PLASMA MEMBRANE AND INTERNALIZED IMMUNOGLOBULINS OF LYMPH NODE CELLS STUDIED WITH CONJUGATES OF ANTIBODY OR ITS FAB FRAGMENTS WITH HORSERADISH PEROXIDASE

JEAN-CLAUDE ANTOINE, STRATIS AVRAMEAS, NICHOLAS K. GONATAS,
ANNA STIEBER, and JACQUELINE O. GONATAS

From the Unite d'Immunocytochimie, Departement de Biologie Moleculaire, Institut Pasteur, Paris, France and the Division of Neuropathology, Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19174

ABSTRACT

Normal rat and mouse lymphoid cells were incubated at 0°–4°C for 1 h with purified rabbit or sheep antirat (mouse) immunoglobulin (Ig)-horseradish peroxidase (PO) conjugates or with Fab fragments of antibody coupled with peroxidase. Cells were subsequently washed and incubated in fresh medium, without labeled antibody or Fab fragments for 5–30 min at 20° or 37°C. With the use of the diaminobenzidine (DAB) method, distribution of peroxidase was studied in the light and electron microscopes. Fab fragments of antirat Ig antibody were iodinated with ¹²⁵I and subsequently coupled with horseradish PO. Plasma membrane and internalized immunoglobulins were detected by electron microscope autoradiography and peroxidase cytochemistry. Single- (Fab-PO), and double- ([¹²⁵I]Fab-PO) labeled lymphoid cells showed identical patterns of surface or internal distribution of immunoglobulins.

In the electron microscope, Fab-PO conjugates at 0°–4°C resulted in a diffuse specific staining of the plasmalemma of lymphocytes and plasma cells. Most of the small dark lymphocytes (T cells?) did not show plasma membrane Ig. Macrophages did not show plasmalemma staining, but displayed nonspecific cytoplasmic staining after incubation at 20° or 37°C with antibody or Fab-PO conjugates. Lymphocytes and plasma cells, after incubation with antibody-PO conjugates at 0°–4°C, had patchy deposits of oxidized DAB on their plasma membranes. Macrophages, similarly treated, had no plasmalemmal staining.

Patch and cap formation on the plasma membrane of lymphocytes and plasma cells was seen regularly after antibody-PO incubation at 37°C.

Internalization patterns were different in lymphocytes and plasma cells. In lymphocytes, peroxidase staining was observed in small round or oval vesicles clustered at one pole of the cell (30 min at 37°C). In plasma cells, peroxidase staining was seen in clusters of tubules resembling the Golgi apparatus. Internalization of plasma membrane IgG was less pronounced after antibody-PO labeling as compared to Fab-PO labeling.

Recent immunocytological studies, utilizing various markers, have demonstrated the presence of immunoglobulins (Ig)¹ on the plasma membrane of lymphoid cells (1-5). Variations in experimental conditions and in the labeled antibody or its Fab fragment have resulted in different staining patterns (1-9). It is generally agreed that incubation of unfixed cells with monovalent antibody fragment at 4°C results in a diffuse staining of the plasma membrane of lymphoid cells, while at 4°C, the bivalent antibody molecule produces a patchy surface staining. When cells, prelabeled with antibody at 4°C, were incubated at 37°C, segregation of surface immunoglobulin to patches or caps, and internalization (endocytosis) was observed (6, 9, 10). It has been argued that the observed changes in distribution of surface immunoglobulins reflect rearrangements of antigens by the labeled antibody, which are consistent with current views of the fluid mosaic model of the plasma membrane (11).

In this paper we present electron microscope observations on rat lymph node cell plasma membrane immunoglobulins, obtained with the use of peroxidase (PO)-labeled rabbit and sheep antirat Ig purified monospecific antibody or their Fab fragments. In order to determine whether labeling of Fab fragments of antibody with horseradish PO alters the distribution and internalization (endocytosis) patterns of immunoglobulins, we have utilized [¹²⁵I]Fab-PO conjugates.

We have confirmed previously reported observations by other investigators on the differential distribution of immunoglobulin molecules resulting from incubations of lymphocytes at various temperatures with bivalent or monovalent antibody labeled with fluorescein, ferritin, or ¹²⁵I. In addition, we have been able to demonstrate that (a) lymphocytes and plasma cells have different internalization patterns of surface immunoglobulins, (b) there is a morphologically different class of unlabeled lymphocytes, and (c) labeling with monovalent antibody is followed by massive internalization.

These experiments demonstrate the feasibility of the peroxidase labeling for the study of surface and internalized plasma membrane (immunoglobulins)

moieties. Peroxidase cytochemistry, because of its better resolution than autoradiography, allows a more precise localization of the internalized (endocytosed) molecules.

MATERIALS AND METHODS

Animals

Male or female 5-mo old Sprague-Dowley rats were used. 3-mo old C₃H mice were also used.

Preparation of Antisera and of Purified Antibodies

Rabbit and sheep antirat Ig antisera were prepared according to procedures already described (5, 12). These antisera, after immunoelectrophoresis, revealed IgG 2a and IgG 2b lines, and contained anti-Fab antibodies. To prepare anti-Fcγ and anti-Fab antibodies, the antisera were passed on a rat Fab fragment immunoabsorbent prepared with glutaraldehyde (13). The adsorbed anti-Fab antibodies were isolated by acid buffer elution. After several passages on the Fab immunoabsorbent, the antiserum was found to contain only anti-Fcγ antibodies. These latter were then isolated by passage on a rat IgG immunoabsorbent.

Monospecificity of these antibodies was tested by double immunodiffusion. Fab fragments which were used for the preparation of the immunoabsorbent were obtained by papain digestion of rat IgG according to the method of Porter (14).

Preparation of Fab Antibody Fragments

Two procedures for the preparation of Fab fragments were followed:

PREPARATION OF FAB FRAGMENTS ACCORDING TO THE METHOD DESCRIBED BY PORTER (14): Fab fragments were prepared by papain digestion of isolated sheep antirat Ig antibodies. On immunoelectrophoresis and double immunodiffusion, the fragments were immunologically pure.

PREPARATION OF FAB FRAGMENTS BY AN IMMUNOABSORBENT: Globulins of sheep antirat Ig antiserum were precipitated by 40% ammonium sulfate, dissolved in distilled water, and dialyzed against 0.1 M potassium phosphate buffer, pH 7.5. The dialysate was digested with papain as described (14). After dialysis against phosphate-buffered saline (PBS), the papain digest was passed on a rat IgG immunoabsorbent. Proteins eluted from the immunoabsorbent were filtered on a Sephadex G-100 (2.5 × 91 cm) equilibrated with PBS. According to immunological tests and ultracentrifugal analysis, these fragments were pure.

¹ *Abbreviations used in this paper:* DAB, 3,3'-diaminobenzidine; Ig, immunoglobulin; IgG, immunoglobulin G; PBS, phosphate-buffered saline; PO, peroxidase.

Coupling of the Isolated Antibodies and of Their Fab Fragments with Peroxidase

COUPLING WITH PEROXIDASE: Purified antibodies or their Fab fragments were labeled with peroxidase (Boehringer Mannheim RZ = 3) following a two-step procedure (15). In order to remove unreacted peroxidase and antibodies or Fab fragments, conjugates were filtered on a Sephadex G-200 column equilibrated with PBS (2.5×100 cm). The labeled Fab preparation was passed through a Sephadex G-200 column calibrated for molecular weight determinations. Tubes corresponding to a molecular weight of 80,000 and containing Fab-PO conjugate in 1:1 molar ratio were pooled and used in the present experiments. This preparation did not precipitate the corresponding antigen.

Normal rabbit and sheep IgG and their Fab fragments were prepared following procedures already described (14, 16). Normal IgG and Fab fragments were conjugated with peroxidase following procedures described above and were used as controls.

Iodination of Fab Fragments

Lactoperoxidase-catalyzed iodination was performed at room temperature in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) according to the method of Marchalonis (17). The reaction mixture contained 1.0 mg Fab, 100 μ g of lactoperoxidase (Calbiochem, San Diego, Calif.), and 0.5 mCi of carrier-free [^{125} I]Na (New England Nuclear, Boston, Mass.) in a total volume of 300 μ l. The reaction was initiated by the addition of 10 μ l of 8.8 mM H_2O_2 followed by nine sequential additions at 15-s intervals to maintain the reaction. The reaction was stopped by the addition of 100 μ l of 10^{-2} M KI. The preparation was dialyzed overnight at 4°C against PBS and then applied to a Sephadex G-200 column and developed with PBS. The specific activity of [^{125} I]Fab obtained was 0.33 mCi/ml.

Preparation of Cell Suspensions and Their Incubation with Conjugates

Cell suspensions from cervical or pool of lymph nodes of normal rat were prepared and washed by successive centrifugations as described in a previous paper (5). Conditions of incubation for each experiment are given in Results. Briefly, cells (5×10^7 cells/ml) were incubated for 1 h at 4°C with various concentrations of conjugates (25–400 μ g/ml), washed four times in the medium, then incubated at different times at 20° or 37°C before observation.

Before and after each experiment, the percentage of dead cells was determined by trypan blue exclusion.

Light Microscope Preparations for Immunoperoxidase

After the cells were incubated with peroxidase conjugates, they were washed four times with the cold medium

(4°C). Histochemical detection of peroxidase was carried out following procedures already described (5, 12).

Electron Microscope Preparations for Immunoperoxidase

Histochemical detection of peroxidase was performed according to the method of Graham and Karnovsky (18). For electron microscope studies, cells were fixed for 2 h at room temperature in 4% paraformaldehyde in 0.1 M cacodylate buffer. After the peroxidase stain, cells were left overnight in a 0.1 M cacodylate buffer, pH 7.4, 0.2 M sucrose, then postfixed in osmium tetroxide, dehydrated, and embedded in Epon according to standard procedures. Endogenous peroxidase activity in lymph node cells was detected with the identical method. For the study of uptake of horseradish PO or of "activated" peroxidase (peroxidase after exposure to glutaraldehyde), unfixed cells were incubated for 1 h at room temperature or at 4°C in buffered physiologic saline (pH 7.3) containing 100 μ g/ml of peroxidase or activated peroxidase, washed, fixed, and processed as above.

Electron Microscope Autoradiography

Cells ($3-5 \times 10^7$) from cervical lymph nodes of normal Osborn-Mendel white rats, weighing 150–200 g, were suspended in 5 ml of Earle's balanced salt solution containing 100 μ g of [^{125} I]Fab PO per 1 ml. After incubation for 1 h, at 4°C, cells were washed three times in Earle's at 4°C and subsequently fixed for 30 min at room temperature in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.35. For internalization experiments, cells after the initial incubation at 4°C with [^{125} I]-Fab-PO and the three washed in Earle's, were transferred to Earle's solution or to medium 199 (Grand Island Biological Co., Grand Island, N. Y.) for 5, 15, and 30 min at 37°C. After fixation in paraformaldehyde and three washes in Earle's, cells were incubated with diaminobenzidine tetrahydrochloride (DAB) for 10 min at room temperature (5 mg DAB, 10 ml 0.1 M Tris buffer, pH 7.35, 1 drop H_2O_2 30% vol/vol [18]). After postfixation for 1 h in 1% osmium tetroxide in 0.1 M phosphate or cacodylate buffer, pH 7.35, cells were embedded in Araldite. Sections with light-gold interference color (about 1,000 Å thick) were processed for ultrastructural autoradiography according to the method of Salpeter (19–22).

RESULTS

Study at the Light Microscope Level

PATTERNS OF STAINING PRODUCED BY ANTIRAT Ig ANTIBODIES: Cells were incubated for 1 h at 4°C with the conjugate, washed in order to eliminate the excess of reagent, and then incubated at 20° or 37°C for 1 h. Positive cells showing a diffuse staining of the plasma membrane were observed with preparations incubated at 4°C. In addition to the diffuse staining of the

plasma membrane, small spots were sometimes visible on a few cells; cells could be stained with anti-Fab and anti-Fc γ antibodies, indicating that the two IgG fragments are exposed on the cell membrane. Many of the cells incubated at 4°C and then postincubated for 1 h at 20° or at 37°C showed peroxidase staining localized at one pole of the cell in the form of caps, or in the form of randomly distributed small spots.

PATTERNS OF STAINING PRODUCED BY FAB ANTIBODY FRAGMENTS AGAINST ANTIRAT Ig: At 4°C, staining was uniformly distributed, but occasionally small spots were sometimes visible. Frequently, the staining was concentrated at one pole of the cell. Under all conditions tested, the staining with Fab fragments was lighter than that obtained with whole anti-Ig antibodies. After 5 min at 37°C, a great number of anti-Ig antibody-treated cells showed a cap or spot staining, while with Fab fragments incubation for at least 30 min at 37°C was needed to obtain a notable percentage of cells having spots randomly distributed or localized at one pole of the cell. These spots were shown by electron microscopy to correspond mostly to endocytosed material; in the elec-

tron microscope, clear spots or caps were not seen on the surface of cells incubated with Fab fragment of antibody conjugated with peroxidase.

Ultrastructure

Only normal rat lymph node cells were examined in the electron microscope. Since DAB cytochemistry may suffer from limitation of diffusion of reaction product and from the problem of endogenous peroxidasic activity, a study of these two parameters was preformed.

Endogenous peroxidase activity was detected regularly in granules of the few polymorphonuclear leukocytes and eosinophiles found in our lymph node cell preparations and in macrophages; however, none of these cell types had deposits of oxidized DAB-osmium complexes on its plasma membrane.

The validity of the "double" marking ($[^{125}\text{I}]\text{Fab} + \text{PO}$), and marked coincidence of the two labels, are illustrated in Fig. 1. In this experiment, normal rat lymph node cells were incubated with iodinated Fab fragments of antirat Ig which were also coupled with peroxidase. There is a diffuse staining of the entire plasma membrane by DAB,

FIGURE 1 Autoradiography on a DAB-stained rat lymphocyte, incubated at 0°–4°C for 1 h, with $[^{125}\text{I}]\text{Fab}$ fragments of antibody-conjugated with peroxidase. Note surface DAB staining and almost regular distribution of grains at plasma membrane. $\times 11,000$.

and the heavy labeling of the plasma membrane by ^{125}I , in an almost regular pattern, suggests that diffusion artifact of the DAB staining may not be important. The diffusion of the oxidized DAB will be further discussed in regards to the spotty staining obtained by antibody-PO (Fig. 6).

Small and large lymphocytes, and plasma cells had no endogenous peroxidase except in mitochondria. When lymph node cells were incubated at 4°C for 1 h with the Fab-PO of sheep antirat Ig, continuous plasma membrane staining of lymphocytes and plasma cells resulted (Figs. 2, 3). The thickness of the oxidized DAB deposit was on the average 250–350 Å (Fig. 4). The staining pattern was homogeneous or globular, but no standard size of the positive globules could be distinguished. There was no diffusion of staining within the cytoplasm of the cell, and frequently “unit” structure of plasma membrane formed the innermost barrier of the stained region (Fig. 4). It is impossible to define precisely the diffusion of the reaction product along the surface of the positive cells; if diffusion of oxidized DAB along the plasma membrane exists, this does not prevent the visualization of a “patchy” or interrupted pattern of

staining of the surface of lymphocytes incubated with the entire bivalent antibody molecule (Fig. 6).

Occasionally, cells stained with DAB, without any prior incubation with labeled antibody, showed a fine accentuation of the plasma membrane, measuring about 75–100 Å (Fig. 5); this density was not seen when DAB or H_2O_2 was omitted from the incubation medium. The nature of this staining is unknown, but it is clearly distinguishable from the plasma membrane labeled with antibody or its Fab fragment-PO conjugate (compare Figs. 4 and 5).

Comparison between Rabbit Antirat Ig-PO and Fab-PO (Figs. 2, 3, 6)

At 4°C , surface staining was continuous with Fab-PO and almost always in patches or spots after incubation in rabbit antirat Ig-PO and, only occasionally, plasma membrane labeling by the entire antibody-PO was continuous at 4°C . Almost all labeled cells were large lymphocytes with numerous ribosomes, and a sparse endoplasmic reticulum, and other organelles. Under the conditions of fixation used in these experiments, a large number of small lymphocytes appeared darker

FIGURE 2 Rat lymphocyte: continuous plasma membrane staining. Sheep antirat Fab-PO. 1 h at 4°C . Preparation stained with DAB, but not with lead or uranyl. $\times 19,000$.

FIGURE 3 Rat plasma cell incubated with Fab fragment of rabbit antirat Ig-PO for 1 h at 4°C (stained only with uranyl acetate). Plasma membrane is stained with DAB. $\times 18,000$.

FIGURE 4 Fab fragments of sheep antirat Ig, coupled with peroxidase (Fab-PO), incubated for 1 h at 4°C with rat lymph node cells, fixed, and subsequently stained with DAB. Note that trilaminar structure of plasma membrane is discernible, despite density of oxidized-DAB-osmium black precipitate. Thickness of floccular DAB staining is 250–350 Å. Note the absence of diffusion of stain in cytoplasm. $\times 75,000$.

FIGURE 5 Rat lymphocyte fixed and stained only with DAB. Occasionally, staining of plasma membrane 75–100 Å in thickness, results from this treatment. $\times 65,000$.

than the positively stained larger and paler lymphocytes (Figs. 7, 8); the plasma membrane of the darker and smaller lymphocytes was rarely stained with the entire antibody or its Fab fragment (Table I). About 17% of rat plasma cells had surface IgG, while about 40% of lymphocytes had surface Ig. Less than 3% of the small dark lymphocytes had surface Ig (Table I).

Internalization

LYMPHOCYTES: When lymphocytes incubated with Fab-PO were transferred to 37°C for 5–30 min, intense staining was seen in round or tubular vesicles, usually clustered near a nuclear invagination (Fig. 9). “Internalization” of stain occurred while surface staining was retained, diminished in intensity, or completely absent. Spot or cap formation was not seen with Fab-PO treatment of cells and it seemed that cap formation was not a prerequisite for internalization; the surface staining obtained after incubation at 37°C of lymphocytes labeled with Fab-PO was thin, and clear patches or caps of stain were never observed. At 30 min in 37°C, internalization was more pronounced than after 5 min in 37°C, but no significant difference in the pattern of staining was

observed between cells incubated at 5 and 30 min. By comparison with the Fab-PO, the label of cells incubated with the entire antibody internalized poorly (Fig. 10), while spots or caps were seen very frequently (Figs. 10, 11).

Internalization of surface immunoglobulins, studied with [¹²⁵I]Fab-PO, yielded results identical to those obtained by Fab-PO (Fig. 12). Frequently, clusters of grains were noted in one pole of the lymphocyte, and grains were in an area in which DAB-positive vesicles or vacuoles were present. Iodinated normal Fab-PO gave essentially negative results with a “background” grain count ranging from one grain per 800–1,200 μm^2 with the exception of fields occupied by macrophages.

PLASMA CELLS: The surface labeling and the internalization patterns of plasma cells were quite different from those of lymphocytes. The entire antibody-PO conjugates gave a spotty or patchy staining, while Fab-PO resulted in a continuous staining of the plasma membrane which was somewhat thinner than the label obtained on lymphocytes (Figs. 3, 13). When cells were incubated for 5–30 min at 37°C after an initial 1-h incubation at 4°C in Fab-PO, extensive internal positive tubular or vesicular profiles, usually in

FIGURE 6 Patchy staining of plasma membrane produced by rabbit antirat Ig-PO (30 min at 4°C). Stained with lead and uranyl. $\times 23,000$.

FIGURE 7 Note continuous peripheral staining of large lymphocyte and absence of plasma membrane staining of small dark lymphocyte. Rat lymphoid cells incubated with sheep antirat-Fab-PO. 1 h, 4°C (stained with lead and uranyl). $\times 8,000$.

FIGURE 8 Enlargement of Fig. 7. $\times 32,000$.

TABLE I
Plasma Membrane Staining after Incubation for 1 h at 4°C with Fab Fragment of Sheep Antirat Ig-PO*

Macrophages		Plasma cells		Lymphocytes		Small "dark" lymphocytes	
Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
0	11	21	98	151	204	3	98

* Counts performed in the electron microscope.

clusters, were observed. The tubular profiles were in the nuclear "hof" and probably represented the Golgi apparatus (Fig. 13).

MACROPHAGES: The two types of identified macrophages did not show plasma membrane staining after Fab or whole antibody-PO incubations.

Controls

With the exception of macrophages, which showed only internal staining not distinguishable from endogenous peroxidasic activity, lymphocytes and plasma cells never showed intracytoplasmic or surface stain with normal rabbit IgG-PO or with its Fab fragment-PO.

DISCUSSION

Immunocytological studies of the plasma membrane Ig of lymphocytes have introduced new

insights into the organization and redistribution of surface immunoglobulins. Originally, Taylor et al. (6) and subsequently de Petris and Raff (10, 23), with the use of fluorescein or ferritin-labeled antibodies, found that at incubation temperatures of 0°–4°C, a patchy diffuse pattern of staining of Ig molecules is obtained, while at temperatures of 22°–22°C but a redistribution of the label in caps and internalization (endocytosis) occur. Essentially similar results were obtained by Perkins et al. (24) and Unanue et al. (9) with the use of ultrastructural autoradiography of ^{125}I -labeled antibodies.

The following data of others, pertinent to our findings and to the subsequent discussion, are cited:

(a) Diffuse labeling of Ig of lymphocyte plasma membranes is obtained with monovalent Fab-anti-Ig, at all temperatures (6, 7, 23).

(b) Labeling of Ig of lymphocyte plasma membranes by bivalent antibody results in patch and cap formation (6, 7, 10, 23).

(c) Patch and cap formation is not prerequisite for internalization (endocytosis) of the label (23).

(d) Both lymphocytes and, to a lesser degree, plasma cells have surface Ig (24, 25).

(e) Although endocytosis of labeled plasma membrane has been observed, its exact fate (i.e. lysosomes, Golgi apparatus, etc.) has not been ascertained because of resolution limitation of the employed autoradiographic methods (3), while peroxidase labeling of whole antibody showed only in lymphocytes, the marker to be associated with large vesicles near the Golgi apparatus (25).

Our findings with peroxidase labeling of purified antibody or its Fab fragments confirm certain results obtained by others with different techniques; Fab-PO results in a diffuse staining of the plasma membrane and no significant patch or cap formation is seen when Fab-PO-labeled cells at 4°C are transferred to 37°C (10, 23). To the

contrary, antibody-PO results in a patchy staining, even at 4°C, and patch and cap formation is seen frequently after labeling with bivalent antibody. The internalization (endocytosis) of the peroxidase-labeled membrane has been observed very frequently after Fab-PO treatment and less frequently after antibody-PO treatment. Extensive cross-linking of a purified antibody with the plasma membrane IgG may be responsible for the observed limited internalization of the antibody-PO conjugates; if, instead of a purified antibody, the entire IgG fraction is used for peroxidase labeling, internalization of antibody-PO is observed (25).

The cross-linking of antibody or Fab molecules with peroxidase with glutaraldehyde, as used in these experiments, results in a covalent bond (15), and it is therefore unlikely that the observed peroxidasic staining is due to dissociated peroxidase molecules from antibody or Fab fragments. Furthermore, the double-labeling experiments with [¹²⁵I]Fab-PO have shown a similar distribu-

FIGURE 9 Rat lymphocyte, incubated 1 h at 0°–4°C with sheep antirat Fab-PO, washed, and incubated for 30 min at 37°C. Note intense intracytoplasmic staining in the form of vesicles, tubules, and larger granules. Unstained with lead or uranyl. $\times 16,000$.

FIGURE 10 Rat lymphocyte, incubated for 1 h at 4°C with rabbit antirat Ig-PO, washed, and transferred to 37°C for 30 min. Note absence of plasma membrane staining, except in lower left part of cell periphery, where staining is patchy. Compare internal staining with Fig. 9 (not stained with lead or uranyl). $\times 22,000$.

FIGURE 11 "Cap" formation on rat lymphocyte after incubation with rabbit antirat Ig-PO at 4°C and then transfer to 37°C for 30 min. Stained with lead and uranyl. $\times 10,000$.

tion of the autoradiographic and cytochemical marker. It is, therefore, unlikely that the observed surface or intracytoplasmic peroxidasic staining results from the interaction of the cell with the enzyme rather than with the labeled molecule. With the use of peroxidase conjugates of purified antibody or Fab fragments, we have detected significant staining of the plasma membrane of plasma cells (Fig. 3), while with the use of radio-labeled or peroxidase-labeled immunoglobulin fractions, plasma cells were weakly labeled (9, 25). We believe that the labeling of the plasma membrane IgG of plasma cells is valid, and this view is supported by the recognition of internalization of the label (Fig. 13). Furthermore, the internalization pattern in lymphocytes is different from that in plasma cells (Figs. 3, 13). This difference may be related to the secretory function of the plasma cell, and to the more complex functions of the B lymphocytes.

FIGURE 12 Rat lymphocyte incubated for 1 h at 0°–4°C with [^{125} I]Fab fragment of sheep antirat IgG antibody coupled with PO. Washed and incubated at 37°C for 30 min. Note intracytoplasmic localization of certain but not all grains. $\times 10,000$.

FIGURE 13 Rat plasma cell incubated with Fab fragment of rabbit antirat Ig-PO and subsequently transferred to 37°C for 30 min. Note internal staining of peroxidase in the form of elongated tubules or vesicles. Stained only with uranyl. $\times 20,000$.

Three additional results obtained with the peroxidase labeling of surface IgG are worth commenting upon. (a) Surface Ig receptors on macrophages were not identified; (b) most small dark lymphocytes (Figs. 7, 8) were not stained by rabbit antirat Ig-PO or by sheep antirat Ig-PO (Table I). This observation raises the possibility that T cells, lacking surface Ig detectable by these morphologic methods, are among the population of the small dense lymphocytes (24, 26). In that respect, our findings are similar to those of Perkins et al. who described a type of thymic lymphocyte with surface Ig which displayed a lower nuclear-cytoplasmic ratio as compared to other lymphocytes, a paler cytoplasm, and fewer ribosomes than most thymus lymphocytes (24). (c) Surface Ig must be easily accessible to antibodies since Fab and Fc γ fragments of the molecules are detected by corresponding antibodies. However, it is not known if one molecule has its two types of fragments exposed or if there is preferential masking of Fab or Fc γ fragments.

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