances, the staining pattern represents a synthesis of preceding events, i.e. of both the initial wave of receptor redistribution and

endocytosis, and any further cycles that may have occurred during culture, with or without intervening receptor resynthesis. Some typical results are presented in Table IX. With the lower antigen concentration, the proportion of antigen-binding cells fell after 24 h incubation. In both the groups, the percentage of cells showing caps fell, and those showing label only in internalized patches rose until such cells were in the majority by 24 h. In fact, the overall results were surprisingly similar to those where a pulse of antigen had been given, except that the proportions of labeled cells at 24 h were higher. However, at 24 h the labeling was light and patchy even with the higher antigen concentration, and many cells had only internal patches and no surface label.

There seemed to be three possible explanations for the paucity of visible cell surface labeling at 24 h. The first is that the initial phase of receptor ingestion was not followed by a phase of resynthesis and redisplay. The second is that the extracellular rhodaminated reagent was progressively consumed or degraded in the cultures. The third is that receptor resynthesis did occur, only to be followed by virtually immediate antigen binding and pinocytosis, so that the concentration at the cell surface at any one time was low. Further experiments were performed to test these possibilities.

In the first instance, nonlabeled NIP-POL was used as the continously present antigen, again at either 0.3 or 30 μg/ml. Then, to test the receptor status of NIP-specific cells at various times, the cells were washed and exposed for 10 min at 37°C to NIP-POL-Rho. The results are given in Table X. They show that with the lower antigen concentration, there was a transient and partial reduction in the receptor coat, with a complete reappearance by 24 h. With the higher antigen concentration continuously present, labeling was extremely faint at all time points, indicating either site occupancy of both original and re-expressed receptors, or a failure of receptor resynthesis.

The intact receptor coat at 24 h in cells held with immunogenic NIP-POL contrasted with the relative paucity of persistent cell surface labeling in cells held for 24 h with a similar concentration of NIP-POL-Rho (Table IX). One possibility was a consumption of NIP-POL-Rho during 24 h of culture with NIP-specific cells, thus leaving the supernate reduced in available labeling reagent. This was investigated by exposing fresh, fractionated cells to supernates from cultures of fractionated cells and NIP-POL-Rho. The capacity to stain the new NIP-specific cells was indeed found to be significantly reduced.

Further results on Table X warrant comment. Both the low and the high concentrations of antigen caused obvious blast transformation within 24 h. The low concentration caused blasts to appear which were mainly antigen binding, i.e., presumably it predominantly stimulated the original antigen-binding subset in the population. The high concentration caused blastogenesis of equivalent degree, but not specifically amongst antigen-binding cells, presumably because at this concentration NIP-POL is a polyclonal B-cell activator. Furthermore, the results did not suggest a "freezing" of residual receptors in any of the groups. Whereas the percentage of caps was somewhat lower at 24 h than at earlier time points, the labeling was certainly not linear, but rather of a particularly patchy nature.

A further experiment was performed in which cells were exposed for 24 h to 30

TABLE IX

The Localization of Antigen on and in Hapten-Specific Lymphocytes in the Continuous Presence of NIP-POL-Rho

Time of incubation with antigen	Concentration of NIP-POL-Rho/ml	Percent of cells with red fluorescence	Percent of fluorescent cells showing:		
			Some capping	Some endocytosis	Only internal label
	μg				
0*	0.3	18.5	Not recorded	0	0
	30	38.8	48.3	0	0
15 min	0.3	24.5	78.5	42.9	7.1
	30	50.6	79.4	10.6	0
3 h	0.3	19.0	31.8	86.4	50.0
	30	33.8	47.1	62.7	13.7
24 h	0.3	11.4	0	85.7	71.4
	30	44.3	21.5	70.3	54.1

* Cells stained at 4°C for 30 min and not incubated.

TABLE X

*The Labeling of Hapten-Specific Lymphocytes by NIP-POL-Rho after Preincubation of Cells in NIP-POL**

Duration of preincubation in unlabeled antigen	Concentration of unlabeled antigen	Concentration of NIP-POL-Rho	Percent of cells showing fluorescence‡	Percent of fluorescent cells showing capping
h	*μg/ml*	*μg/ml*		
0	0	10	30.6	53.6
		30	39.2	50.0
1	0.3	10	24.3	76.0
	30	30	12.8	44.4
3	0.3	10	17.3	69.2
	30	30	12.7	46.7
24	0.3	10	43.2§, ‖	40.6
	30	30	21.7§, ¶	23.5

* Cells were incubated in microcultures at 10^5 cells/10 μl with unlabeled NIP-POL. They were then washed and incubated for 10 min at 37°C with NIP-POL-Rho.

‡ The average intensity of fluorescence in all groups preincubated with 30 μg of NIP-POL was considerably lower than in controls. At the 1 and 3 h points in the 0.3 μg groups, labeling was also faint.

§ Labeling very patchy; particularly in noncapped cells.

‖ Blast transformation 43%; 74.3% of large cells and 19.6% of small cells labeled.

¶ Blast transformation 43%; 23.1% of large cells and 20.6% of small cells labeled.

μg/ml of NIP-POL-Rho and either examined or re-exposed to fresh NIP-POL-Rho at 30 μg/ml. There was virtually no difference in labeling between the two samples, both showing faint and patchy labeling as in Table IX.

Taking the results of Tables IX and X together, it appears that the immunogenic antigen concentration never completely cleared the membrane of unoccupied Ig receptors, whereas the tolerogenic concentration cleared most receptors from the surface by patching, capping, and endocytosis, later antigen-binding depending entirely on resynthesis. The relatively faint labeling at 24 h in the continuous presence of 30 μg/ml of NIP-POL-Rho suggested that resynthesis may not have been very extensive. Therefore, the capacity of short-term exposure of cells to antigen to modulate receptors was tested more directly.

Modulation of NIP Receptors after 2 h Incubation with NIP-POL and 20 h Culture without Antigen. Table XI shows that brief incubation with antigen at high concentration, followed by culture without antigen caused a severe reduction in both the proportion of fluorescent cells and their median intensity of staining. As in Table X, the immunogenic antigen concentration caused more blastogenesis amongst antigen-binding cells, though to a lesser degree than continuously present antigen. This was less evident for the high antigen concentration. There was a marginal reduction of labeling amongst cells briefly incubated with the immunogenic concentration. This may have reflected incomplete recovery from the partial receptor reduction which the brief incubation would have occasioned (cf. Table X) or may have represented chance variation. Finally, in neither case did antigen exposure at these concentrations "freeze" the membrane, as shown by the percentages of capped cells.

Next, a dose-response study, covering the range 0.3–100 μg/ml of NIP-POL in 0.5 $\log_{10}$ steps, was performed using the above modulation protocol. This showed that 10 μg/ml did not cause significant reduction in NIP-POL-Rho binding at 24 h, and that 100 μg/ml caused an even more profound reduction than 30 μg/ml.

To determine whether the apparent nonreversed modulation could have been due to persistent, surface-adherent antigen, cells that had been modulated as above by 30 μg/ml NIP-POL were examined at 24 h by anti-NIP-Flu. Some light, patchy fluorescence was noted, but not sufficient to make persistent site occupancy an adequate explanation for the results.

Behavior of Spleen Cells from 8-Day-Old Mice. It has recently been reported (20, 21) that treatment of spleen cells from immature mice with anti-Ig antibodies is followed by a disappearance of lymphocyte surface Ig and a failure of its reappearance. It seemed of interest to test the reaction of NIP-GEL-fractionated cells from immature mice for modulation by antigen. Accordingly, spleen cells from mice 8 days old were studied. It was found that 23% of unfractionated cells were fluorescent when stained with anti-μ-Flu. When fractionation on NIP-GEL dishes was performed, about 28,000 bound cells were recovered from 10^8 cells fractionated, or approximately a third of the proportion obtained with adult spleen. These were incubated with or without NIP-POL for 2 h at 37°C, washed, and reincubated without antigen for 20 h. Results are given in Table XII.

The first noteworthy point is that this population labeled significantly better than did equivalent adult cells, and gave a higher percentage of capping. Secondly, the immunogenic concentration of antigen caused no reduction in

TABLE XI
The Modulation of Receptors on Hapten-Specific Lymphocytes after Brief Incubation with NIP-POL

Concentration of NIP-POL/ml during preincubation*	Percent of cells showing fluorescence after staining with 10 μg/ml NIP-POL-Rho			
	Strong	Moderate	Trace	Total
μg				
0	17.5	16.7	7.1	41.3
0.3‡	8.9	14.3	8.0	31.3
30.0§	0.8	5.7	6.6	13.1

* Cells were incubated for 2 h at 37°C with unlabeled antigen, washed twice, recultured for 20 h without antigen, and then stained with NIP-POL-Rho.
‡ 14% blastogenesis; 74% of blasts labeled; and 61% of labeled cells capped.
§ 16% blastogenesis; 32% of blasts labeled; and 50% of labeled cells capped.

TABLE XII
The Behavior of Receptors on Hapten-Specific Lymphocytes from 8-Day-Old Mice

Concentration of NIP-POL/ml during preincubation	Incubated before staining	Percent of cells showing fluorescence after staining with 10 μg/ml NIP-POL-Rho				Percent fluorescent cells showing caps
		Strong	Moderate	Trace	Total	
μg						
0	No	14.2	24.8	15.9	54.9	69.8
0	Yes	25.7	21.9	10.5	58.1	90.0
0.3	Yes	29.0	20.6	8.4	57.9	88.5
30	Yes	4.0	13.5	8.7	26.2	60.4
100	Yes	1.1	2.2	1.1	4.5	75.0

receptors available at 22 h. In contrast, the higher antigen concentration caused profound, nonreversed modulation.

Discussion

The simple Haas and Layton fractionation procedure (11) yielded spleen cell suspensions consisting of 90% B lymphocytes and 37 ± 3% hapten-binding cells. This availability of enriched cell populations rendered practicable a range of studies on antigen-induced receptor movement which would have been very difficult without fractionation. Using the fluorescent polymeric antigens NIP-POL-Rho and DNP-POL-Flu, four basic experimental designs were followed, namely: (*a*) brief labeling of enriched cells with fluorescent antigen followed by incubation without further antigen (pulse design); (*b*) continuous labeling with fluorescent antigen during varying incubation periods (continuous labeling design); (*c*) incubation with nonlabeled antigen for various periods followed by brief labeling with fluorescent antigen (receptor status design); and (*d*) incubation with unlabeled antigen for 2 h, followed by 20 h incubation in the absence of

further antigen, and brief labeling with fluorescent antigen (modulation design). Furthermore, studies were performed with putatively immunogenic or tolerogenic concentrations of antigen. From this combination of approaches, an overview of receptor behavior during early stages of immunogenesis or paralysis has been obtained. Simple inspection of antigen-labeled cells has been aided by the use of a fluorescent anti-NIP reagent to discriminate between cell surface and endocytosed label, and fluorescent antiglobulin reagents to determine the position of receptors unoccupied by antigen.

The pulse studies showed a sequence of events that might have been predicted from the highly polyvalent nature of the antigen, and also showed some significant differences from events after attachment of antiglobulin. With either low or high antigen concentrations, patching and capping of Ig receptors and partial endocytosis of antigen commenced within a few minutes. However, some cell surface antigen persisted for at least 6 h in the majority of cells in residual patches and caps, showing that the cells were less able to clear their surface of this polymer than of divalent antiglobulin molecules which disappeared from the cell surface in less than 1 h. While cells initially exposed to the high concentration of antigen obviously bound much more and became nonfluorescent more slowly, the sequence of events was essentially similar to that after binding of small amounts of antigen. By 24 h most cells had lost all detectable antigen. The pulse studies also revealed that the surface Ig of most cells was probably homogeneous in terms of binding specificity, as when the cells were labeled at high concentration of fluorescent antigen, all stainable surface Ig was pulled into the cap in many cases. However, at low concentration of extracellular antigen, caps of Ig and antigen formed that contained only a minority of the total surface Ig and left a residual population of unoccupied, randomly distributed receptors. It seems likely that the proportion of Ig capped within a given time depends on the concentration of extracellular antigen and the affinity of the surface receptors.

Interpretation of the continuous-labeling design was rendered difficult by the fact that during culture, some of the fluorescent antigen was removed from the extracellular fluid by adherence to cells and endocytosis, and probably by enzymic degradation. Some cells maintained surface caps for 24 h, but most exhibited only patchy external label together with endocytosed material, or, at later times, endocytosed material only.

The receptor status design experiments were the first to show substantial differences between the low and high antigen concentrations. They revealed a massive fall in the capacity of cells exposed to high concentrations of antigen to bind further antigen. This design did not exclude the possibility that the fall was due to prior occupancy of large numbers of surface sites by unlabeled antigen. However, such a possibility was negated by the results of the continuous-labeling design, as surface labeling in the continuous presence of 30 μg/ml NIP-POL-Rho was light and unimpressive.

The results strongly suggested that the immunogenic antigen concentration never entirely cleared the membrane of Ig receptors; after a transient reduction in numbers, these must have been resynthesized at a sufficient rate at least to replace losses from patching and endocytosis. In contrast, tolerogenic concentra-

tions not only nearly saturated the available antigen-binding sites (Table X) but led to a dynamic equilibrium situation where, at 24 h, cells showed few occupied (Table IX) or unoccupied (Table X) surface Ig receptors.

The modulation design revealed that tolerogenic concentrations may actually have depressed receptor resynthesis. After only 2 h exposure to high (but not to low) antigen concentrations, and 20 h culture without antigen, the lymphocytes were left virtually bereft of available Ig receptors. The pulse design clearly showed that this could not have been due to residual surface site occupancy by unlabeled antigen, and the anti-NIP-Flu studies confirmed this. In contrast, low antigen concentrations did not completely remove the Ig receptor coat and allowed the persistence of plentiful unoccupied receptors. The continued availability of free, unoccupied surface receptors for antigen at all stages of putative immunogenesis is of interest, particularly as significant triggering can be obtained by amounts of antigen 30-fold lower than the 0.3 μg/ml used as our lowest concentration. In this context, it is of interest that hormones can exert their maximal biological effects on cells while binding to only a minority of available receptors (22, 23). In our case immunogenic concentrations cap the receptors to which they attach but leave some of the cells' Ig unoccupied and in a random distribution. The purpose of this apparent excess of surface Ig remains problematical.

The results differ in some respects from those of Ault et al. (10), who studied interaction of B cells with another strong tolerogen, DNP-poly(D-glutamic acid-D-lysine). They found impaired capacity for interiorization and prolonged extracellular persistence. As with NIP-POL, however, the net result was an absence of available new unoccupied receptors for antigen. Our findings do not support the idea that tolerogenic antigen concentrations lead to the "freezing" of the Ig receptors on the membrane (5). We found that for those few unoccupied cell surface receptors which we could detect at 24 h after the high antigen concentration in either the receptor status or modulation designs, patching occurred rapidly after exposure to new labeled antigen and capping sometimes occurred. In the continuous-labeling studies, a minority of cells also showed continuing caps, though most showed extensive endocytosis. We suggest that the absence of caps after incubation of lymphocytes with tolerogenic concentrations of ^{3}H-POL noted by Diener and Paetkau (5), using a continuous-labeling design, may also have reflected endocytosis and modulation of receptors rather than immobilization.

We have difficulty in keeping microcultures with so few cells alive long-term without the use of "filler" cells, so we have not measured the duration of modulation in this system. If it is long lasting, it could well be the basis of the tolerance observed in vitro after high concentrations of POL (24) or hapten-POL (14). It will also be of interest to determine whether antigen-antibody complexes, capable of in vitro tolerogenesis at much lower concentrations (25), succeed in modulating Ig receptors on hapten-specific cells.

The failure of re-expression of receptors after exposure to high antigen concentrations resembles an effect obtained when immature but not mature B lymphocytes are exposed transiently to antiglobulins (20, 21). Accordingly, it was of some interest to examine hapten-fractionated B cells from 8-day-old mice. Apart from appearing to contain fewer contaminating, nonantigen-binding cells, and

showing uniformly strong capping behavior, these cells behaved like adult cells. It is possible that the hapten-fractionation procedure selects out a relatively mature subset of the 8-day spleen cells, thus militating against a special susceptibility for modulation. This question and other qualities of B cells from suckling mice are under investigation.

While these studies suggest that modulation of B-cell Ig receptors through exposure to high concentrations of polymeric antigens may be one causative mechanism for B-cell tolerance, much further work remains to be done before this can be accepted as a unifying hypothesis. For example, as yet we know nothing of the receptor events occurring in immature B cells undergoing "clonal abortion" tolerogenesis (26) by oligovalent antigens. A noncapping ligand, namely the F $(ab)_1$ fragment of antiglobulin, can irreversibly modulate receptors of immature B cells (20). If the targets for clonal abortion could be isolated by some development of the present technology, it would be of interest to study the interaction of their emerging receptors with NIP_1-human gamma globulin or similar tolerogens.

The suggestion has been made (27) that patching of surface receptors is involved in lymphocyte triggering. The present experiments have added to the accumulating evidence that patching is not a sufficient condition for B-cell activation, as both immunogenic and tolerogenic concentrations initiated patching. Furthermore, preliminary experiments (data not shown) with nonimmunogenic conjugates such as NIP-GEL-Flu have shown that these also cause rapid patching and capping. Other studies,[2] using microcultures of single clones of hapten-fractionated cells (13), have revealed that binding of small amounts of antigen to the cell surface at a single point in time induces only suboptimal immunogenesis. Clearly, the molecular biology of B-lymphocyte triggering involves complex processes and a longer time dimension than is afforded by the present studies of receptor movement. Nevertheless, a description of these events under putatively immunogenic conditions is useful for the development of a conceptual framework. Therefore, studies similar to the above should be initiated for other immunogens and, particularly, for T-dependent systems of B-lymphocyte activation.

Summary

Mouse spleen cells were subjected to a fractionation procedure designed to enrich for 4-hydroxy-3-iodo-5-nitro-phenylacetyl (NIP)- or DNP-specific B lymphocytes, which depended on adherence of specific cells to a layer of hapten-gelatin at 4°C, recovery of bound cells by melting, and digestion of adherent antigen by collagenase. A population of cells resulted which contained 90% typical B cells and 37% of cells capable of binding a fluorescent, haptenated polymeric protein.

Fractionated cells were reacted in vitro with fluorescent conjugates of the specific haptens with polymerized flagellin [NIP-polymerized flagellin (POL)-tetramethylrhodamine isothiocyanate conjugate or DNP-POL-fluorescein isothiocyanate conjugate] under a variety of conditions, with the aim of investigating the behavior of Ig receptors on B lymphocytes after exposure to antigen. Experi-

[2] Pike, B. L., and G. J. V. Nossal. Manuscript in preparation.

ments were performed with immunogenic and tolerogenic concentrations of antigen. Furthermore, four experimental designs were used, namely: (*a*) brief labeling with fluorescent antigen followed by culture without antigen (pulse design); (*b*) culture in the continuous presence of fluorescent antigen (continuous-labeling design); (*c*) culture in the continuous presence of nonlabeled antigen followed by labeling of unoccupied receptors by fluorescent antigen (receptor status design); and (*d*) culture with nonlabeled antigen for 2 h followed by incubation without further antigen for 20 h and labeling with fluorescent antigen (modulation design). Further insight into receptor occupancy and distribution was gained by the use of fluorescent antihapten and antiglobulin reagents.

It was found that both immunogenic and tolerogenic antigen concentrations caused rapid patching and capping of the receptors to which they attached, followed by endocytosis and probably some shedding of Ig receptors. However, a proportion of cells continued to bear some cell surface antigen for 24 h. The immunogenic antigen concentration failed to completely remove the receptor coat from the cell surface. At all stages of immunogenesis, plentiful unoccupied receptors could be demonstrated. The tolerogenic concentration nearly saturated available receptors, and in its continuous presence, only few unoccupied or antigen-occupied surface receptors could be detected after 24 h of culture. Experiments of the modulation design showed that brief incubation with the tolerogenic concentration appeared to suppress receptor resynthesis, as few new receptors could be demonstrated after 20 h of further culture without antigen.

Experiments were performed to determine whether fractionated cells prepared from spleens of 8-day-old mice showed an unusual tendency for modulation, even with immunogenic antigen concentrations. They were found to behave essentially like adult fractionated cells. The results are discussed in the framework of current theories of B-lymphocyte activation and tolerization.

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