Emergence of a Surface Immunoglobulin Recycling Process during B Lymphocyte Differentiation

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ABSTRACT Surface immunoglobulin (Ig)-mediated endocytosis has been investigated in rat B lymphocytes and plasma cells, using horseradish peroxidase (HRP)-labeled sheep anti-rat Ig Fab' fragment of antibody and HRP as monomeric ligands, respectively. Quantitative estimates of HRP activity associated either with plasma membrane or with endomembrane compartments were made in several experimental conditions. Binding of HRP-conjugate on B lymphocytes was followed by its endocytosis in combination with surface Ig, as shown by the progressive disappearance of plasma membrane-associated HRP activity. Between 1 and 6 h at 37°C in presence of conjugate the total amount of cell-associated activity was constant. These results indicate that during this time no reappearance of surface Ig occurred by neosynthesis, by the expression of an intracellular pool or by the recycling in a free form of the previously internalized molecules. On the contrary, at saturating doses, internalization of HRP by anti-HRP plasma cells increased linearly with time at 37°C in presence of antigen, when, during the same time, the plasma membrane HRP-binding capacity remained constant. Cycloheximide did not affect continuous HRP uptake. The existence of a large intracellular pool of receptors has been ruled out by experiments of removal of binding sites with pronase. In addition, monensin caused a progressive decrease in the number of surface receptors on plasma cells but not on B lymphocytes. Our data then indicate that, unlike B lymphocytes, plasma cells were able to recycle their surface Ig.

Receptor-mediated endocytosis of ligands has been described in various cell types (reviewed in references 18 and 36), including B lymphocytes (3, 27, 35, 45) and plasma cells (3, 20). In some cells and/or with some ligand-receptor systems, a temporary loss of membrane receptors is observed after internalization of the ligand-receptor complexes. The so-called down regulation process has been principally documented for receptors whose major role is to convey a signal to a target cell, such as receptors for polypeptide hormones or neurotransmitters (reviewed in references 11 and 26). The most widely accepted explanation of this phenomenon is that, after internalization, ligand-receptor complexes are trapped within the lysosomes and degraded. Reexpression of new receptors appears to require *de novo* synthesis. In other cell types and/or with other ligand-receptor systems, a distinct behavior of the receptors has been described. After internalization of the complexes, receptors are not catabolized, but return to the plasma membrane, either complexed with the ligand (43) or in a free form (18, 25, 37, 43). This process was termed receptor recycling and allows for an efficient delivery of ligands to intracellular sites (mainly the lysosomes), either to provide nutritional molecules inside the cells or to degrade injurious agents.

How some receptors escape from intracellular degradation whereas others are degraded remains poorly understood. The aim of this study was to determine whether the cellular differentiation state can affect receptor behavior. We have addressed this question with immunocytes of the B lineage. Immunocompetent resting B lymphocytes express surface immunoglobulins (Ig) on their plasma membrane, through which they recognize antigens. Up to now, surface Ig-mediated endocytosis has been principally studied by means of ligands (antigen or anti-Ig antibodies) that induce redistribution (patching and capping) of surface Ig (27, 35, 45). Internalization of ligand-surface Ig complexes leaves the cell free of surface Ig and their reexpression requires protein synthesis (45). However, it has been recently reported that divalent antibodies directed against low density lipoprotein receptor of fibroblasts inhibit the recycling of receptor, whereas Fab fragments do not (2). It was then of interest to further investigate endocytosis of a monomeric ligand in B lymphocytes. We present here a study on the internalization of Fab' frag-

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 APRIL 1984 1238-1246

ments of anti-Ig antibodies by B lymphocytes removed from lymph nodes of nonimmunized rats.

Binding of antigen or anti-Ig antibodies on the surface Ig delivers a signal that, in combination with other signals, activates B lymphocytes. Once activated, B lymphocytes proliferate and differentiate in plasma cells. In these latter cells, the majority of Ig molecules, instead of participating in the membrane recognition mechanisms are now secreted in large amounts. However, throughout the process of differentiation, B cells continue to express surface Ig, although their number progressively decreases (31) and plasma cells still exhibit detectable numbers of surface receptors for antigen (23, 29). In the present study, we have investigated receptor-mediated endocytosis of antigen in plasma cells using horseradish peroxidase (HRP)[1] as monomeric antigen and popliteal lymph node cells of HRP-immunized rats as source of plasma cells.

We present evidence of a very different kinetics of monomeric ligand uptake in B lymphocytes and plasma cells. Our data suggest that the differences observed can be explained by a surface Ig recycling in plasma cells, but not in B lymphocytes.

MATERIALS AND METHODS

Sources of Materials: HRP was obtained from Boehringer (Grade I, Mannheim, Federal Republic of Germany [FRG]) and Incomplete Freund's adjuvant from Difco Laboratories (Detroit, MI). Cycloheximide, monensin, and rabbit γ-globulins (B grade) were purchased from Calbiochem (La Jolla, CA), Pronase E (70,000 PUK/g), 30% H_2O_2 and 2-mercaptoethanol from Merck-Schuhardt (Darmstadt, FRG), DNase II (880.5 U/mg) and pepsin (2900 U/mg) from Worthington Biochemicals (Freehold, NJ). Nonidet P-40 (NP-40), *O*-dianisidine DiHCl (OD), *O*-phenylenediamine DiHCl (OPD), and 3-amino-9-ethyl carbazole were obtained from Sigma Chemical Co. (St. Louis, MO), iodoacetamide from Aldrich Chemical Co. (Milwaukee, WI), Ultrogel AcA 44, and DEAE-trisacryl M from IBF (Villeneuve-La-Garenne, France) and Sephacryl S-200 from Pharmacia Fine Chemicals (Uppsala, Sweden). 1,4-benzoquinone was purchased from Fluka AG (Buchs, Switzerland) and L-[^{3}H]leucine (50 Ci/mmol) from Commissariat à l'Energie Atomique (Saclay, France).

Animals: Male rats of the Fischer 344 strain were used. They were reared in specific pathogen-free conditions in the breeding center of the Pasteur Institute (Dr. J. L. Guénet).

Antigens and Immunizations: 5–6-mo-old rats were immunized by subcutaneous injections, in each hind footpad, of 0.5 mg of HRP or rabbit γ-globulins emulsified in IFA. 2 mo to 1 yr later, secondary immunizations were carried out in the same way with 0.25 mg of HRP or rabbit γ-globulins in each hind footpad. Rats were killed 4 d later using a lethal dose of ether.

Preparation of HRP-conjugates: Immunization of sheep with rat IgG and isolation of specific antibodies by affinity chromatography on immunoadsorbents were described elsewhere (5, 41). Sheep anti-rat Ig antibodies were pepsin-digested according to Nisonoff et al. (30), reduced with 2-mercaptoethanol and alkylated with iodoacetamide. Sheep anti-rat Ig Fab' were purified by filtration through a Sephacryl S-200 column calibrated for molecular weight determinations. Fab' fragments of normal sheep IgG were prepared as followed. Normal sheep serum was chromatographed on DEAE-trisacryl M equilibrated with 0.025 M Tris, HCl buffer, pH 8.8, 0.035 M NaCl. The IgG eluted at this ionic strength were concentrated by precipitation with 40% ammonium sulphate, pepsin-digested as above and the Fab' fragments were purified by filtration on Sephacryl S-200.

Sheep anti-rat Ig Fab' and normal sheep Fab' were covalently linked to HRP by a two-step procedure (6). The labeled Fab' preparations were passed through a Sephacryl S-200 column and fractions corresponding to a molecular weight of 80,000 containing Fab'-HRP conjugate in a 1:1 molar ratio were pooled and used in the present experiments.

In some experiments, HRP-labeled normal sheep $(Fab')_2$ and sheep anti-rat Ig $(Fab')_2$ conjugates were used. $(Fab')_2$ fragments were prepared by pepsin digestion and covalently linked with HRP (6). After coupling, these conjugates were used without any further purification or after filtration through a Sephacryl S-200 column as described above. In this latter case, fractions containing $(Fab')_2$-HRP conjugate free of unlabeled $(Fab')_2$ fragments were selected. The HRP/$(Fab')_2$ molar ratios of the selected conjugates were 1.8–1.9.

Preparation of Cells: Normal lymphoid cells were prepared from cervical and mesenteric lymph nodes of 9–10-mo-old rats. After bleeding by cardiac puncture, organs were removed and teased in Hanks' medium (Institut Pasteur Production, Paris, France) containing 10% Haemaccel (Laboratoires Hoechst, Puteaux, France). To deplete macrophages, we passed lymphoid populations through AcA 44 acrylamide-agarose beads as previously described (21). Recovered cells were resuspended in modified Click's medium (12) buffered with 20 mM HEPES (Flow Laboratories, Irvine, UK) supplemented with 3×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum.

Anti-HRP and anti-rabbit γ-globulin lymphoid cells were prepared as described above from popliteal lymph nodes of HRP-or rabbit γ-globulin-immunized rats that had received a booster injection 4 d earlier. Cell viability, as tested by trypan blue exclusion, was at least 90% in our experiments.

HRP Binding and Uptake Assays: The binding of HRP or HRP conjugates to cells was measured by incubating them (5×10^7/ml) for 1 h at 4°C with various concentrations of HRP or HRP conjugates in modified Click's medium. After three washings, cell-bound HRP was estimated quantitatively by measuring enzymatic activity. 0.1 ml of the cell suspension was added to 2.9 ml of isotonic buffer containing enzyme substrate. The binding of HRP conjugates to B lymphocytes was studied using OD substrate prepared as described earlier (4, 21). The binding of HRP to plasma cells was studied using OPD (46). OPD and H_2O_2 were dissolved in 0.15 M phosphate buffer, pH 6.0 (0.5 mg OPD/ml and 0.015% H_2O_2). The reaction was allowed to proceed for 15 min in the dark at room temperature and was stopped by adding 300 μl of 6 N HCl. The suspension was then centrifuged at 350 *g* for 7 min and the absorbance of the colored supernatant was read at 492 nm.

The uptake of HRP or HRP conjugates by cells was measured by incubating them at 37°C with HRP or HRP conjugates for various periods of time in modified Click's medium (5×10^6 cells/ml). After four washings, 0.05 ml of the cell suspension was lysed with 0.05 ml of 1% NP-40 in solution in phosphate-buffered saline (0.01 M potassium phosphate buffer pH 7.4; 0.15 M NaCl). After incubation for 10 min at room temperature, HRP activity was measured using OD or OPD as described above.

Quantities of cell-bound HRP were calculated by referring to a standard curve of HRP activity.

Pronase Treatment of Lymphoid Cells: To remove cell surface-bound HRP or HRP conjugates, we incubated cells for 20 to 30 min at 37°C in modified Click's medium without fetal calf serum, and with 2 mg/ml pronase (previously heated 45–60 min at 37°C) and 50 μg/ml DNase II. After two washings in Hanks' Haemaccel, cell-associated activity was measured. To remove membrane receptors on plasma cells, we incubated cells for 15 min at 4°C with the same concentrations of pronase and DNase II. Cells were then washed four times in Hanks' medium containing 10% FCS before reincubation at 37°C.

Cytochemical Detection of Cell-bound HRP: After incubation of normal or immune lymphoid cells with HRP conjugates or HRP, respectively, cells were spread on a microscopic slide using a cytocentrifuge (Shandon Southern Products, Inc., Sewickley, PA). Fixation of cells and detection of HRP using 3-amino-9-ethyl carbazole as chromogenic substrate were previously described (21, 22). Percentages of stained cells were calculated after counting 1,000–1,500 cells on each slide.

Detection of Antibody-secreting Cells: Anti-HRP antibody-secreting cells were detected using the plaque-forming cell assay described by Cunningham and Szenberg (13). In this assay, sheep erythrocytes (SRBC), covalently linked to HRP with 1,4-benzoquinone, were used as targets (42) and a polyspecific rabbit anti-rat Ig serum was used as enhancing serum. A guinea pig serum previously adsorbed on SRBC was used as a source of complement.

Biosynthetic Labeling: To determine the effect of cycloheximide on protein synthesis, we incubated lymph node cells from anti-HRP rats for 1 h at 37°C in modified Eagle's culture medium free of L-leucine (Flow Laboratories) containing 60 μCi/ml of L-[^{3}H]leucine. Trichloroacetic acid–precipitable radioactivity was then counted.

RESULTS

Binding of HRP-labeled Anti-rat Ig Fab' Fragment on B Lymphocytes

With B lymphocytes, the surface Ig-mediated endocytosis process was studied using HRP-labeled sheep anti-rat Ig Fab' fragment as monomeric ligand. The binding of this ligand was measured by incubating macrophage-depleted normal

[1] *Abbreviations used in this paper:* HRP, horseradish peroxidase; NP-40, Nonidet P-40; OD, *O*-dianisidine DiHCl; OPD, *O*-phenylenediamine DiHCl; SRBC, sheep erythrocyte.

lymph node cells for 1 h at 4°C with various concentrations of conjugate. The amount of cell-bound HRP was then estimated as described in Materials and Methods. As shown in Fig. 1, the binding was a specific saturable process. A plateau was reached with 120 μg/ml of conjugate. The nonspecific binding, measured by incubating cells with HRP-labeled normal Fab' fragment, represented 3–11% of the specific one. At the light microscopic level the maximum number of surface Ig-positive cells which varied between 36 and 46% of the cells, was obtained with 50–100 μg/ml of conjugate. No positive cells were observed with the control conjugate.

After incubation at 4°C with conjugate, the amount of cell-bound HRP was also determined after lysis of cells with NP-40 and compared to the amount of HRP detected on intact cells. Difference between the two values, termed NP-40 releasable HRP, was found to be very small, as shown in Fig. 1. In addition, the pronase treatment of living labeled cells removed most of the activity previously titrated on intact cells, without significantly affecting the low amount of NP-40 releasable activity (Table I, experiments 1 and 2). This indicates that at 4°C, most of the HRP activity was associated to the cell surface.

Internalization of HRP-labeled Anti-rat Ig Fab' Fragment by B Lymphocytes

HRP conjugate uptake was studied by incubating normal lymphoid cells at 37°C with 200 μg/ml of conjugate. As shown in Table I (experiment 3), pronase removed most of the HRP activity titrated on intact cells. Furthermore, the proteolytic digestion did not significantly modify the amount of NP-40 releasable activity. We have then attributed the HRP activity detected on intact cells to plasma membrane-bound HRP conjugate molecules, and the NP-40 releasable activity to internalized HRP conjugate molecules.

Normal lymphoid cells were incubated 1 h at 4°C or 1, 3, and 6 h at 37°C with anti-rat Ig conjugate and the HRP activity associated with different compartments of the cells was estimated. With time of incubation at 37°C, we observed

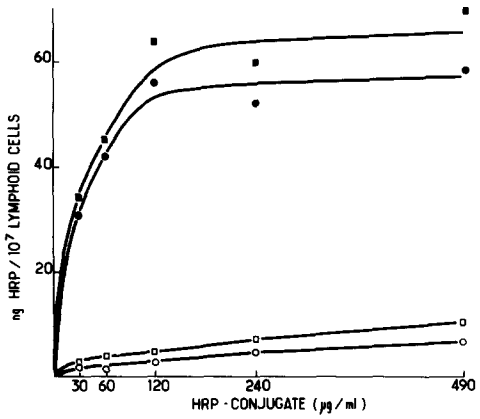

FIGURE 1 Binding of HRP-labeled sheep anti-rat Ig Fab' fragment on B lymphocytes. Macrophage-depleted mesenteric and cervical lymph node cells (5 × 10^7/ml) were incubated 1 h at 4°C with various concentrations of conjugate in modified Click's medium. Titration of cell-associated HRP was performed with OD chromogenic substrate either on living cells (●) or after lysis of cells with NP-40 (■). Nonspecific binding was measured by incubating cells with HRP-labeled normal sheep Fab'. Titration of cell-associated HRP was performed either on living cells (○) or on cell lysates (□).

TABLE I

Binding and Internalization of HRP-labeled Sheep Anti-rat Ig Fab' by B Lymphocytes

Experiment	Temperature of incubation with conjugates	Amount of HRP detected on 10^7 living cells	Amount of HRP detected on 10^7 living cells treated with pronase	Amount of NP-40 releasable HRP/10^7 cells	
				Before pronase treatment	After pronase treatment
	°C	*ng*	*ng*	*ng*	
1	4	59.3	1.6	11.7	10.2
2	4	48.1	2.6	7.4	8.4
3	37	27.7	4.2	73.1	66.7

Normal lymphoid cells were incubated for 1 h at 4°C or 37°C with 200 μg/ml HRP-labeled sheep anti-rat Ig Fab'. After washing, one portion of cells was treated 20 to 30 min at 37°C with pronase. Then, HRP activity was titrated on living cells (third and fourth columns). Amounts of NP-40 releasable HRP before and after treatment of cells with pronase are indicated in the fifth and sixth columns.

a progressive clearance of plasma membrane-bound HRP (Fig. 2). After 6 h at 37°C the surface-binding capacity of the cells was ~20% of the binding capacity at 4°C. This decrease was due to an internalization of (surface Ig-HRP conjugate) complexes because the amount of NP-40 releasable HRP simultaneously increased to reach a plateau after 3 h of incubation. In these conditions, the percentage of HRP-labeled cells was constant and similar to the percentage of conjugate-binding cells counted at 4°C (Fig. 2). As for the results obtained at 4°C, the nonspecific binding and internalization measured by incubating cells with HRP-labeled normal sheep Fab' were very low (on the order of 5–10% of the values found with the specific conjugate) regardless of the incubation time at 37°C, and no positive cells could be detected with this conjugate.

As shown in Fig. 2 both the binding and the internalization process were unaffected by cycloheximide which at the dose used inhibited protein synthesis by 93%. The most interesting point was that from 1 to 6 h at 37°C the total amount of cell-associated HRP was constant. This probably indicates that once internalized under the form of immune complexes the surface Ig do not come back at the cell surface in a free form to be recycled. No adjustment for the degradation process of HRP was needed in these conditions since we had previously demonstrated that HRP can remain a long time (at least 6 h) in intracellular sites of lymphocytes without any alteration of its enzymatic activity, and even after this lag, the rate of inactivation was low (50% in 18 h) (21). An observation more difficult to interpret was the difference measured for the total amount of cell-associated HRP activity between cells incubated 1 h at 4°C and 1 h at 37°C (Fig. 2). The average ratio between the values obtained at 37°C and those obtained at 4°C was 2.2. This might be due to a rapid neosynthesis of surface Ig during the first hour of incubation, or to the rapid expression of an intracellular pool of surface Ig. The first hypothesis was ruled out as cycloheximide-treated cells showed the same increase between 1 h at 4°C and 1 h at 37°C (Fig. 2). The second explanation was also unlikely because after a 15-min exposure of lymphoid cells to pronase, which removed 88% of the surface Ig, followed by a 1 h postincubation at 37°C, no reexpression of surface Ig was observed.

We then performed experiments with the bivalent conjugate HRP-labeled sheep anti-rat Ig $(Fab')_2$. As shown in Table II, the average ratio between the values obtained at 37°C and those obtained at 4°C was between 1.04 and 1.26 which indicates that most, if not all, bivalent conjugate-binding sites were accessible at 4°C on the cell surface. Taken together, the above results suggest that a part of the surface Ig molecules or determinants were probably not accessible at 4°C to the monovalent conjugate.

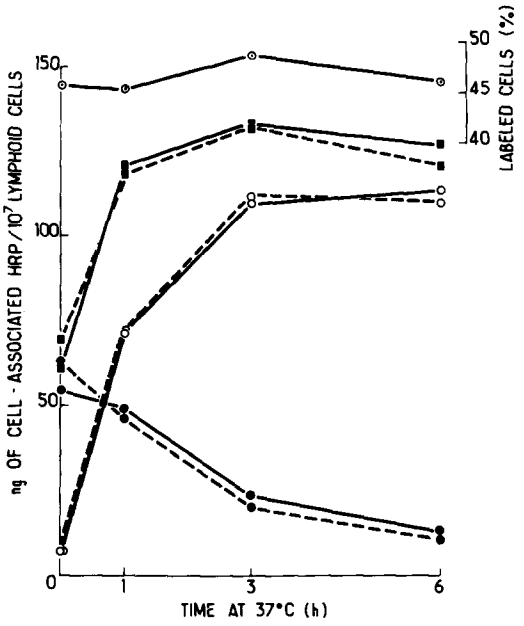

FIGURE 2 Uptake of HRP-labeled sheep anti-rat Ig Fab' fragment by B lymphocytes. Macrophage-depleted mesenteric and cervical lymph node cells (5×10^7/ml) were incubated at 4°C (time 0) or 37°C with 200 μg/ml conjugate in modified Click's medium and with (——) or without (-----) 20 μg/ml cycloheximide. After 1 h at 4°C and 1, 3, and 6 h at 37°C, aliquots of cells were withdrawn and cell-associated HRP activity estimated by using OD as chromogenic substrate. (●) Nanograms of plasma membrane-bound HRP/10^7 cells; (○) nanograms of internalized HRP/10^7 cells; (■) total amount of cell-associated HRP in ng/10^7 cells. Results shown in this figure are the means of two separate experiments. Corrections for nonspecifically bound or internalized HRP conjugate are already made in the presented data. Nonspecific binding and internalization were estimated by incubating aliquots of the same cell suspensions with HRP-labeled normal sheep Fab'. In parallel, aliquots of cells were prepared for cytochemical detection of HRP-labeled cells. After cytocentrifugation and fixation, cell preparations were dipped in 3-amino-9-ethyl carbazole substrate. Results are expressed in percent of lymphoid cells labeled with HRP-conjugate (⊙).

Binding of HRP on Anti-HRP Plasma Cells

With plasma cells, the surface Ig-mediated endocytosis process was studied using HRP as the monomeric ligand. The cells used for this study were popliteal lymph node cells taken from anti-HRP rats, having received a booster injection 4 d earlier. We have previously demonstrated that at this stage of the immune response ~80% of the antigen-binding cells were proplasmacytes and plasmacytes and only these cells internalized large amounts of HRP (20). All the results reported below were obtained using macrophage-depleted cell populations.

Fig. 3 illustrates the antigen-binding capacity of popliteal lymph node cells incubated 1 h at 4°C with various concen-

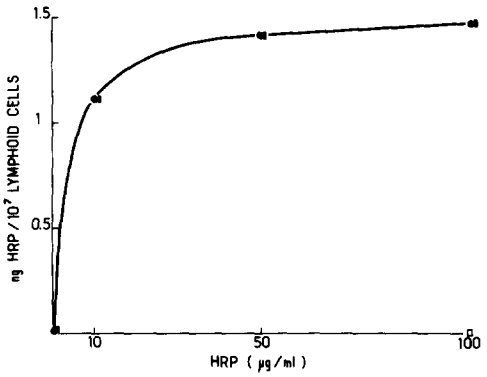

FIGURE 3 Binding of HRP on anti-HRP plasma cells. Macrophage-depleted popliteal lymph node cells (5×10^7/ml) were incubated 1 h at 4°C with various concentrations of HRP in modified Click's medium. Titration of cell-associated HRP was performed with OPD chromogenic substrate either on living cells (●) or after lysis of cells with NP-40 (■). As a control, popliteal lymph node cells from rabbit γ-globulin-immunized rats were prepared as above and incubated with 100 μg/ml HRP. No binding of HRP could be detected on these cells (□).

TABLE II

Binding and Internalization of HRP-labeled Sheep Anti-rat Ig $(Fab')_2$ by B Lymphocytes

Conjugate	Temperature of incubation with conjugate	Amount of HRP detected on 10^7 living cells	Amount of NP-40 releasable HRP/ 10^7 cells	Total amount of cell-associated HRP (ng)/10^7 cells	Total amount of cell-associated HRP at 37°C / Total amount of cell-associated HRP at 4°C
	°C	*ng*	*ng*		
Not filtered	4	8.4 ± 1.2*	3.3 ± 1.1	11.7 ± 2.0	1.042 ± 0.165
	37	2.6 ± 0.1	9.6 ± 1.7	12.2 ± 1.8	
Sephacryl S-200 filtered	4	64.9‡	11.5	76.5	1.26
	37	13.9	83	96.9	

Normal lymphoid cells were incubated 1 h at 4°C or 37°C with a saturating concentration (100 to 400 μg/ml) of HRP-labeled sheep anti-rat Ig $(Fab')_2$ conjugate. Then, HRP activity was titrated on living cells (third column) or on cell lysates (fifth column). Differences between the two sets of values are indicated in the fourth column. The ratios of the total amounts of cell-associated HRP titrated at 37°C and 4°C are present in sixth column. Nonspecific binding and internalization were estimated by incubating aliquots of the same cell suspensions with HRP-labeled normal sheep $(Fab')_2$ fragments not filtered or filtered on Sephacryl S-200. Values obtained with these conjugates did not represent >5–10% of the values obtained with the specific conjugates.

* Means ± one standard deviation of five experiments. The conjugate used in these experiments was not filtered on Sephacryl S-200 column. With the glutaraldehyde coupling procedure, ~20% of the $(Fab')_2$ fragments are usually linked to HRP (8). So the presence in this conjugate of high amounts of unlabeled $(Fab')_2$ fragments competing with the labeled molecules explains the lower quantities of cell-associated HRP compared to values obtained with the filtered conjugate.

‡ Means of two experiments. In these experiments, we have selected HRP-$(Fab')_2$ conjugate free of unlabeled molecules. Its HRP/$(Fab')_2$ molar ratio was 1.8–1.9.

trations of HRP. As both the percentage of ligand-binding cells and the number of surface receptors were lower than in the experiments on B lymphocytes, the more sensitive OPD substrate was used to measure the cell-associated HRP activity. A plateau was reached at 50 μg/ml HRP, but if saturation of the binding sites was always obtained with this concentration, the maximum amount of HRP bound to 10^7 cells ranged between 0.3 and 1.8 ng/10^7 cells. This was due to differences in the percentage of HRP-binding cells present in popliteal lymph nodes (Fig. 4*A*). Binding of HRP on anti-HRP plasma cells occurred through specific receptors since no binding could be detected on anti-rabbit γ-globulin lymphoid cells incubated 1 h at 4°C with 100 μg/ml HRP (Fig. 3). It is also obvious from Fig. 3 that no endocytosis of antigen took place at this temperature as no HRP activity was released by treatment of cells with NP-40. Pronase treatment of cells previously incubated 1 h at 4°C with HRP confirmed this point. Indeed, in these conditions, the proteolytic enzyme removed 95 to 100% of the cell-associated HRP activity (Fig. 5).

Internalization of HRP by Anti-HRP Plasma Cells

In a previous paper (21), we demonstrated that HRP uptake in plasma cells was a saturable process over 50 μg HRP/ml of culture medium. In the present experiments, HRP uptake was studied as a function of time. Fig. 5 shows a typical experiment in which one portion of cells was incubated for 1 h at 4°C with 100 μg/ml HRP to estimate their binding capacity and the other portion for 1, 3, and 6 h at 37°C with the same dose of antigen. Uptake of HRP, measured after lysis of cells with NP-40, increased linearly with time at 37°C and exceeded by several times the binding at 4°C. This increase was not explained by the fluid-phase endocytosis of HRP. Indeed, at the same concentration of HRP, anti-rabbit

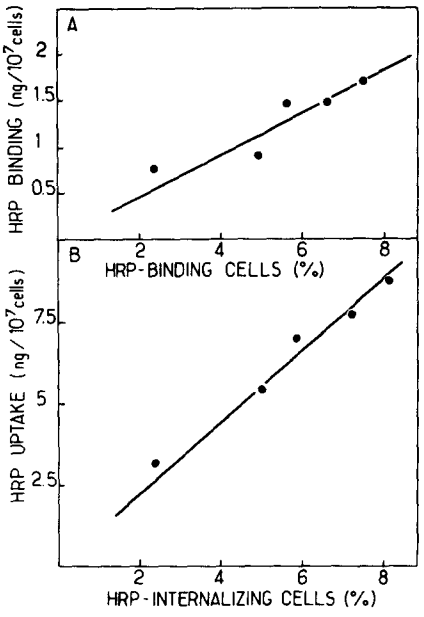

FIGURE 4 Correlation between the amount of cell-bound HRP and the percentage of HRP-binding cells (*A*) or HRP-internalizing cells (*B*). (*A*) Abcissa: nanograms of HRP bound by 10^7 cells incubated 1 h at 4°C with 100 μg/ml HRP. Ordinate: percentage of HRP-binding cells in the same cell suspensions calculated after counting 1,000–1,500 total lymph node cells. (*B*) Abcissa: uptake of HRP by 10^7 cells incubated 3 h at 37°C with 100 μg/ml HRP. Ordinate: percentage of HRP-internalizing cells in the same cell suspensions.

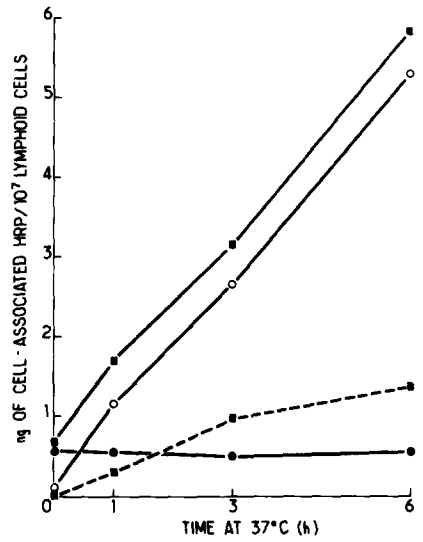

FIGURE 5 Uptake of HRP by anti-HRP plasma cells. Macrophage-depleted popliteal lymph node cells were incubated at 4°C (time 0) or 37°C with 100 μg/ml HRP in modified Click's medium. After 1 h at 4°C and 1, 3, and 6 h at 37°C, aliquots of cells were withdrawn and cell-associated activity estimated by using OPD as chromogenic substrate. (●) Nanograms of HRP/10^7 cells removed by pronase (plasma membrane bound-HRP); (○) nanograms of internalized HRP/10^7 cells; (■——■) total amount of cell-associated HRP in ng/10^7 cells. Uptake of HRP by fluid phase endocytosis was measured by incubating anti-rabbit γ-globulin lymphoid cells as above with 100 μg/ml HRP and total cell associated activity titrated with OPD substrate (■----■).

γ-globulin lymphoid cells internalized 5 to 10 times smaller amounts of HRP than specific plasma cells (Fig. 5). The amount of cell-surface bound HRP was estimated after treatment of living cells with pronase. As shown in Fig. 5, the number of HRP molecules removed by pronase on living cells was constant for 6 h at 37°C and remained equal to that found at 4°C. This indicates that, on the contrary to what happened in B lymphocytes (Fig. 2), the surface binding capacity of plasma cells was unchanged even after 6 h of incubation at 37°C with HRP. The amount of HRP associated to a given number of cells depended on the percentage of cells in which HRP was detected cytochemically (HRP-internalizing cells) (Fig. 4*B*). HRP-binding cells and HRP-internalizing cells were very likely the same cells because their number was roughly the same (Fig. 6), and they belonged to the same cell types (20, 21). Furthermore in most HRP-labeled cells both plasma membrane and intracytoplasmic staining could be detected. An average value of 37°C uptake/4°C binding ratio was calculated from data of five separate experiments. It was found to be of 6.5 and 11.5 after 3 and 6 h of incubation at 37°C, respectively. No adjustment for degradation of HRP was needed in our calculations, since no significant decrease in HRP activity was noticeable before 6 h after initial penetration (21).

Taken together, these data then demonstrate an ability of plasma cells to endocytose HRP far exceeding the number of available surface-binding sites. However, it was possible that values obtained at 4°C underestimated the real binding capacity. To test this hypothesis, we incubated cells for 1 h at 37°C with various concentrations of HRP in the presence of sodium azide (10^{-2} M), which is a good inhibitor of the endocytic process of antigen by plasma cells (19). Under these condi-

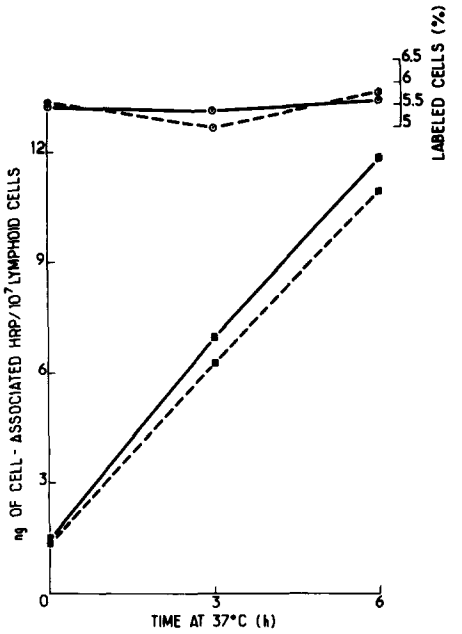

FIGURE 6 Uptake of HRP by anti-HRP plasma cells in presence or absence of cycloheximide. Macrophage-depleted popliteal lymph node cells were preincubated for 1 h at 37°C with or without 20 μg/ml cycloheximide before adding 100 μg/ml HRP. After 1 h at 4°C and 3 or 6 h at 37°C, samples of cells were washed and total amount of cell-associated HRP determined on cell lysates with OPD substrate. (■——■) total amount of cell-associated HRP in ng/10^7 cells not treated with cycloheximide; (■----■) total amount of cell-associated HRP in ng/10^7 cycloheximide-treated cells. In parallel, aliquots of cells were prepared for cytochemical detection of HRP-labeled cells. After cytocentrifugation and fixation of cells, HRP was revealed using 3-amino-9-ethyl carbazole substrate. Results are expressed in percent of lymphoid cells labeled with HRP. (⊙——⊙) percent of HRP-labeled cells in control group not treated with cycloheximide; (⊙----⊙) percent of HRP-labeled cells in cycloheximide-treated group.

tions, no change in the amount of cell-bound HRP was found compared to 4°C, suggesting no effect of temperature on the affinity or amount of binding (data not shown).

Effect of Cycloheximide on HRP Binding and Uptake in Plasma Cells

The effect of cycloheximide was tested to determine whether continuous uptake of HRP depended on *de novo* synthesis of new receptors during the 6 h of incubation at 37°C. The rate of HRP receptor turnover was first investigated. Cells were maintained at 37°C in the presence of cycloheximide. At various times, aliquots of cells were cooled and assayed for HRP binding. No decrease in number of surface receptors was observed up to 6 h. After 6 h, an increase of cellular mortality was noted. The following experiments were then designed to directly assess the effect of cycloheximide on HRP uptake. Cells were preincubated for 1 h at 37°C with or without (control cells) 20 μg/ml cycloheximide. A portion of cells was then cooled to 4°C to determine the number of binding sites. The other portion was incubated at 37°C up to 6 h with 100 μg/ml HRP in the presence or absence of cycloheximide. After 3 and 6 h, uptake was determined in control and cycloheximide-treated cells. Fig. 6 clearly indicates the absence of effect of cycloheximide on HRP uptake.

In addition, it might be argued that the presence of soluble anti-HRP antibody secreted by plasma cells in the culture medium could affect antigen internalization. This can be ruled out from the results presented in Fig. 7. Cells from anti-HRP rats were preincubated for 1 to 6 h at 37°C with 20 μg/ml cycloheximide and then tested for the presence of anti-HRP antibody-secreting cells by a local haemolysis plaque assay. After 1 h of preincubation with the drug the secretion of antibody was already drastically reduced and after 2 h only few cells still secreted detectable amount of antibody. In these conditions uptake of HRP was found to be normal (Fig. 6).

HRP Binding and Uptake in Pronase-treated Plasma Cells

Continuous HRP uptake could be due to a large intracellular pool of receptors replacing those which mediated endocytosis of HRP. To test this hypothesis, we treated cells with pronase to eliminate receptors for HRP. These experiments were performed at 4°C to avoid a possible proteolysis of cycling receptors. In the experiment shown in Fig. 8*A*, cells were incubated for 15 min at 4°C with pronase. About 85–95% of the binding activity was lost after treatment with pronase. Cells were then incubated at 37°C with 100 μg/ml HRP with or without cycloheximide. After 3 h, HRP uptake was found to be about 20% of control values. Between 3 and 6 h, we observed a rapid recovery of uptake capacity. However, such a recovery could be prevented by cycloheximide, suggesting that uptake was mediated by new receptors emerging on the cell surface between 3 and 6 h of incubation. The following experiments support this hypothesis. Pronase-treated cells were incubated at 37°C in the absence of HRP and assayed at varying times for HRP binding. As shown in Fig. 8*B*, only 10 to 20% of binding sites reappeared after 3 h of incubation. At 6 h, cells had recovered fully their binding capacity and even more, but this recovery was not observed in the presence of cycloheximide. In control experiments it was verified that pronase treatment of lymphoid cells did not alter the fluid-phase endocytosis of HRP (data not shown).

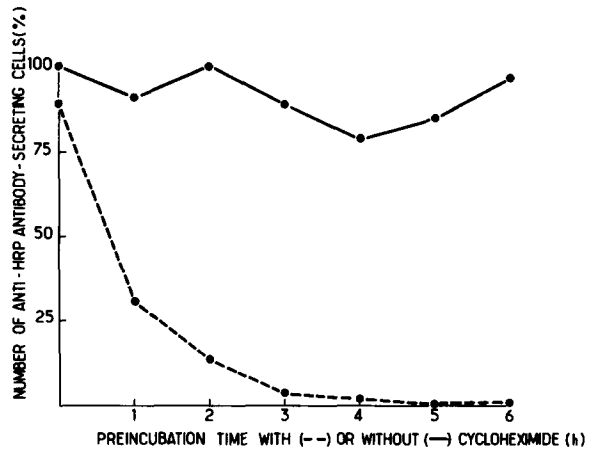

FIGURE 7 Inhibition of Ig secretion by cycloheximide. Popliteal lymph node cells from anti-HRP rats were incubated at 37°C for various periods of time with (●----●) or without (●——●) 20 μg/ml cycloheximide. After 0, 1, 2, 3, 4, 5, and 6 h, aliquots of cells were withdrawn and the number of anti-HRP antibody-secreting cells determined by a haemolytic plaque assay. Results are expressed as percent of the number of antibody-secreting cells found before incubation at 37°C (36,500/10^6 lymphoid cells). Each point is the mean of triplicate determinations.

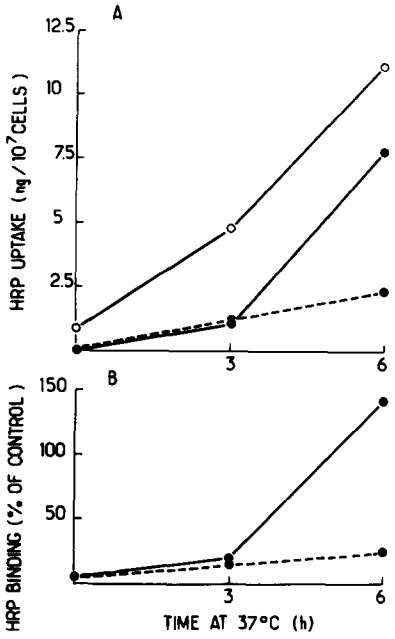

FIGURE 8 Binding and uptake of HRP by pronase-treated anti-HRP plasma cells. Macrophage-depleted popliteal lymph node cells were incubated for 15 min at 4°C in modified Click's medium without fetal calf serum containing 2 mg/ml pronase and 50 μg/ml DNAse II (●). Control cells (○) were maintained in the same medium without enzyme. The treatment with pronase led to approximately 90% decrease in HRP binding. (*A*) Cells were incubated at 37°C with 100 μg/ml HRP. Pronase-treated cells were cultured with (-----) or without (——) 20 μg/ml cycloheximide. After 3 and 6 h, aliquots of cells of each group were lysed with NP-40 to determine HRP uptake. (*B*) Cells were incubated at 37°C without HRP. Pronase-treated cells were cultured with (-----) or without (——) 20 μg/ml cycloheximide. After 3 and 6 h, aliquots of cells were assayed for HRP binding. The data are presented in terms of the percentage of HRP binding on pronase-treated cells to control cells.

This indicates that inhibition of HRP uptake induced by pronase digestion of anti-HRP-specific cells was not due to a general decrease of plasma membrane activity.

Effect of Monensin

The carboxylic ionophore monensin has been shown to interrupt the recycling of low density lipoprotein receptors in human fibroblasts (7). In our study the effect of this drug on the number of surface Ig and of antigen-binding sites expressed on B lymphocytes and plasma cells, respectively, has been tested. Cells were maintained for various times at 37°C in the presence of monensin and then assayed for HRP-anti-rat Ig Fab' conjugate or HRP binding. As shown in Fig. 9, no effect of monensin was found on B lymphocyte–binding activity. On the contrary, monensin induced a progressive loss of HRP-binding sites from plasma cell surface (Fig. 9). In addition, HRP uptake was inhibited by 50% after 3 h of incubation at 37°C in the presence of 10^{-6} M monensin. However, the inhibition of HRP uptake was not due to the blockade of the first step of endocytosis of HRP as shown by the following experiment. After a short preincubation for 15 min at 37°C with or without monensin, cells were incubated at 4°C with HRP, washed and transferred at 37°C for 30 min and 1 h to allow internalization of previously bound HRP. The amount of NP-40 releasable HRP increased at the same rate whether monensin was present or not in the medium (Table III).

DISCUSSION

The aim of this study was to determine whether cell differentiation state can affect receptor behavior during the receptor-mediated endocytosis process. B lymphocytes and plasma cells represent a convenient system to address this question. Indeed, B cell differentiation results in the development of endoplasmic reticulum and enlargement of the Golgi appa-

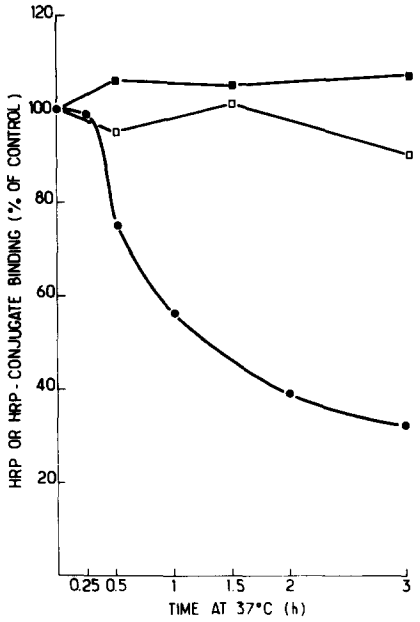

FIGURE 9 Effect of monensin on the number of surface Ig of B lymphocytes and on the number of anti-HRP surface receptors of plasma cells. Macrophage-depleted lymph node cells from nonimmunized and anti-HRP rats were maintained at 37°C without monensin (control cells) or with 10^{-6} M (□, cells from nonimmunized rats; (●) cells from anti-HRP rats) or 10^{-5} M (■, cells from nonimmunized rats) monensin. After various times at 37°C, aliquots of cells were washed and incubated 1 h at 4°C with HRP-labeled anti-rat Ig Fab' fragments (B lymphocytes) or HRP (plasma cells). After further washings, HRP binding was then determined. The data are presented in terms of the percentage of binding on monensin-treated cells to control cells. Results are the means of two separate experiments.

TABLE III

Effect of Monensin on the First Step of HRP Internalization

Monensin	Amount of HRP detected on 10^7 living cells incubated at 4°C	Amount of NP-40 releasable HRP/10^7 cells transferred at 37°C		
		0 min	30 min	1 h
	ng		*ng*	
–	0.82	0	0.28	0.55
+	0.80	0	0.21	0.55

Cells were preincubated with or without 10^{-6} M monensin for 15 min at 37°C. They were then incubated for 1 h at 4°C with 100 μg/ml HRP in the presence or absence of monensin. After three washings, a portion of cells was assayed for HRP binding. The other portion was transferred at 37°C in HRP-free medium with or without 10^{-6} M monensin. After 30 min and 1 h, HRP activity was measured before and after lysis of cells with NP-40. The differences between these two values give the amount of HRP liberated with NP-40. Lysis of cells incubated for 1 h at 4°C with HRP did not increase the amount of titrable cell-bound HRP (time 0 min) which indicates that no internalization occurs at this temperature.

ratus, allowing biosynthesis and secretion of antibody at a high rate. We took advantage in this study of the fact that both B lymphocytes and plasma cells express surface Ig that mediate endocytosis of antigen or anti-Ig antibodies.

Because the frequency of antigen-specific B lymphocytes in normal and immunized animals is extremely low, we used anti-rat Ig Fab' fragment as a model ligand, because of its capacity to react with all B lymphocytes. The binding of HRP-anti-Ig Fab' conjugate was followed by its progressive internalization and simultaneously the plasma membrane became progressively unable to bind the conjugate. Between 1 and 6 h of incubation at 37°C in the presence of conjugate, the total amount of cell-associated HRP remained constant. That indicates that during this time, no reappearance of free surface Ig occurred by neosynthesis, recycling, or the expression of an intracellular pool. However, after 1 h at 37°C in presence of conjugate, cell-associated HRP activity was twice greater than that titrated on cells incubated for 1 h at 4°C with the same saturating dose of conjugate. Experiments performed with cycloheximide or on cells treated with pronase allowed to exclude a rapid neosynthesis or the expression of an intracellular pool of surface Ig to explain this increase. Evidence for the absence of a large intracellular pool of membrane Ig in B lymphocytes has been already obtained by Andersson et al. (1). Table II shows that rise in cell-associated HRP activity between 1 h at 4°C and 1 h at 37°C was absent or much less pronounced when we used HRP-labeled anti-rat Ig $(Fab')_2$ bivalent conjugate instead of the monovalent conjugate. One explanation could be that a cross-linking ligand induces at 4°C membrane modifications leading to the recruitment of new receptors or antigenic determinants otherwise inaccessible. Similar data have been obtained by Bornens et al. (9). Indeed, these authors have found that rat thymocytes were able to bind twice as many molecules of concanavalin A as of succinyl-concanavalin A which, unlike the native molecule does not have the capacity to induce patches and caps on lymphocytes.

Recycling of small and progressively decreasing amount of surface Ig always complexed with the ligand cannot be totally discarded by our experiments. However, the main effect of the internalization of monomeric ligand-surface Ig complexes in B lymphocytes is a temporary down-regulation of surface Ig, which leads to a rapid shut off of the ligand uptake. These results confirm those previously obtained with multivalent ligands (antigen or anti-Ig antibodies) that induced redistribution of surface Ig on the plasma membrane before internalization (27, 35). In B lymphocytes, regeneration of surface Ig appears then to require *de novo* synthesis (45).

Plasma cells exhibit striking differences in the uptake of monomeric ligand. The first difference concerns the constant binding capacity of plasma cells with time of incubation at 37°C with HRP. The second difference concerns the continuous uptake of antigen with time of incubation at 37°C, that exceeds surface binding capacity of cells.

Antigen uptake proceeds continuously for at least 24 h (unpublished results). This indicates that, after one round of receptor-mediated endocytosis, anti-HRP receptors were again present on the cell surface to mediate new cycles of internalization.

The experiments with cycloheximide show that, in the absence of ligand, the rate of receptor turnover was slow and that the maintenance of binding activity was not due to *de novo* synthesis of receptors. Also, HRP uptake was not affected by blockade of protein synthesis. These data clearly demonstrates that receptors replacing at the cell surface those internalized with HRP were not resynthesized by plasma cells. In addition, in the presence of cycloheximide, antibody secretion was drastically reduced. Some effect on antigen internalization of soluble antibody present in the culture medium can then be ruled out.

Alternatively, continuous HRP uptake could be due to a progressive externalization of a large pool of pre-synthesized receptors inside the cells. However, after removal of surface-binding sites with pronase and in the absence of protein synthesis, plasma cells recovered after 6 h at 37°C only 10 to 20% of their binding activity. It should be noted that pronase-treated plasma cells were able to resynthesize new receptors faster than expected from the rate of receptor turnover found in untreated cells (no decrease in binding activity during 6 h of incubation with cycloheximide). Such a phenomenon has been already described for other receptors after treatment with a proteolytic enzyme (10). HRP uptake was also considerably lowered in pronase-treated cells and the remaining uptake could be attributed for the most part to fluid-phase endocytosis. These data then indicate that intracellular pool, if exists, was very small in comparison of the surface pool. Therefore, the best explanation for continuous HRP uptake is that antigen surface receptors that enter the cells are dissociated from their ligand and are reexported to the cell surface to be reused.

As shown in Fig. 9, monensin caused a progressive loss of receptors from the cell surface of plasma cells, but not of B lymphocytes. Given that the first step of the endocytic process was not affected, this could explain the inhibition of continuous HRP uptake we observed in monensin-treated cells. These data suggest that a constitutive endocytosis of receptors (or of a part of receptors) takes place in plasma cells. In secretory cells, monensin probably disrupts an ion gradient that is crucial for budding of vesicles from the Golgi complex, which leads to a blockade of secretion (38, 39, 44). In plasma cells this drug causes the immunoglobulins to accumulate within the Golgi complex, whose cisternae become dilated (38). In our experiments, a 10^{-6} M concentration of monensin diminished the number of anti-HRP antibody-secreting cells, detected by a local haemolysis plaque assay, by 80% in 2 h. These data raise then the possibility of a coupling between endocytosis and exocytosis in plasma cells. Such a coupling is thought to be necessary for the remodeling of cell surface after exocytosis in other secretory cells (15, 24, 33). In addition, secreted Ig is not concentrated in plasma cells, but is very likely released by continual exocytosis (38), which suggests that an extensive membrane traffic probably occurs in plasma cells (32, 40). However, given that monensin may act at several points along the endocytic pathway (14, 28, 34), further investigation is needed to firmly establish that membrane recycling takes place in plasma cells.

As mentioned in the introduction, evidence exists that some features of the receptor may determine whether it is degraded along with the ligand or recycled back to the plasmalemma. Membrane Ig of B lymphocytes seems to behave like receptor of hormones or neurotransmitters that are not recycled in the presence of the ligand (17) or recycled at a very slow rate (16). However plasma cells seems to differ in the cycling of surface Ig from their progenitors, B lymphocytes. As far as studied, ligands (antigen, monovalent, or divalent anti-Ig antibodies) are delivered to the same intracellular sites in B lymphocytes

and plasma cells (3, 20, 21). One possibility to explain surface Ig recycling in plasma cells could be that a new membrane traffic or the amplification of a membrane traffic already existing in B lymphocytes is established in plasma cells between sites of receptor-mediated endocytosis and plasmalemma. This emphasizes that cell differentiation state can affect endocytic receptor recycling.

We thank Colette Jouanne for excellent technical assistance.

This work was supported by research grant 80-7-0189 from Délégation Générale à la Recherche Scientifique et Technique.

Received for publication 21 June 1983, and in revised form 29 November 1983.

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