EVENTS AFTER THE BINDING OF ANTIGEN TO LYMPHOCYTES

Removal and Regeneration of the Antigen Receptor*

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B lymphocytes express on their surface about 10^5 molecules of immunoglobulin (Ig) that serve as receptors for antigen (1). It has been possible to study extensively the events after binding of anti-Ig antibody to this surface Ig. Part of the interest in such experiments results from the hope that by studying B cells in general it might be possible to infer something about the behavior of the clonally selected antigen-binding B cells. Thus, we now know that binding of anti-Ig antibody to B cells is followed by redistribution of the surface anti-Ig Ig complexes, endocytosis, shedding of complexes, degradation of the ligand (2), and as described most recently, the triggering of cell movement (3). Indeed, the study of these events that follow the binding of anti-Ig has contributed greatly to our knowledge of the structure and function of the lymphocyte membrane. The mobility of the surface macromolecules, as vividly demonstrated by the capping phenomenon, has been the most striking conclusion from such studies. In addition, the ability of the lymphocyte to endocytose and degrade the bound antibody has raised important questions concerning the way in which a B cell might participate in the handling of antigen. Since such speculations are based largely on information derived from studies using anti-Ig, it is important to investigate further the events after the binding of antigens to their specific binding cells.

Binding of antigens to lymphocytes has been demonstrated in several ways. In the case of most antigens, even those with multiple antigenic determinants, the proportion of lymphocytes in any selected population which bind is less than 1%; moreover, in the case of small antigens with a limited number of determinants, it is usually on the order of 0.01–0.1% (4, 5). Thus, although the biological function of these few specific lymphocytes is relatively easy to demonstrate both by specific depletion on antigen-coated columns (6) and by deletion using radioactive antigen in "suicide" experiments (7), it has been difficult to directly evaluate their requirements for binding antigen and the events which follow such binding.

In these studies we have further explored the behavior of Ig on B cells by quantitating the turnover and subsequent reappearance of surface Ig after

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binding antibody as well as by studying the dynamics of the turnover of the receptors after binding to antigen. We have been able to show that in most cases binding of antigen to the receptors causes the prompt loss and then later reappearance of the receptor with about the same time course as that after binding by anti-Ig antibody. The conclusion is that the lymphocyte possesses a remarkable ability to rapidly clear its surface of bound antigen and then to regenerate its receptors.

Materials and Methods

Cells.—Splenic lymphocytes from A/St mice (West Seneca Laboratories, Buffalo, N. Y.) were used in all experiments. The lymphocytes were collected from teased spleens and purified in Ficoll-Hypaque gradients as previously described (8).

The mice had been immunized at least 1 mo before with dinitrophenyl- (DNP)[1] conjugated keyhole limpet hemocyanin (KLH) 100 μg intraperitoneally in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). The mice were boosted 1–2 wk before use with 100 μg of DNP-KLH in incomplete Freund's adjuvant or in some cases with 50 μg of DNP-KLH in saline intraperitoneally.

Antigens and Antibodies.—Antigens used were guinea pig albumin (GPA) obtained from Pentex, Inc., Kankakee, Ill; mouse serum albumin (MSA), mouse gamma globulin (Cohn fraction II) (MGG), both from Miles Laboratories, Kankakee, Ill.; and KLH prepared from the hemolymph of the keyhole limpet (*Megathura crenulata*) by ultracentrifugation and stored under sterile conditions in 3% NaCl. These proteins were conjugated to DNP by standard procedures. The DNP-KLH used for immunization had 15 DNP residues/100,000 mol wt. The molar ratios of DNP used for the other conjugates are indicated below. DNP-lysine was obtained from Sigma Chemical Co., St. Louis, Mo. Antigens were radioiodinated using the lactoperoxidase method of Marchalonis (9) with ^{125}I (New England Nuclear Corp., Boston, Mass.) to a specific activity of 10–30 μCi/μg.

Also used in these experiments was a polyvalent rabbit antimouse immunoglobulin antibody (anti-Ig) both as a DEAE gamma globulin fraction and as a fluorescein-conjugated label. The specificity of this antibody has been described before (10).

Immunocytochemical Procedures.—For immunofluorescence, 5×10^6 lymphocytes were incubated with 50 μg of a fluorescent anti-Ig (fluorescein to protein molar ratio of 4) in a total volume of 100 μl at 4°C for 30 min, then washed three times, and examined in the fluorescence microscope (10).

Surface Ig was quantitated by an antigen inhibition test using mouse Fab as antigen. Details of the procedure have been given before (1, 11). Results are expressed as ng of Ig/10^7 cells.

Autoradiography.—Autoradiography was carried out by incubating 10^7 cells for 30 min at 1–4°C with 0.5–1 μg of the radioactive antigen in 200 μl of medium consisting of 5% fetal calf serum (FCS) (Associated Biomedic Systems, Inc., Buffalo, N. Y.) in Hank's balanced salt solution (Microbiological Associates, Inc., Bethesda, Md.) with 10 mM N-2-hydroxyethylpiperazone-N-2 ethane sulfonic acid (HEPES) (Microbiological Associates). In the case of labeling with [^{125}I]DNP-GPA, a similar medium containing 5% GPA instead of FCS was used. After incubation with the antigen, the cells were washed three times in medium, then placed on top of a 1-ml layer of 100% FCS and centrifuged through it (300 *g* for 8-min). They were then washed again in medium with 5% FCS, and finally resuspended in about

[1] *Abbreviations used in this paper:* DNP, dinitrophenyl; FCS, fetal calf serum; GPA, guinea pig albumin; HEPES, N-2-hydroxyethylpiperazone-N-2 ethane sulfonic acid; KLH, keyhole limpet hemocyanin; MGG, mouse gamma globulin; MSA, mouse serum albumin.

40 μl. Samples of 10 μl each were smeared onto acid-cleaned microscope slides previously coated with 1% gelatin. The slides were air-dried, fixed in methanol, coated with Kodak NTB-2 emulsion (Eastman Kodak, Inc., Rochester, N. Y.), and exposed for 7–14 days. Slides were developed in Kodak D-19 developer and stained with Giemsa stain. The slides were scanned at 200 and 400 × and only intact lymphocytes having ten or more grains overlying them were scored as positive. Under these conditions, the number of background grains was negligible and macrophages were not significantly labeled. In control experiments, the removal of cells adherent to a culture dish did not change the proportion of antigen-binding cells, despite the reduction of macrophages by tenfold. From 30,000 to 100,000 cells were examined on each slide, the number being estimated from the mean count in 10–20 random fields. Counts were compared using a Chi-square test modified for a Poisson distribution (12). The results are reported as the proportion of positive cells/100,000 small lymphocytes plus or minus the standard deviation based on Poisson statistics. The P values are reported only where important differences depend on them.

Lymphocyte Cultures.—In many experiments, cells were placed into culture before or after exposure to antigen. For incubations of less than 4 h, this was done in the above-mentioned medium in a 37°C water bath. For longer incubations, the cells were placed into minimal essential medium (Microbiological Associates) containing 0.15% $NaHCO_3$ and 10% FCS, as well as penicillin and streptomycin, 50 U of each/ml. The cells were cultured at 10^7/ml with 4 ml placed in a 60 × 15 mm plastic petri dish (Falcon Plastics, Division of Bio Quest, Oxnard, Calif.) and rocked in an atmosphere of 7% O_2, 10% CO_2, saturated water, and the balance nitrogen. Thus, the cultures were done under conditions corresponding to those for an in vitro immune response as described by Mishell and Dutton (13).

Experimental Design.—The majority of experiments took one of two forms. In the first type, the lymphocytes were initially incubated with labeled antigen, washed, then after culture for various times, processed for autoradiography. This experiment gave information on the persistence of label on or in the lymphocyte. In the second type, the cells were initially incubated with antigen or anti-Ig antibody, washed, cultured, then labeled with radioactive antigen and processed for autoradiography. This experiment indicated whether there were functional receptors present after the first exposure to the ligands. In most experiments in which the cells were incubated with unlabeled or labeled antigen, or anti-Ig antibody, this incubation was done for ½ h on ice as described above, the cells washed three times, then placed into the culture medium.

After overnight culture, dead cells were removed by Ficoll-Hypaque gradient. This greatly facilitated accurate counting of antigen-binding cells. Significant bias of the results by thus selecting the population of lymphocytes was ruled out in experiments to be described. In cultures of less than 18 h, such repurification was not necessary.

RESULTS

Reappearance of Surface Ig.—Binding of anti-Ig to surface Ig of the B lymphocyte leads to a rapid redistribution and endocytosis of the immune complex. We were interested in establishing the time of reappearance of surface Ig in culture after treatment with anti-Ig. This was done both by the technique of fluorescent labeling using a fluorescein-conjugated anti-Ig to detect cells bearing surface Ig and by the quantitative inhibition technique to measure the total amount of surface Ig as a function of time.

The results of studies on the time course of the reappearance of surface Ig after treatment with anti-Ig are shown in Table I. The assay for quantitation of surface Ig gives results which vary slightly from experiment to experiment

TABLE I

Regeneration of Surface Immunoglobulin After Treatment with Anti-Ig

Exp.	Treatment	Incubation	Ig-positive cells	Surface Ig
		h	%	*ng/10^7 cells*
I	None (three washes)	—	ND	97·4
	Nine washes	—	ND	85·9
II	NRG	2	46	ND
	NRG	6	45	ND
	NRG	24	42	ND
	NRG	48	40	ND
	Anti-Ig	2	6	ND
	Anti-Ig	6	33	ND
	Anti-Ig	24	46	ND
	Anti-Ig	48	43	ND
III	None	24	45	60.0
	Anti-Ig	2	10	29.5
	Anti-Ig	24	46	52.5
	Anti-Ig	48	52	49.6
	Anti-Ig-24 h-anti-Ig	24	48	56.2

Cells were treated for ½ h with medium alone, normal rabbit gamma globulin (NRG), or anti-Ig (5 mg for 2×10^8 cells), then washed and placed into culture as described. After the indicated period of incubation, the cells were reacted with fluorescein-conjugated anti-Ig (50 μg for 10^7 cells), and counted for percent of Ig-positive cells or they were used in the quantitative assay for surface Ig or both. In the last example, the cells were recovered after 24 h of culture, treated again with anti-Ig, and recultured. ND, not done.

but, as shown in exp. I, the result was independent of extensive washing of the cells. In exp. II, the reappearance of Ig-positive cells, as determined by fluorescent labeling, is shown. This phenomenon had been described previously (14, 15). First, it should be noted that after exposure to normal rabbit Ig the proportion of Ig-positive cells is not changed by prolonged culture at 37°C. Thus, although cells are lost in these cultures, it seems that T and B cells are lost in approximately equal proportions. After exposure to anti-Ig, the number of labeled lymphocytes at 2 h is small (about 15% of B cells); at 6 h, there is a significant rise in the number of labeled cells. At this time, about 80% of the B cells have re-expressed surface Ig. In exp. III, it can be seen that the surface Ig as quantitated by this method is nearly completely replaced at 24 h of culture and that this recovered Ig is stable in culture for an additional 24 h. Treatment of cells with anti-Ig on two successive days does not decrease the ability of the cells to recover their surface Ig (Table I, exp. III). It is usually found that the degree of loss of fluorescent-positive cells is greater than the loss of total surface Ig by the quantitative method. This probably reflects differences in the sensitivities of the two methods. It is possible that those cells which

remain Ig positive belong to a subclass of cells which display more Ig on their surface.

Having established both quantitatively and qualitatively that there is a profound loss of surface Ig after treatment with anti-Ig and that there is essentially complete recovery of surface Ig within 24 h of incubation under these conditions, we turned to investigate the behavior of the antigen receptor after binding specific antigen.

Specificity of Antigen Binding.—Experiments were done to establish the specificity of the antigen-binding assay that we were using. The binding of [^{125}I]DNP$_{12}$GPA in the presence of unlabeled excess GPA was inhibited by DNP-lysine (at concentrations of 10^{-3}–10^{-5} M). Since 10^{-3} M DNP-lysine reduced binding to undetectable levels, this inhibition was used as a criterion of specificity; and in subsequent experiments, controls were run in its presence. In most cases, no labeling was detected. Because of this total inhibition by free hapten, it seemed very likely that the majority of the binding cells detected in this assay were specific for the DNP determinant rather than the GPA carrier or new determinants resulting from the conjugation of DNP to the GPA. It must be recognized, however, that by thus arbitrarily defining DNP-binding cells as those which are inhibited by 10^{-3} M DNP-lysine an unknown number of low-affinity cells are not being counted. The same type of selection is made when one fixes a particular dose of antigen to use in such binding studies. Although we did not study it systematically, it was clear that using larger doses of antigen yielded more binding cells. This observation has been made by others (16). The doses used in these experiments were chosen by trial and error to give clearly positive cells with little background radioactivity and without so heavily labeling the cells that their morphology was obscured.

As shown in Table II, the binding of [^{125}I]DNP$_{12}$GPA was totally inhibited by pretreatment with anti-Ig and was only slightly inhibited by pretreatment with a high concentration of the unrelated antigen KLH. Conversely, KLH binding to lymphocytes was not inhibited by DNP-lysine but was totally inhibited by pretreatment with KLH or anti-Ig antibody.

TABLE II

Specificity of DNP- and KLH-Binding Cells

Preincubation	Cells binding [^{125}I]DNP$_{12}$GPA per 10^5	Cells binding [^{125}I]KLH per 10^5
Medium alone	112 ± 21	190 ± 32
10^{-3}DNP-lysine	<6	170 ± 50
KLH 500 μg/ml	46 ± 13*	<5
Anti-Ig 250 μg/ml	<5	<5

Cells were preincubated as indicated for ½ h at 2°C, washed, then assayed for antigen-binding cells as described in the Materials and Methods.

* Inhibition at the $P < 0.025$ level.

Persistence of Antigen in the Cell.—In this set of experiments, our purpose was to determine the fate of antigen bound to lymphocytes. Most experiments were done in conditions where one does not expect T-cell activity. Thus, exposure to antigens was done briefly and at 4°C. Furthermore, in the case of DNP-binding cells, these were exposed to DNP on carrier proteins to which the mice were not immunized. Mice were immunized with DNP-KLH, and the cells were exposed to DNP bound to GPA, or the mouse proteins albumin and Ig.

The labeled antigen was first incubated with the cells in the cold, the excess washed off, and a sample of the cells was smeared immediately for autoradiography. The remaining cells were put into culture at 37°C for various times and then removed for autoradiography. In some experiments, the cells were incubated for a second time with labeled antigen.

[^{125}I]DNP$_{12}$GPA bound to 40–150 cells/100,000 under the conditions of these experiments. It could be shown that on incubation at 37°C it rapidly disappeared from the cell so that by 30 min of incubation, labeled cells could no longer be detected (Fig. 1). In all experiments, by 1 h there was no label persisting in these cells. By 2 h, it was not possible to relabel them with fresh [^{125}I]DNP$_{12}$GPA (Table III, exp. I); but by 4 h, there was some relabeling possible (Table III, exp. II). The dissociation in time between the loss of the original label and the ability to relabel the cells makes it seem likely that when the first antigen is lost from the cell the receptor is either lost or becomes nonfunctional. We will later examine the reappearance of the receptor function.

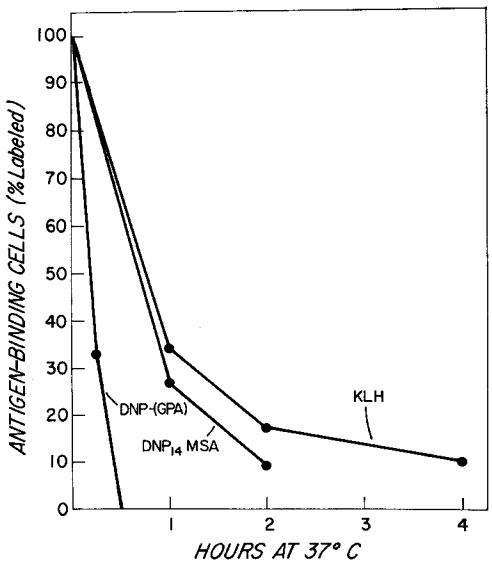

FIG. 1. Comparison of the rate of disappearance of labeled antigens from binding cells expressed as a percent of the original number of binding cells. See text for discussion.

TABLE III

Binding of $DNP_{1.5}$ and $DNP_{12}GPA$ to Cells With Subsequent Loss of Receptor Function

Exp.	First label		Time in culture	Second label	
	Antigen	Cells labeled per 10^5		Antigen	Cells labeled per 10^5
			h		
I	[^{125}I]$DNP_{12}GPA$, 5 μg/ml	46 ± 6	2	[^{125}I]$DNP_{12}GPA$, 5 μg/ml	2 ± 2 $P < 0.0005$
II	[^{125}I]$DNP_{12}GPA$, 5 μg/ml	145 ± 17	4	[^{125}I]$DNP_{12}GPA$, 5 μg/ml	33 ± 9 $P < 0.0005$
III	[^{125}I]$DNP_{1.5}GPA$, 25 μg/ml	<7	—	—	—
	[^{125}I]$DNP_{12}GPA$, 5 μg/ml	73 ± 12	—	—	—
	$DNP_{1.5}GPA$, 1 mg/ml	—	1	[^{125}I]$DNP_{12}GPA$, 5 μg/ml	43 ± 11 $P > 0.10$
	$DNP_{12}GPA$, 1 mg/ml	—	1	[^{125}I]$DNP_{12}GPA$, 5 μg/ml	<5

Cells were first incubated with either labeled or unlabeled antigen as indicated in the first two columns ("First label"). After washing, they were cultured at 37°C, then some were relabeled. [^{125}I]$DNP_{12}GPA$ did label cells directly and produced an effective loss of receptor function even at the low dose of 5 μg/ml. Since this dose of [^{125}I]$DNP_{12}GPA$ remains in the cells only about ½ h, the loss of receptor function persists beyond the time that the original antigen could still be occupying receptors. In the case of $DNP_{1.5}GPA$, there is no direct labeling at 25 μg/ml; and even at a dose of 1 mg/ml, receptor function is not significantly reduced.

[^{125}I]DNP_{14}MSA when used at the standard concentration of 5 μg/ml and without the addition of unlabeled MSA bound to about 1% of the cells. Thus, it seems likely that this antigen was binding not only to DNP-specific cells but also to cells with receptors for specificities on the MSA or for new specificities produced by the DNP conjugation procedure. Despite such heterogeneity of binding, the antigen was also rapidly cleared from the cells as shown in Fig. 1. An attempt was made to study the persistence of DNP_{14}MGG by similar experiments, but this substance bound to about one-half of the lymphocytes, possibly due to binding to Fc receptors on B cells or due to other nonspecific mechanisms. Thus, study of DNP_{14}MGG by direct labeling was not fruitful. In the case of both DNP_{14}MSA and DNP_{14}MGG for which much of the binding may not have been via specific receptors on B cells such direct-labeling experiments must be carefully interpreted; however, the results of indirect or relabeling experiments to be described shed some light on the persistence of these compounds in the cells and are consistent with the conclusion that all of these antigens are substantially cleared from the cells within a few hours of culture.

It was of interest to determine whether the number of DNP determinants per carrier molecule would affect handling by the lymphocyte. Thus, $DNP_{1.5}$-GPA was evaluated both by direct labeling of the cells with [^{125}I]$DNP_{1.5}$GPA and by using unlabeled $DNP_{1.5}$GPA to block the binding of [^{125}I]DNP_{12}GPA. By directly labeling with [^{125}I]$DNP_{1.5}$GPA, very few cells could be detected using even as much as five times the usual amount of antigen, i.e., 25 μg/ml (Table III, exp. III). When the cells were reacted with 1 mg/ml of unlabeled $DNP_{1.5}$GPA, washed, incubated for 1 h at 37°C, and then relabeling was attempted with [^{125}I]DNP_{12}GPA, a large proportion of the cells could be labeled. This is in marked contrast with the findings for similar blocking by DNP_{12}GPA (Table III, exps. I, II, and III) and can be explained by saying that the affinity of binding of $DNP_{1.5}$GPA to the DNP-specific cells was so low that it was to a great extent removed by the manipulations of washing. The fact that receptors could be identified on the cells after only 1 h in culture suggests that much of the $DNP_{1.5}$GPA dissociated from the cells, leaving the receptor intact. This was not the case with other antigens tested. [^{125}I]KLH bound to 100–200 cells/100,000 in these experiments and on incubation at 37°C disappeared somewhat more slowly (Fig. 1).

During the course of these experiments in which labeled cells were being cultured at 37°C, some cells seemed to show typical semilunar cap formation of the labeled antigen. Due to relatively poor localization of the label on the cell by autoradiography, it was not possible to obtain accurate counts of the proportion of cells undergoing capping; but in general terms, capping was seen only with KLH and DNP_{14}MSA as antigens, not with DNP_{12}GPA. Caps were noted only during the early stages of incubation within the first hour and never involved more than 10% of the labeled cells.

Reappearance of Receptor Function.—Our data thus indicate that during the loss of antigen from the cells receptor function is also lost. We have, therefore, studied the reappearance of receptor function during prolonged culture. Initial studies confirmed that there was a progressive increase in the number of cells which could be labeled after treatment with unlabeled antigen. This recovery took place after initial treatment with either antigen or anti-Ig and the rate of recovery was comparable for both.

When overnight or longer incubations were used to demonstrate recovery of receptor function, it was difficult to count antigen-binding cells due to the presence of dead cells and large, seemingly transformed cells as well as some plasma cells in the culture. Thus, the technique of Ficoll-Hypaque centrifugation was used to select out a population of viable, small lymphocytes. Control experiments convinced us that this step made counting much easier and more accurate but did not significantly alter the results since careful counting of the cultured cells without repurification yielded approximately the same numbers. In all subsequent experiments involving 18 h or more of culture, the technique of repurification on Ficoll-Hypaque was used.

The results of experiments showing the reappearance of receptors for DNP_{12}-GPA are shown in Table IV. Treatment of the lymphocytes with $DNP_{12}GPA$, $DNP_{14}MSA$, $DNP_{14}MGG$, and anti-Ig all resulted in early loss followed by extensive reappearance of receptor function by 18 h. In some cases, the proportion of binding cells did not return completely to the original levels. The reason for this variability is not known, but in most cases it is of only marginal statistical significance and may be explained by variations in culture conditions.

TABLE IV

Recovery of DNP Receptor Function After Various Treatments

Exp.	Length of culture	Treatment with				
		Medium	$DNP_{12}GPA$	Anti-Ig	$DNP_{14}MSA$	$DNP_{14}MGG$
	h					
I	0	68 ± 15	ND	ND	ND	ND
	1	ND	ND	11 ± 6	9 ± 9	6 ± 6
	4	ND	22 ± 8	ND	ND	ND
	18	ND	31 ± 8	53 ± 20	33 ± 11	56 ± 18
II	18	51 ± 10	44 ± 13			
	42	59 ± 14	41 ± 10			

Cells were treated with the various unlabeled antigens or with anti-Ig as indicated, all at 1 mg/ml. They were then washed and placed into culture. After the period of culture, the cells were assayed for binding of ^{125}I-$DNP_{12}GPA$. Recovery after each type of treatment is present by 18 h. The variation in the degree of recovery, varying from 31 to 56 binding cells per 10^5, is of marginal statistical significance and is within experimental error. Prolonged culture for 42 h does not alter the proportion of binding cells which recover after treatment with $DNP_{12}GPA$ (Exp. II). ND, Not done.

Exp. II in Table IV is included to demonstrate that there was no significant change in the proportion of binding cells on prolonged culture regardless of previous exposure to antigen. It is our belief that the reemergence of receptors takes place on the same cell which originally binds the antigen. There is no evidence of cell division in our experimental setup nor of new recruitment of cells from a precursor pool.

Similar results with KLH-binding cells are shown in Table V. In these experiments, there was usually at least 50% recovery of binding cells. In this case, there was 100% recovery in the KLH-treated cells and about 50% recovery in the anti-Ig treated group.

In all of these experiments, the lymphocytes were treated with antigen for ½ h on ice. In order to test whether prolonged exposure at 37°C would in

TABLE V

Recovery of KLH Receptor Function After Treatment with KLH and Anti-Ig

Time in culture	Medium	KLH	Anti-Ig
h		*(0.5 mg/ml)*	*(1 mg/ml)*
0	199 ± 30	ND	ND
1	ND	25 ± 7	<10
2	ND	38 ± 9	ND
4	ND	73 ± 15	ND
8	ND	110 ± 32	ND
18	ND	188 ± 63	90 ± 27

Recovery of KLH-binding activity after treatment with unlabeled KLH or anti-Ig. Recovery is not always to the original level as shown in the anti-Ig column here, although it is always to at least 50% of the original level: such differences are within experimental errors.

any way change the ability of the cells to subsequently reexpress their receptor function, they were incubated overnight with the antigen and then washed five times. Immediately after incubation with the antigen, a sample of cells was labeled with [^{125}I]$DNP_{12}GPA$ and processed for autoradiography. The remainder were placed again in culture for 18 h with fresh medium free of antigen and then were labeled and processed for autoradiography. The results in Table VI show that receptor function was not being expressed immediately after the 18-h exposure to the antigen but was nevertheless fully manifested after the second culture period. In the case of cells incubated originally with $DNP_{15}KLH$, which is the hapten-carrier combination to which they had been primed, there was extensive blast transformation and relatively few positive cells were detected.

It should be mentioned that cells that had recovered their receptor function did not seem to be either more or less heavily labeled than before. In one experiment, counting silver grains over the cells failed to reveal any difference in the distribution of labeled cells before and after receptor regeneration.

TABLE VI

Effect of Prolonged Treatment of Cells

Treatment for 18 h at 37°C	Binding cells at end of treatment	Binding cells after 18-h recovery
Medium alone	56 ± 10	45 ± 11
$DNP_{12}GPA$, 200 μg/ml	<5	34 ± 10
Anti-Ig, 200 μg/ml	14 ± 4	54 ± 12
$DNP_{15}KLH$, 200 μg/ml	<5	13 ± 7 (30% blasts)

Effects of long-term incubation with various antigens or anti-Ig present with the cells in culture overnight. The cells were washed, examined immediately for [^{125}I]$DNP_{12}GPA$-binding cells and then allowed to recover in culture for 18 h. There is complete recovery except in the case of treatment with $DNP_{15}KLH$ in which case there is the beginning of an immune response which significantly altered cell morphology and made counting difficult.

DISCUSSION

The binding of anti-Ig induces a redistribution of the surface Ig of B cells that is dependent upon cross-linking by the antibody. This redistribution indicates that the surface Ig is mobile within the plane of the plasma membrane in accordance with the fluid mosaic model of cell surfaces proposed by Singer (17). During redistribution and/or after it, the surface complexes are interiorized in vesicles and subsequently catabolized; a small portion of them, however, are shed off (2). The end result is a B lymphocyte rendered bare of its surface Ig for some period of time. The present series of experiments depict and quantitate the re-expression of surface Ig subsequent to one or several episodes of clearance with anti-Ig. Since antigen binds to the surface Ig receptor at the combining site, it was of concern that the events after binding of antigen might conceivably be different from that of anti-Ig which binds to either the constant region of Fab or to Fc determinants of surface Ig. For this reason, we studied the handling by DNP-specific cells of DNP conjugated to several carrier proteins. In general, results with anti-Ig or with specific antigen were comparable and demonstrated the capacity of the lymphocyte to clear its surface of complexes and then to regenerate functional-binding receptors within a relatively short span of time.

The clearing of antigen-Ig complexes depended on a cross-linking multivalent antigen. $DNP_{1.5}GPA$, for all practical purposes a monovalent antigen, rapidly dissociated from the cell, leaving the receptor intact. In contrast, $DNP_{12}GPA$ disappeared by ½ h, leaving a cell without functional receptors for about 4 more h. This was true even when the original amount of $DNP_{12}GPA$ used in labeling the cell was as low as 5 μg/ml. This sequence of events with $DNP_{12}GPA$ is, of course, exactly what happens when B lymphocytes are treated with anti-Ig. Since autoradiography cannot distinguish between label on the cell and label in the cell, it may be assumed that when the cells have

lost the label the antigen has either been shed or endocytosed and degraded. We are unable to distinguish between these two pathways. We can only say that KLH, which is much larger than the albumins, is lost more slowly, perhaps reflecting the time required for degradation. Thus, the size or chemical composition of an antigen must alter its handling by the lymphocyte. It is possible also that more heavily substituted proteins may be handled differently from the multivalent conjugate we have used. These studies with DNP conjugates are in agreement with the study of Wilson and Feldman who observed the reappearance of receptors for DNP red cells after an initial binding to the cells of DNP proteins (18).

In the course of the experiments on antigen disappearance, it was noted that KLH and $DNP_{14}MSA$ frequently redistributed into a cap formation. The extent to which capping occurs is not clear, but there seems little doubt that it can take place with these antigens. Capping has also been reported with erythrocytes bound to murine lymphocytes (19), and with other large soluble antigens (20, 21). We did not see capping with $DNP_{12}GPA$, perhaps because of the rapidity with which it is lost from the cell, or due to differences in the amount of cross-linking induced by this compound.

After clearance of the antigen from the lymphocyte, there is progressive reappearance of the Ig receptor function. Our data showing regeneration of receptor function correlates well with the reappearance of surface Ig after anti-Ig treatment. The degree of regeneration in terms of the number of labeled cells was variable, although usually quite near 100%. Prolonged incubation with the blocking antigen, up to 18 h and in subsequent experiments to 48 h (manuscript in preparation), did not affect the regeneration. It should be noted that during the period of exposure to antigen it is not possible to detect receptors. This is probably due to binding of antigen and endocytosis as the receptors are regenerated. When excess antigen is removed, the cell proceeds to regenerate a full complement of receptors.

We were unable to detect any differences in the amount of antigen-binding capacity of the individual cells after receptor regeneration. Thus, either in terms of the total number of binding cells or in terms of labeling per cell, we were unable to confirm the contention of Diener and Paetkau (22) that cells regenerating receptors have increased binding capacity. It should be noted that their studies were done using a T-independent antigen (flagellin). Most thymic-independent antigens are polyclonal mitogens for B cells (23). These classes of antigens clearly have intrinsic properties different from the antigens used herein. Our studies have been made with antigens that by themselves do not stimulate immunity.

One point that emerges from these studies is the capacity of the lymphocyte to clear and eliminate antigen-receptor complexes and to regenerate free receptors both in the absence of any apparent "helper" function of thymic cells or macrophages. This clearing of surface complexes or aggregates seems to

represent a generalized biological phenomenon found in all types of cells, be they T or B cells, macrophages, or other. In the case of B lymphocytes, the extent to which helper cells influence both clearance and regeneration of receptors needs to be determined. In the presence of helper thymic function, immune induction takes place leading to a change in the status of the B lymphocyte which then proliferates and differentiates. The changes that have been described herein and in other reports take place in the absence of helper function and result in a limited response of the cell. This response consists of some degree of proliferation seen in species like the rabbit (24) but not, for example, in the mouse; it also includes the stimulation of random movement described before (3). Whether a B lymphocyte after the period of clearance and regeneration of receptors is capable of complete stimulation (i.e., with helper cells) is a critical question that may bear on mechanisms of tolerance as well as immunity.

SUMMARY

The behavior of the immunoglobulin antigen receptor on lymphocytes was studied using both fluorescent antiimmunoglobulin antibody to detect B cells and autoradiography with radiolabeled antigens to detect antigen-binding cells. It was shown that after binding of antiimmunoglobulin antibody to the lymphocyte there was a rapid loss of surface immunoglobulin and then a progressive reappearance over 18 h. This could be quantitated using an inhibition assay for surface immunoglobulin. Similarly, after binding various dinitrophenyl-conjugated proteins or keyhole limpet hemocyanin to their specific antigen-binding cells, there was a loss of the antigen receptor from the surface and then a progressive reappearance of the receptor. The reappearance of surface immunoglobulin and of the antigen receptor proceeded at about the same rate. Repeated exposure to antibody or prolonged exposure to antigen did not diminish the capacity of the lymphocyte to re-express its receptor. These events, which follow the interaction of antigen and its receptor, are of possible importance in understanding the mechanism of triggering of the immune response and of tolerance.

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