

QUANTITATIVE ULTRASTRUCTURAL AUTORADIOGRAPHIC STUDIES OF IODINATED PLASMA MEMBRANES OF LYMPHOCYTES DURING SEGREGATION AND INTERNALIZATION OF SURFACE IMMUNOGLOBULINS

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

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

ABSTRACT

Rat spleen lymphocytes were iodinated (^{125}I) with lactoperoxidase. Quantitative autoradiographic studies on cells fixed immediately after iodination showed 19–24% of intracytoplasmic grains at 3HD and over from the plasma membrane. Normalization of grain density distribution and comparison of resulting curves with the universal curve of grain scatter of ^{125}I showed that a significant percentage of intracytoplasmic grains (36%) originates from intracytoplasmic labeled sources rather than from scattering from the heavily labeled plasma membrane. Damaged cells had a threefold grain density than intact cells.

Radioactivity counts in sliced I^1A lyacrylamide gels of iodinated cells revealed 65–72% of total radioactivity in five peaks of apparent mol wt of 44, 50, 57, 90 and 195 thousand daltons. Segregation and internalization of anti-immunoglobulin-Ig-horseradish peroxidase (HRP) complexes from the iodinated plasma membrane proteins of lymphocytes was studied with quantitative autoradiography (^{125}I) and peroxidase cytochemistry; 64% of grains at 1.5HD (1,500 Å) from the plasma membrane were within the cap zone, and 36% of grains remained outside the capped immunoglobulins; 45–57% of grains internalized together with Fab-anti-Ig-Ig-HRP, and 68% of grains internalized together with anti-Ig-Ig-HRP.

These studies indicate that (a) iodination of rat spleen lymphocytes results in a significant internal labeling and that (b) immunoglobulins segregate into caps and internalize together with other iodinated plasma membrane proteins while a significant percentage of iodinated proteins (36%) are excluded from the immunoglobulin caps or internalization sites (32–55%).

In 1971, Taylor et al. (31) reported that plasma membrane (surface) immunoglobulins of lymphocytes incubated with fluorescein-labeled anti-immunoglobulin antibodies aggregate into patches, form a polar cap, and undergo endocytosis (inter-

nalization). Energy and temperature (22–36°C) are necessary for capping and endocytosis of plasma membrane immunoglobulins (7, 20, 25, 33, 34). Capping of anti-immunoglobulin-immunoglobulin complexes is independent of cell move-

ment (34). Endocytosis of surface immunoglobulins of lymphocytes and plasma cells has been observed after incubation with monovalent (Fab) antibody fragments, but capping of surface immunoglobulins requires previous incubation with the bivalent antibody (2, 7). Immunoglobulins as well as several other surface antigens of lymphocytes show redistribution and endocytosis after exposure to appropriate antibodies or ligands (4, 6, 13, 24, 25, 26, 37); furthermore, certain plasma membrane antigens segregate together after exposure to antibody against either one of the antigens, while other antigens move quite independently (6, 24, 25). These experiments have suggested that plasma membrane proteins of lymphocytes segregate from the rest of the membrane and move independently through the fluid matrix of the plasma membrane (27). It has not yet been established whether (a) the segregation and the internalization of plasma membrane Ig is induced by the anti-Ig antibody or whether (b) other plasma membrane proteins are associated with the migration of the Ig molecules. In order to gain further information regarding these two questions, we have examined with ultrastructural methods the segregation of plasma membrane immunoglobulins (Ig) from lactoperoxidase-iodinated plasma membrane proteins. Lactoperoxidase is a nonpermeant probe which catalyzes the iodination of exposed membrane proteins of intact cells, and has been used for biochemical studies of plasma membranes (11, 16, 17, 21, 23, 29, 32, 35). Since a systematic quantitative ultrastructural autoradiographic study of lactoperoxidase-iodinated cells is not available, we have performed this study, with the methods described by Salpeter, on cells fixed for autoradiography immediately after iodination or after incubation with anti-Ig antibody (8, 9, 12, 17, 21, 23, 28). Rat spleen cells iodinated with lactoperoxidase were incubated with conjugates of anti-Ig antibody and horseradish peroxidase (HRP).

Subsequently, cells were incubated at 36°C, and the redistribution of plasma membrane immunoglobulins and iodinated proteins was studied with peroxidase cytochemistry and quantitative ultrastructural autoradiography (double labeling) (12).

MATERIALS AND METHODS

Preparation of Cells

Spleen cells of young adult Osborn-Mendel white rats obtained from West Jersey Biological Supply Farms, Wenonah, N. J., were prepared on a Hypaque-Ficoll

gradient. Spleens, dissected free of fat and adjacent pancreatic tissue, were minced at room temperature in Earle's balanced salt solution BSS buffered with 10 mM of HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*-ethano sulfonic acid; Schwarz-Mann Div., Becton, Dickinson, & Co., Orangeburg, N. Y.). 64.8 g of Ficoll (Ficoll 400, Pharmacia Fine Chemicals AB, Uppsala Sweden) were dissolved in 720 ml of distilled H₂O (solution A), and 204 ml of 50% hypaque (Hypaque sodium 50%, Winthrop Laboratories, Div. of Sterling Drugs, Inc., New York), were mixed with 96 ml of distilled water (Solution B). The two solutions, A and B, were thoroughly mixed, and 5 ml of the mixture were placed in a 10-ml centrifuge tube. The spleen cell suspension, filtered through a 100-mesh nylon gauze and washed three times in Earle's BSS buffered with HEPES buffer, in a vol of 5 ml (maximum concentration 10⁹ cells per 5 ml), was layered on top of the Ficoll-Hypaque gradient, and centrifuged for 40 min at 100 g in a desk-top centrifuge at room temperature. Cells were taken in Earle's BSS buffered with 10 mM of HEPES buffer and counted with 0.5% trypan blue. Preparations with damaged cells (trypan blue positive) exceeding 2% were not used for iodination.

Iodination of Cells

The method of Marchalonis with some modifications was adopted (10, 23). 1 × 10⁸ cells in 2 ml of buffered Earle's BSS, 1 mCi of ¹²⁵I (New England Nuclear, Boston, Mass.), and 100 μg of lactoperoxidase 412 nm/280 nm = 0.83 (Calbiochem, La Jolla, Calif.) were gently mixed at room temperature; 10 μl of a 0.0075% solution of hydrogen peroxide (H₂O₂) was added twice at 5-min intervals. Iodination was also performed at 4°C with two additions of H₂O₂ at 10-min intervals. The reaction was terminated by the addition of 20 vol of cold (4°C) buffered Earle's BSS containing 10⁻³ M sodium iodide. The entire procedure took 1 h. In separate experiments, H₂O₂ was produced with glucose and glucose oxidase according to the method of Hubbard and Cohn (16). Cells labeled by both methods showed similar incorporation of iodine and similar viability as judged by trypan blue exclusion. The amount of lipid labeling was determined by extracting, with ether-ethanol (3:2), aliquots that were precipitated by 10% trichloroacetic acid (TCA) onto Whatman 3 filter disks. Less than 5% of the TCA-precipitable count was found in the solvent phase.

To exclude the possibility that extraneous proteins were adsorbed to cells, 1 ml of fetal calf serum iodinated with the chloramine T method of Hunter and Greenwood (18) (2.6 × 10⁶ cpm/ml) was incubated with 4 × 10⁷ splenic lymphocytes at 25°C for 10 min and subsequently washed with cold buffered Earle's solution. After three washes, only 0.2% of the radioactivity was associated with the cells. Low-level labeling was also observed when lactoperoxidase was omitted from the complete reaction system (1.1% of the TCA-precipitated label).

Preparation of Anti-immunoglobulin Antibodies, Their Fab Fragments, and Conjugates with HRP

A description of the employed methods has been given in previous papers (2, 14). HRP was covalently bound to antibodies with glutaraldehyde (3). The purity of Fab fragments was confirmed by double immunodiffusion showing no precipitation between antigen (rat IgG) and HRP-labeled Fab fragments. Also, the purity of Fab fragments was confirmed by ultracentrifugal analysis in a Beckman model E ultra-centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 59,780 rpm with Schlieren optics at 23°C. At a concentration of 10 mg/ml, Fab fragments showed only a single symmetrical peak with a S_{20}° value of 3.5S. In order to remove unreacted peroxidase and Fab fragments, conjugates were filtered on a Sephadex G200 column equilibrated with phosphate-buffered saline (PBS) (2.5×100 cm), and calibrated for molecular weight determinations. Tubes corresponding to a mol wt of 80,000 and containing Fab-peroxidase conjugate in 1:1 molar ratio were used in these experiments. For the ultrastructural detection of HRP, the method of Graham and Karnovsky was applied as previously described (2, 14, 19).

Incubation of Cells with Anti-Ig Antibody, its Fab Fragments, or With Control (Normal Fab Fragments of Sheep IgG)

After iodination and repeated washes, cells were incubated with 25–50 mg of antibody per 1×10^7 cells, at 4°C for 1 h; $5-10 \times 10^7$ cells were suspended in 1 ml of buffered Earle's BSS containing antibody or antibody-HRP conjugates. For internalization of surface Ig, cells were washed at 4°C in buffered Earle's BSS, transferred to buffered Earle's BSS containing 1% fetal calf serum, and incubated at 36°C for 7 or 30 min.

Processing of Cells for Electron Microscope Autoradiography

Cells were processed according to the methods of Salpeter as previously described (8, 9, 12, 28). Silver-to-gold sections were mounted on slides covered with 1% collodion in amyl acetate. Some sections were stained with 8% uranyl acetate in distilled water for 2–3 h at room temperature and in lead citrate for 30 min, also at room temperature. Control sections from noniodinated cells or from iodinated cells without lactoperoxidase were placed next to test sections. In order to exclude grain translocation during processing for autoradiography, murine neuroblastoma cells and iodinated lymphocytes were mixed and processed together. The slides were coated with carbon and then covered with Ilford-L4 emulsion with the use of a dipping machine (V. Ararlaid, Toronto, Ontario). Monolayer (purple) emulsion was

used. The dark room used was equipped with a ceiling safelight (OC filter, 15 W bulb). Sections were developed 1–3 wk later with gold latensification (5 min), Elon-ascorbic acid (4 min) and fixed (1 min) in a non-hardening fixer (8). The collodion film was floated off the slides, and the sections were picked up on 200-mesh copper grids. Sections not stained before coating with emulsion were soaked for 5 min in amyl acetate to remove the collodion and stained with 4% uranyl acetate in 50% ethanol for 20 min at 45°C. Some sections were also stained for 5 s in lead citrate at room temperature. Quantitative autoradiographic studies were done on sections stained after coating with nuclear emulsion, according to established methods (12, 28); half distances (HD) of 900–1,000 Å were used (9). During cell preparation and processing for autoradiography, all washes, dehydrating solutions, etc. were monitored for loss of radioactivity. Significant losses of radioactivity were observed in incubating media, paraformaldehyde, and ethanol (Table I). The final embedded cell pellet had a radioactivity corresponding to that observed in trichloroacetic acid (TCA) precipitates (Table I).

Gel Electrophoresis

The discontinuous sodium dodecyl sulfate (SDS), Tris-glycine buffer system of Laemmli (22) was employed in slabs (100-mm long, 82-mm wide, 2.5-mm thick), using a 10% separating gel (80 mm) and a 5% upper stacking gel (10 mm). The washed cells were pelleted at 500 g for 10 min, suspended in sample buffer, and heated in boiling water for 3 min. Electrophoresis was performed at 25°C at a constant power of 5 W until the bromophenol blue tracking dye reached the bottom of the gel (about 3 h). After completion of the run, the gel was stained for 1 h with 0.25% Coomassie brilliant blue and destained by diffusion as described by Weber and Osborn (36). The slabs were then dehydrated by vacuum and autoradiographed with Kodak X-ray film (NS2T, Eastman Kodak Co., Rochester, N. Y.), or sliced into 1.2-mm sections with a wire slicer and counted in a Searle gamma counter $\{[(EFF)^2/\text{background}] = 405\}$ (Searle Radiographs Inc., Des Plaines, Ill.). Apparent molecular weights were estimated by comparison of the migration distances with representative protein standards. A plot of relative mobility vs. logarithm of molecular weights was linear in the region of 25,000–94,000 daltons. Scanning densitometry was performed with a Helena quick-scan equipped with an integrator (Helena Laboratories, Beaumont, Texas).

RESULTS

Qualitative Electron

Microscope Autoradiography

Similar results were obtained with both methods of iodination. Spleen-derived cells fixed immediately after lactoperoxidase-catalyzed iodination

and processed for ultrastructural autoradiograph showed a large number of grains at the cell periphery (Fig. 1). In general, all types of cells (large and small lymphocytes, plasma cells, macrophages) were evenly labeled. A few isolated intracytoplasmic or nuclear grains were always seen (Fig. 1). Iodinations performed at 4°C for 20 min or at 22–25°C for 10 min gave similar results. Macrophages were also labeled, but because of their small number quantitative studies were not performed on these cells. Damaged cells had many grains evenly distributed over their entire section.

When spleen cells after iodination were incu-

bated with anti-immunoglobulin antibody or its Fab fragments at 4°C and were subsequently incubated at 36°C for 7 or 30 min, the grain distribution was similar to that observed in cells fixed immediately after iodination, with one notable exception: certain lymphocytes and plasma cells showed clusters of grains over cytoplasmic areas, which, in the case of plasma cells, were occupied by the Golgi apparatus. Clusters of internal grains were not seen in cells exposed at 4°C to normal rabbit or sheep immunoglobulin and subsequently incubated at 36°C, or in cells iodinated at 4°C or 22°C and fixed immediately after iodination.

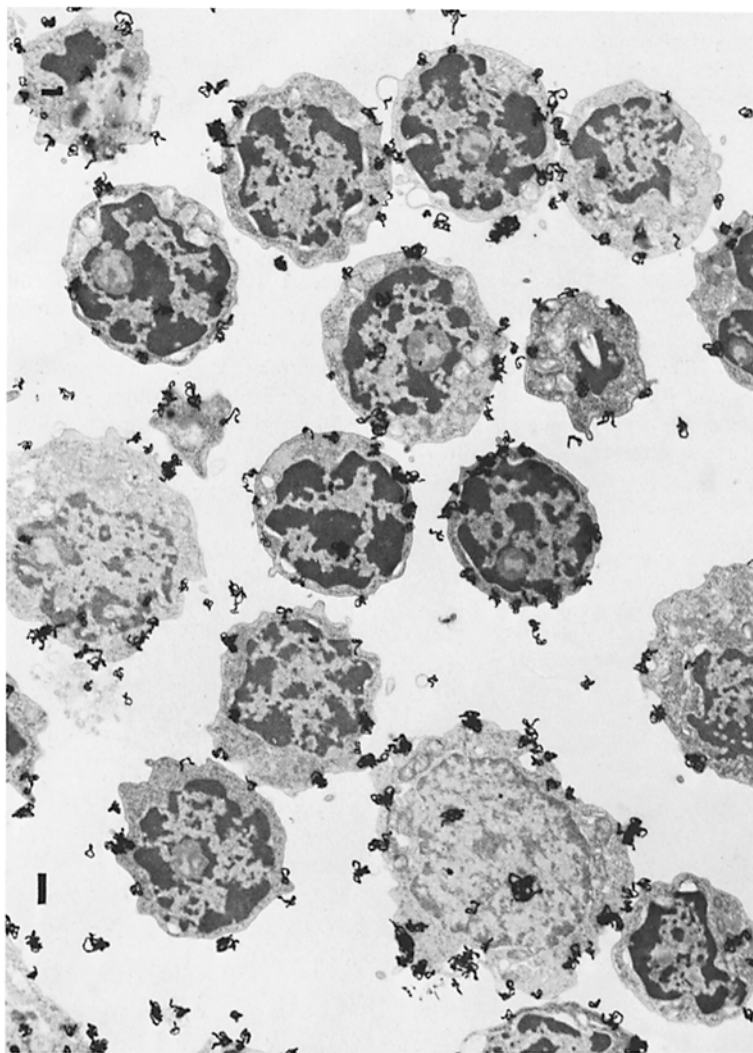


FIGURE 1 Autoradiogram of spleen lymphocytes iodinated with lactoperoxidase and H_2O_2 . Exposure, 14 days. $\times 6,000$.

In another series of experiments, conjugates of antibody or its Fab fragments with HRP were used. The ability of these peroxidase-labeled antibodies to "induce" capping and internalization was checked in separate experiments and before their use with iodinated cells (Fig. 2). Patching and capping of plasma membrane Ig-anti Ig-HRP complexes was seen in the form of a heavy surface or internal peroxidase stain after incubations of cells with the entire antibody-HRP molecules for 1 h at 4°C, washing, and subsequent incubations at 36°C for 7 or 30 min. Patching or capping was not seen with anti-Ig Fab-HRP; internalization of surface Ig-anti Ig Fab-HRP complexes was seen but the internal staining was more in the form of individual vesicles or cisternae than in the form of large aggregates of peroxidase-stained material observed after the use of the entire antibody-HRP (Fig. 2) (also compare Figs. 7 and 8). Cells not exposed to conjugates of antibody with HRP did not show surface or internal peroxidase reaction.

Double Labeling

CAPPING: Spleen lymphocytes without surface Ig (for the major part, T cells) had similar numbers of surface grains when compared with cells with surface Ig (B cells) detected by a positive peroxidase stain (see Quantitative Autoradiography). T cells did not display surface clustering or internalization of grains after incubations at 37°C (Fig. 3). In contrast, B cells showed clear caps of

surface Ig-anti Ig-HRP complexes (Fig. 4). Although most grains were associated with the cap, varying numbers of grains remained at the peroxidase-unlabeled cell periphery (Figs. 4 and 5). Occasionally, masses of peroxidase-positive material and grains appeared to be contiguous with, but at a considerable distance from, the cell surface antibody-HRP material, suggesting a process of elimination by exocytosis of immunoglobulin-anti-immunoglobulin complexes (Fig. 6) (1, 5).

INTERNALIZATION (ENDOCYTOSIS) OF PLASMA MEMBRANE IG: In cells incubated with the entire antibody-HRP conjugate, large focal intracytoplasmic aggregates of HRP stain and of grains were observed (Fig. 7). In these cells, despite a heavy concentration of internal grains, the peripheral (plasma membrane) grains still remained.

Quantitative Autoradiography

CELLS FIXED IMMEDIATELY AFTER IODINATION: In Table I, the radioactivity counts in the TCA precipitate and in the embedded cells are compared. The counts are similar and indicate that autoradiographic grains are due to ¹²⁵I incorporated into proteins rather than into lipid or into other fractions. In Fig. 9 and Table II, the grain distribution of an iodination experiment is illustrated; HD values were 1,000 Å.

STUDY OF INTERNALIZATION WITH DOUBLE LABELING (FAB OF ANTI-RAT IG-HRP):

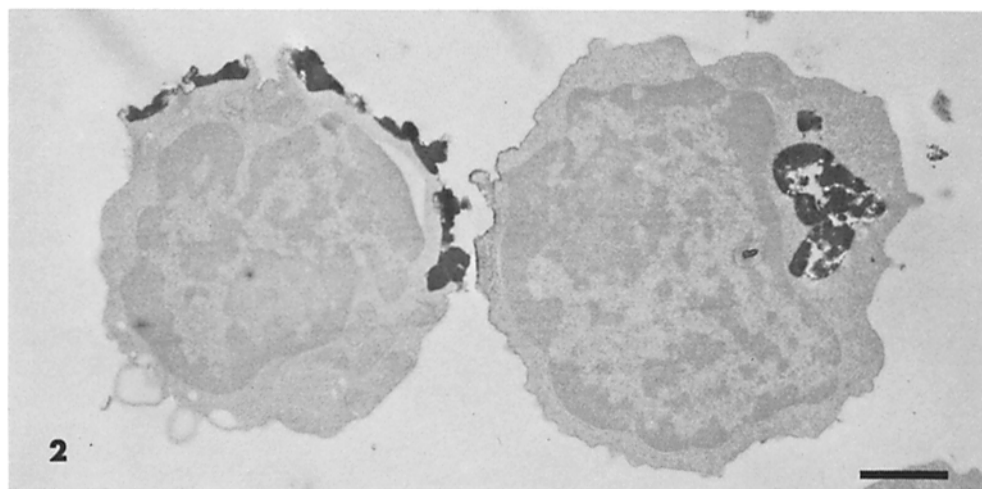


FIGURE 2 Rat spleen cells incubated with anti-rat Ig conjugated with HRP at 4°C, washed and brought to 36°C for 30 min. Note intense internal diamino benzidine-osmium black stain or surface confluent patch-cap-like stain. $\times 11,000$.

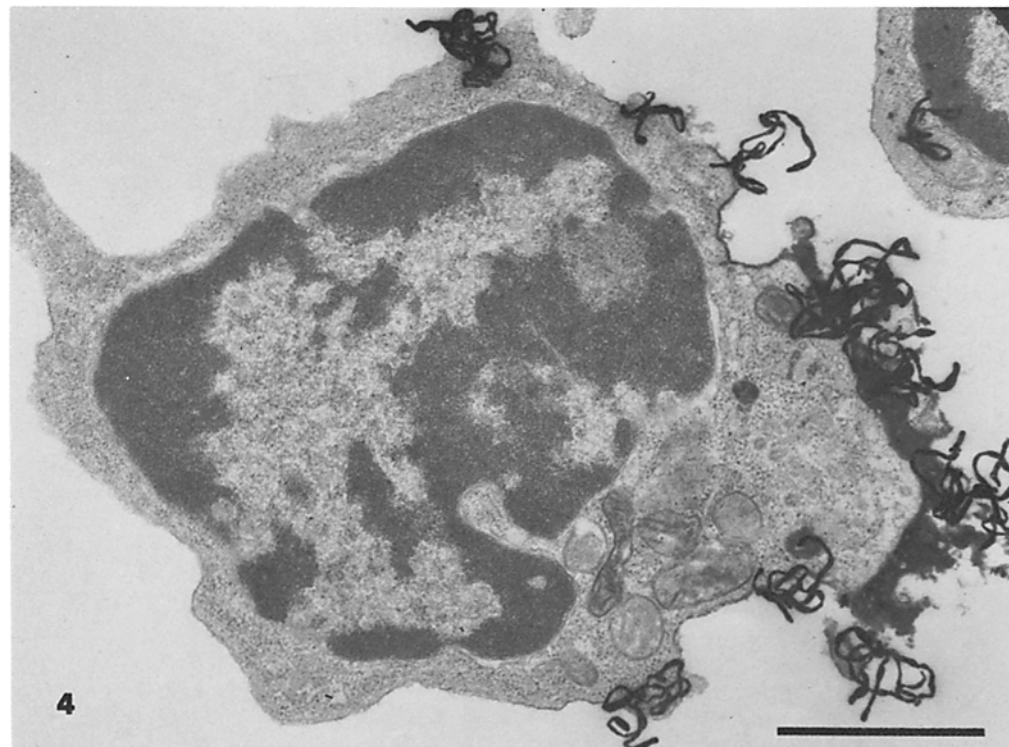
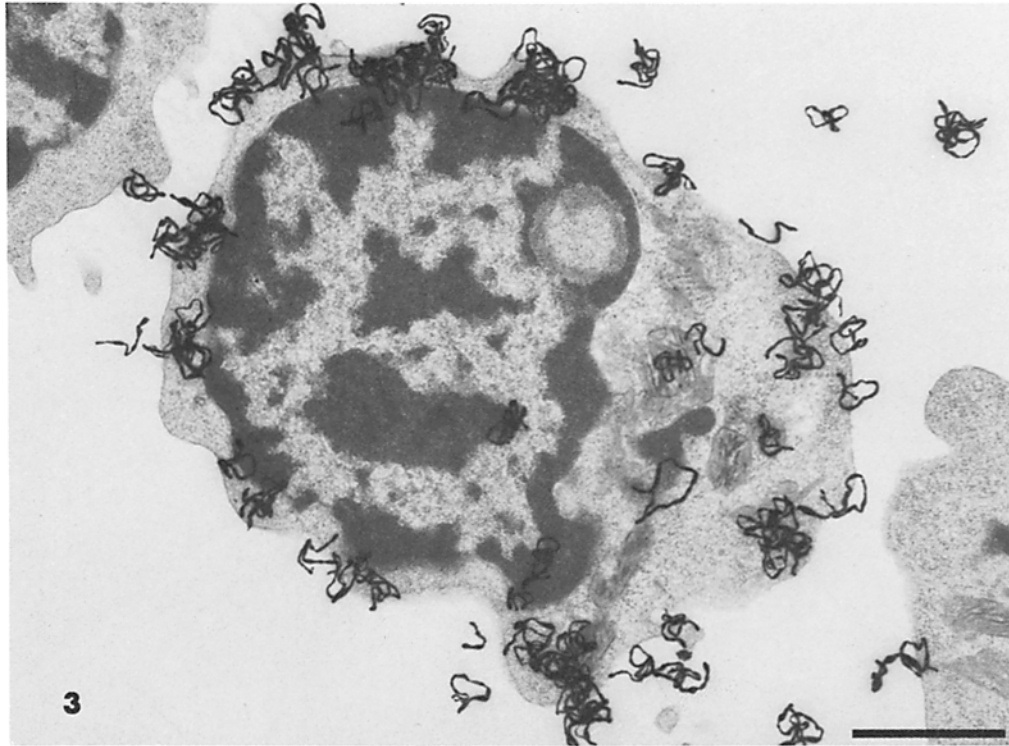


FIGURE 3 Iodinated spleen cell (T) without surface or internal HRP stain after incubation with antirat-HRP at 4°C and 36°C. Exposure, 14 days. $\times 20,000$.

FIGURE 4 Iodinated spleen cell (B) with cap of Ig-anti-Ig HRP complexes. Note surface grains outside cap area. Exposure, 14 days. $\times 27,000$.

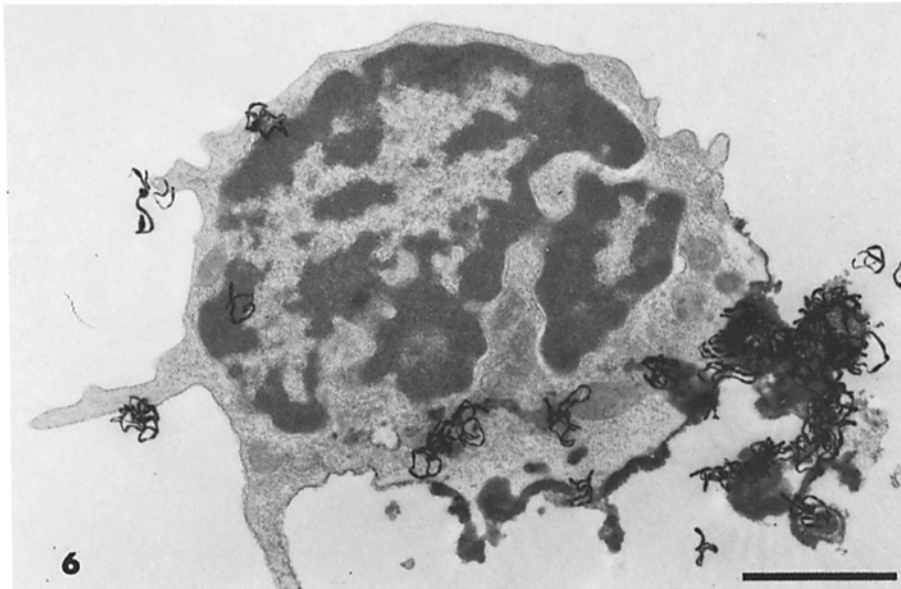
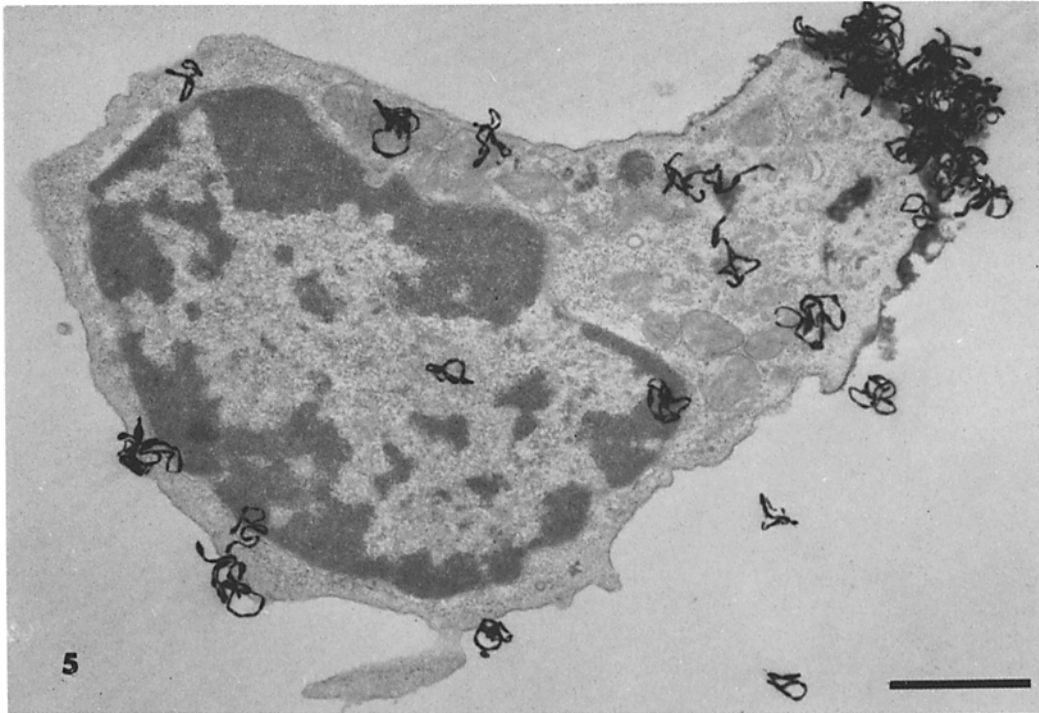


FIGURE 5 Iodinated spleen cell (B) with cap of Ig-anti-ig HRP and significant number of grains at cell periphery without HRP stain. Exposure, 28 days. $\times 19,000$.

FIGURE 6 Iodinated spleen cell with "apparent" exocytosis of Ig-anti-Ig HRP complexes. Exposure, 14 days. $\times 19,000$.

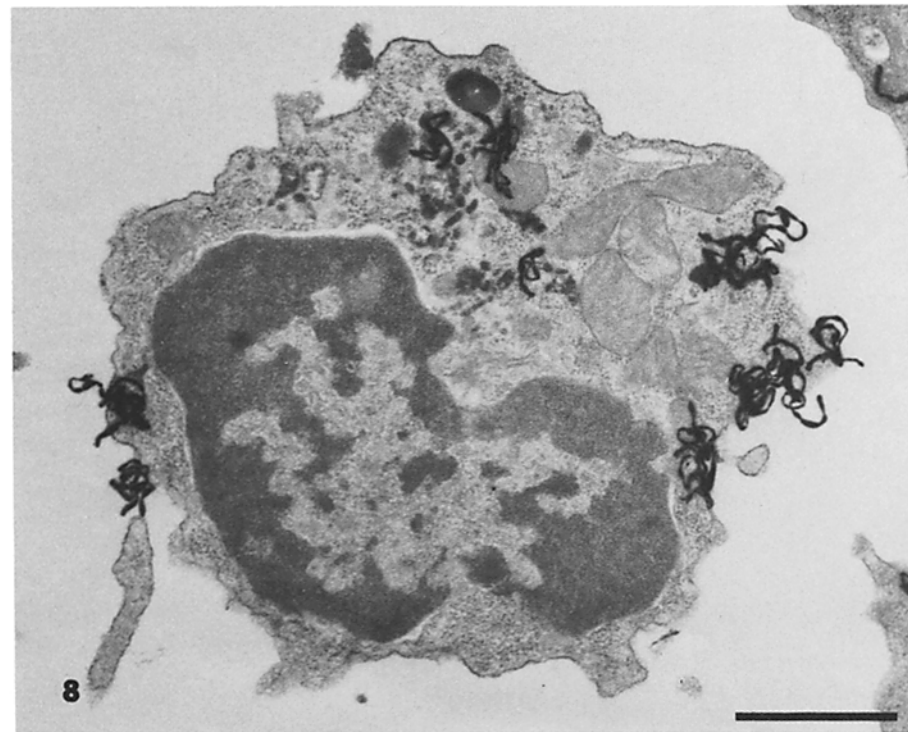
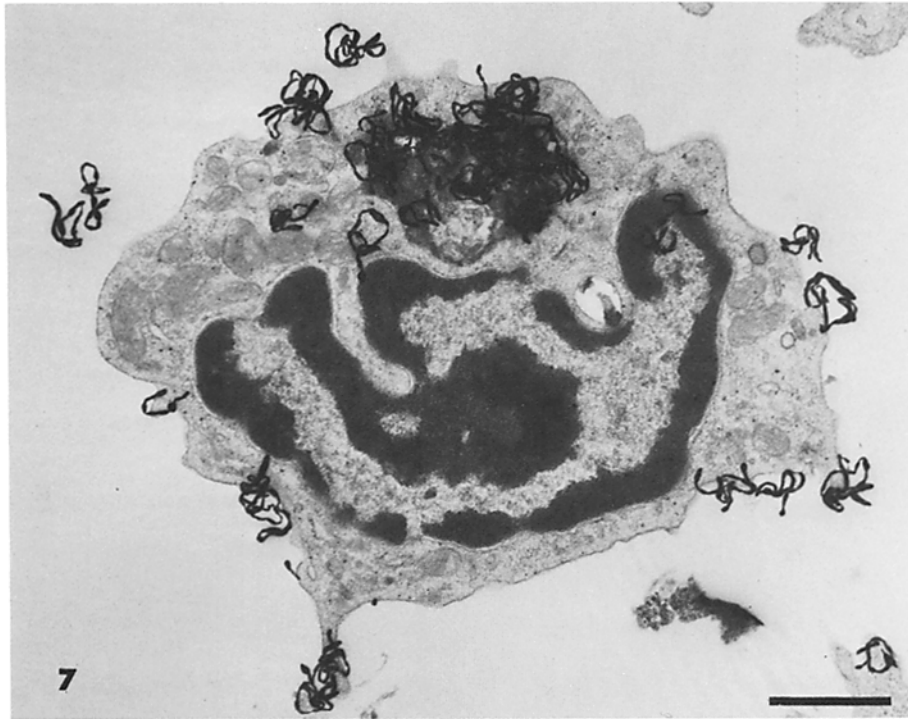


FIGURE 7 Iodinated spleen cell with intense internalization of grains and Ig-anti-Ig HRP complexes. Exposure, 14 days. $\times 20,000$.

FIGURE 8 Iodinated spleen cell with internalization of Ig-Fab-anti-Ig HRP. Compare peroxidase stain and grain density between Figs. 7 and 8. Exposure, 14 days. $\times 21,000$.

TABLE I*
Counts Per Minute

45 min at 4°C with Ab	30 min at 36°C	PF	DAB	O ₂ O ₂	30%-100% ETOH	100% ETOH	Prop. Oxide	50:50 (P:A)	Araldite	TCA	Exp NS.
10 × 10 ⁶	5 × 10 ⁶	2 × 10 ⁶	4 × 10 ⁶	3 × 10 ⁴	1.3 × 10 ⁵	7 × 10 ⁴	3 × 10 ⁴	2 × 10 ⁴	4 × 10 ⁶	5 × 10 ⁶	1990
—	—	39 × 10 ⁶	—	2 × 10 ⁵	4 × 10 ⁵	4 × 10 ⁴	5 × 10 ⁴	3 × 10 ³	3.8 × 10 ⁶	5.4 × 10 ⁶	2018

* Iodination with lactoperoxidase and H₂O₂. In exp 1990, 0.5 mCi was used for 7.3 × 10⁷ cells. In exp 2018, 1 mCi was used for 3.2 × 10⁸ cells; counts are per 1 × 10⁷ cells; Ab, antirat Ig-HRP; PF, paraformaldehyde; DAB, stain in diaminobenzidine tetrahydrochloride; TCA, trichloroacetic acid, P, propylene oxide; A, Araldite; ETOH, ethanol.

TABLE II
Lactoperoxidase (¹²⁵I) Iodination of Rat Spleen Lymphocytes Distribution of Grains/μm²; HD: 1,000 A°

PM	Inside													
	Outside	1HD	2HD	3HD	4HD	5HD	6HD	7HD	8HD	9HD	10HD	11HD	12HD	13HD and Over
3HD	0.488 ± 0.052	0.600 ± 0.051	1.18 ± 0.091	0.676 ± 0.055	0.408 ± 0.047	0.336 ± 0.039	0.280 ± 0.043	0.232 ± 0.043	0.224 ± 0.043	0.164 ± 0.031	0.148 ± 0.031	0.128 ± 0.032	0.132 ± 0.039	0.124 ± 0.015

Based on 2, 182 grains.

Based on 2, 174 μm².

Background 0.003/μm².

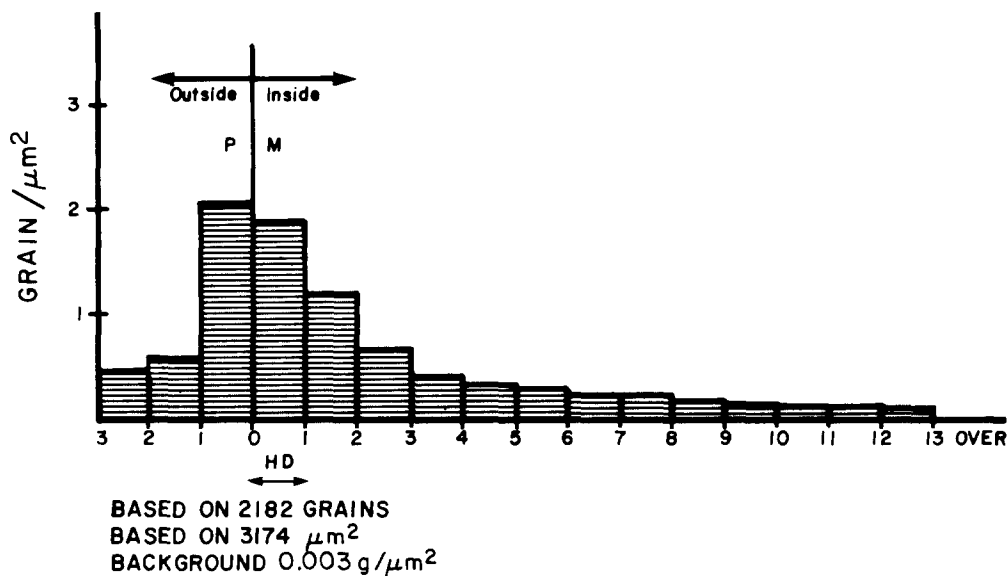


FIGURE 9 Histogram of Table II. Cells fixed after iodination. (HD 1,000 Å)

This experiment is summarized in Tables III and IV, and in Fig. 10 (exp 1952). Grain densities were essentially similar between B and T cells fixed immediately after iodination (Table III, Fig. 10). In B cells with visible internalization of surface Ig, detected by a positive peroxidase stain (Fig. 10) (Table III, or in selected cells, and S in Fig. 10), a significant number of grains were at 3HD or over from the plasma membrane. Thus, in those selected cells with internalization of Fab-anti Ig-HRP-Ig complexes, 45-57% of total grains were within the cell, at distances of 2HD and over from the plasma membrane.

STUDY OF INTERNALIZATION WITH DOUBLE LABELING (ANTI-RAT IG-HRP): In this study, the grain density was counted in selected cells with internalized anti-Ig Ig-HRP complexes (Figs. 7 and 10) (Table V). In these selected cells, the intracytoplasmic grain density at 3HD and over was 68%, somewhat higher than in B cells after incubation with Fab of anti-rat Ig-HRP. Cells without Ig receptors, presumably T cells, did not display a significant increase of internal grains (3HD and over) after incubation at 36°C for 30 min.

STUDY OF CAPPING WITH DOUBLE LABELING (ANTI-RAT IG-HRP): In order to investigate capping of immunoglobulins, conjugates of HRP with the *entire* anti-rat Ig antibody were utilized in another experiment (1990). In this ex-

periment, spleen lymphocytes after iodination with lactoperoxidase and H_2O_2 were incubated for 1 h at 4°C with sheep anti-rat Ig-HRP; subsequently, cells were washed with Earle's BSS and incubated for 30 min at 36°C, washed of medium, fixed, and processed for autoradiography after staining for peroxidase with diaminobenzidine. Quantitative studies of grains at the plasma membrane or 1.5HD (1,500 Å) outside or inside the plasma membrane were performed. In order to evaluate segregation of grains at the cap, the grain density per micron of cell perimeter was counted. Thus, in 140 cell sections studied with definite cap formation, grains in the cap zone had a density of 0.230 ± 0.006 grain per $1 \mu\text{m}$; the grain density over the rest of the periphery of the cell which was unlabeled with peroxidase was 0.080 ± 0.003 grain per $1 \mu\text{m}$; if the grain density per micron was calculated over the entire periphery of the cell, irrespective of peroxidase-labeled areas, the average density was $0.140 \pm .003$. These counts are based on 2,655 grains and on 18,947 μm of cell perimeter (periphery). Thus, the grain density in the cap area was three times greater than the density in the unlabeled periphery of the cells; however, it must be noted that 36% of the grains remained outside the capped immunoglobulins.

DAMAGED CELLS: In our cell preparations, damaged cells represented 1-2%. Damaged cells were identified in the electron micrographs as

TABLE III
Distribution of ^{125}I -Autoradiographic Grains/ μm^2 (Experiment 1952)

		Entire cell			
		Outside		Inside	
		1HD	1HD	2HD	3HD and over
A	T cells	1.60 ± 0.132	1.59 ± 0.124	1.20 ± 0.152	0.120 ± 0.012
	B cells	1.60 ± 0.168	2.20 ± 0.216	1.35 ± 0.204	0.260 ± 0.024
B	T cells	1.33 ± 0.124	1.24 ± 0.104	0.800 ± 0.104	0.180 ± 0.012
	B cells	1.46 ± 0.200	1.38 ± 0.160	1.17 ± 0.200	0.372 ± 0.028

		Cytoplasm only			
		Outside		Inside	
		1HD	1HD	2HD	3HD and over
A	T cells	1.60 ± 0.132	1.56 ± 0.124	1.19 ± 0.156	0.272 ± 0.032
	B cells	1.60 ± 0.168	2.16 ± 0.216	1.42 ± 0.220	0.628 ± 0.08
B	T cells	1.33 ± 0.124	1.24 ± 0.104	.844 ± 0.112	0.416 ± 0.040
	B cells	1.46 ± 0.200	1.40 ± 0.164	1.15 ± 0.212	0.840 ± 0.076

A, Fixed immediately after iodination and incubation with Fab at 4°C.

B, Fixed after "internalization", 30 min at 36°C.

HD = 900 Å.

A, B iodinated with LP and H₂O₂.

Based on 2,568 grains 3,922 μm^2 .

Background 0.0020 grains/ μm^2 .

Control (1933B) 0.0011 grains/ μm^2 .

Exposure time 14 days.

TABLE IV
Selected Cells - Internalization of Fab of Anti Rat Ig-HRP (Exp 1952)

		Cytoplasm only			
		Outside		Inside	
		1HD	1HD	2HD	3HD and Over
B		1.65 ± 0.384	1.38 ± 0.308	0.708 ± 0.268	1.33 ± 0.18

		Total cell			
		Outside		Inside	
		1HD	1HD	2HD	3HD and Over
B		1.65 ± 0.384	1.38 ± 0.308	0.832 ± 0.288	0.644 ± 0.72

For explanation of table see legend of Table III.

either naked nuclei or as cells with disrupted plasma membrane and a pale staining cytoplasm with swollen mitochondria, dilated cisternae of the endoplasmic reticulum, dispersed ribosomes, etc. In two experiments, the grain density over the

entire area of intact cells was compared with the grain density over the area occupied by damaged cells, processes of naked nuclei. In one experiment (1696), we found an average grain density over intact cells of 0.545 ± 0.052 grain per μm^2

and of 1.71 ± 0.090 grains per μm^2 over damaged cells. In the second experiment (1796), the grain density over intact cells was $2.91 \pm 0.149/\mu\text{m}^2$,

and over damaged cells, $6.81 \pm 0.348/\mu\text{m}^2$. In exp 1696, 1,103 grains were counted; in exp 1796, 1,487 grains were counted. Thus, the grain density over damaged cells was three–four times greater than the grain density over intact cells. In intact cells, the majority of grains were near the plasma membrane, while in damaged cells the grains were evenly distributed, especially over naked nuclei.

Polyacrylamide Electrophoresis

The electrophoretic profile of spleen cell membrane proteins revealed more than 30 bands with the Coomassie blue stain (Fig. 11). On the basis of intensity, three major bands, which were also positive with the periodic acid-Schiff reaction, comprised 35–45% of the staining and corresponded to apparent mol wt of 44,000, 29,000 and 26,000 daltons. The incorporation of ^{125}I label into iodinated cells is shown in Fig. 12; 65–72% of the total radioactivity is distributed in five peaks of apparent mol wt of 44,000, 50,000, 57,000, and 90,000 daltons; the slowest moving radioactive band comigrated with myosin (mol wt 195,000). Only one radioactivity peak (44,000 mol wt) corresponded with a major peak stained with Coomassie blue. In all runs, radioactivity of 2–5% of the total remained at the origin.

The electrophoretograms of (a) iodinated spleen cells, (b) iodinated cells incubated at 4°C for 1 h with anti-Ig antibody, and (c) iodinated cells incubated at 4°C for 1 h with anti-Ig (50–100 μg anti-Ig) per 1×10^7 cells, and subsequently brought to 36°C for 30 min were virtually identical. Approximately 3×10^4 iodide atoms were incorporated per cell.

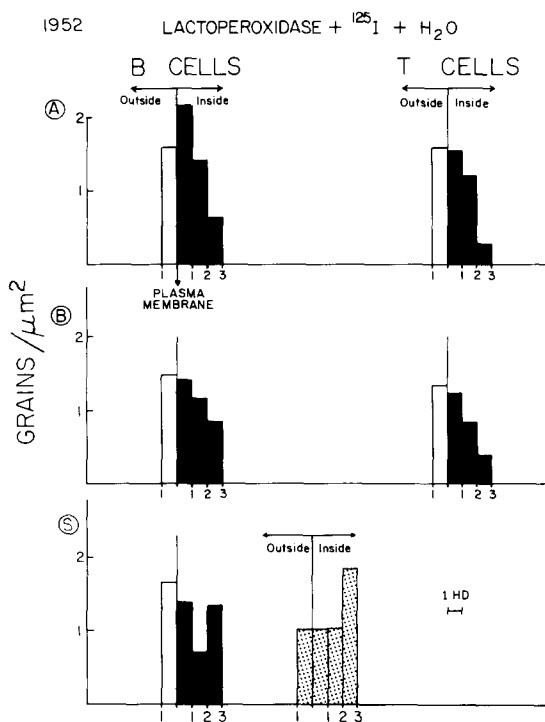


FIGURE 10 Histogram of Tables III, IV, and V. (A) cells fixed for autoradiography after iodination. (B) fixation for autoradiography after incubation with Fab of anti-Ig at 4°C and 30 min at 36°C in medium. (S) selected cells with internalized Ig-Fab-anti-Ig HRP. Dotted column: internalization of anti-Ig-HRP. (HD 900 \AA .)

TABLE V
Selected Cells—Internalization of Anti-Rat-Ig-HRP (Exp 1952)

	Cytoplasm only			
	Outside		Inside	
	1HD	1HD	2HD	3HD and Over
	B	1.02 ± 0.138	1.04 ± 0.131	1.08 ± 0.171
	Total cell			
	Outside		Inside	
	1HD	1HD	2HD	3HD and Over
	B	1.02 ± 0.138	1.04 ± 0.131	0.968 ± 0.151

For explanation of table see legend of Table III.

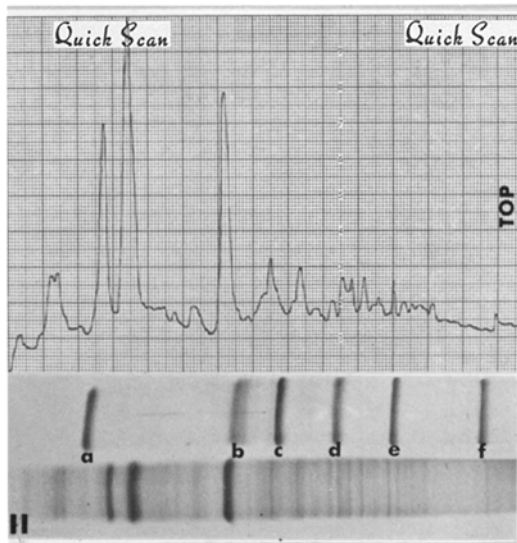


FIGURE 11 SDA-polyacrylamide gel profile and scan at 545 nm of proteins from spleen cells. Electrophoresis was performed according to the procedure of Laemmli (22). Approximately $2-3 \times 10^6$ cells were applied to the gel. Staining was with Coomassie brilliant blue. Standard proteins were (a) chymotrypsinogen (25,700); (b) actin (45,000); (c) glutamic dehydrogenase (53,000); (d) bovine serum albumin (68,000); (e) phosphorylase a (94,000); and (f) myosin (195,000).

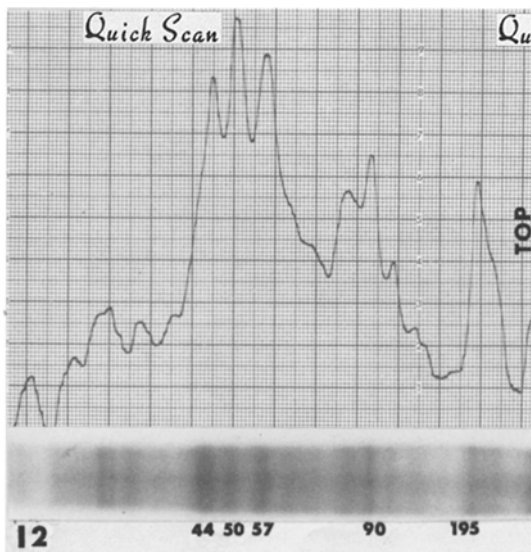


FIGURE 12 X-ray and scan of dehydrated gel obtained from the iodination of spleen cells at 25°C, using lactoperoxidase and hydrogen peroxide. Note major radioactive bands mol wt, 195,000, 90,000, 57,000, 50,000, and 44,000 daltons.

DISCUSSION

In this paper, we have examined (A) autoradiographic grain distributions over spleen cells iodinated with lactoperoxidase and (B) the segregation of specifically labeled lymphocyte plasma membrane immunoglobulins from the iodinated proteins during capping and internalization of immunoglobulin-anti-immunoglobulin complexes.

(A) Quantitative Autoradiography of Iodinated Lymphoid Cells

Previous electron microscope autoradiographic studies of lactoperoxidase-iodinated lymphoma cells, lymphocytes, and L cells reported by Marchalonis et al. (23), Kennel and Lerner (21) and Hubbard and Cohn (17) have shown that most grains are associated with the plasma membrane. In those papers, grain density distributions per unit surface area were not studied; in iodinated lymphocytes, according to Kennel and Lerner (21), only two sections had grains "inside" the cell; in L cells, Hubbard and Cohn (17) found 85-90% of the grains "at the cell periphery," 5-14% in the centrosphere region, and 0-4% over the nucleus. In order to determine whether the intracytoplasmic grains are due to scattering from the adjacent heavily labeled plasma membrane or represent intracytoplasmic incorporation of ^{125}I , we normalized the grain density distribution from Fig. 9 and compared the resulting curves with theoretical grain distribution curves of Fertuck and Salpeter (9) plotted in HD units representing scattering from a known heavily labeled linear source (Fig. 13). The curve in solid line in Fig. 13 is from the ^{125}I autoradiographic studies of Fertuck and Salpeter with Kodak NTE nuclear emulsion with HD of 500 Å and represents the universal curve for ^{125}I scatter. In our system of autoradiography (Ilford L4, Elon-ascorbic acid development), HD values are 900-1,000 Å (9). Normalization of grain distributions in our system (broken line, Fig. 13) and comparison of expected scatter from a linear source (solid line, Fig. 13) shows that 36% of intracytoplasmic grains, at 2-13 HD, represent internal labeling and not scatter from the adjacent heavily labeled plasma membrane. Graphic analysis was performed by cutting out the appropriate segment from photographic prints of Fig. 13 and weighing it on an analytical balance. 39% of internal (cytoplasmic) grains representing genuine labeling (and not scatter) were at 7-13 HD, and 61% at 1-6 HD. Intracytoplasmic labeling must

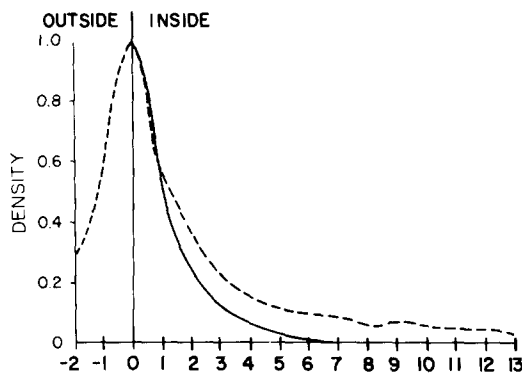


FIGURE 13 Comparison between theoretical grain scatter from ^{125}I of Fertuck and Salpeter (9). Solid line: Kodak NTE, HD values of 500 Å; broken line: actual internal grains of present experiment with Ilford L 4 and HD 1,000 Å.

result from pinocytosis of the enzyme, or from dissociation of iodide radicals from lactoperoxidase, during iodination. Tsai et al. (32) have suggested that during the lactoperoxidase-catalyzed iodination iodide radicals can penetrate the membrane and iodinate cytoplasmic components. They have suggested also that the lactoperoxidase (LP) iodide complex has a finite life and can dissociate into LP and iodide if it will not encounter sites which will accept iodine (32). The exclusion of significant lipid iodination or adsorption of iodinated proteins (see Methods) and the close correspondence of radioactivity values obtained in TCA precipitates of iodinated cells and in the final cell pellet (Table I) indicate that, in these autoradiographs, we have examined grains derived from ^{125}I incorporated into tyrosine or histidine residues of peptides; furthermore, the lack of grains over unlabeled neuroblastoma cells, which were mixed with iodinated lymphocytes and processed with them for autoradiography, indicates that translocation of radioactive sources during processing for electron microscope autoradiography did not occur.

Schmidt-Ullrich et al. (29) have reported that, after lactoperoxidase-catalyzed iodination of thymocytes and subsequent subcellular fractionation, 18% of the radioactivity was in a nuclear fraction, 30% in soluble cytoplasmic proteins, and 52% in a microsomal fraction including the plasma membrane. Our morphologic studies indicate that significant radioactivity is associated with compartments other than the plasma membrane. Another limitation of the lactoperoxidase-catalyzed iodina-

tion is the incorporation of iodine on damaged cells. Our studies have disclosed that damaged cells have a grain density three- to fourfold that of intact cells.

(B) Segregation of Specifically Labeled Plasma Membrane Immunoglobulins

We have shown with peroxidase labeled anti-immunoglobulin antibody or with its Fab fragments that plasma membrane and internalization of immunoglobulins can be demonstrated (2). We have observed that the distribution of peroxidase-labeled antibody and that of iodinated and peroxidase-labeled antibody are identical (2); therefore, the covalently linked HRP is not dissociated from the antibody during internalization of the Ig-anti-Ig HRP complexes (1, 2). Lymphocytes do not have endogenous peroxidase detectable by the cytochemical method of Graham and Karnovsky, and the use of peroxidase as a marker enzyme is valid for our system.

According to Vitetta et al. (35), the plasma membrane Ig of mouse lymphocytes is an Ig M monomer with a mol wt of $214,000 \pm 31,500$ daltons; in lactoperoxidase-iodinated cells, the amount of plasma membrane Ig is 2-3% of the total acid-precipitable radioactivity. In two previous studies of polyacrylamide gel electrophoresis of lactoperoxidase-iodinated human lymphocytes, several iodinated bands have been found. Surface (plasma membrane) Ig was not detected on gel electrophoresis of unfractionated iodinated lymphocytes (11, 29, 30); the low amount of surface Ig is probably responsible for the difficulty of its detection on gel electrophoresis. Tanner et al. (30) have reported in human lactoperoxidase-iodinated lymphocytes four main bands of radioactivity of mol wt of 135,000, 120,000, 111,000, and 92,000 daltons; Gates et al. (11) have found in human lymphocytes subjected to lactoperoxidase-catalyzed iodination a major labeled peak of mol wt of 138,000 daltons. We have found in rat spleen lymphocytes iodinated with lactoperoxidase five major bands of radioactivity of mol wt of 195,000, 90,000, 57,000, 50,000, and 44,000 daltons. One of the bands (44,000 mol wt) that stained positive with Coomassie blue showed incorporation of ^{125}I . These electrophoretograms have been repeatedly obtained in a number of "runs" made from several different iodination experiments. The discrepancies between our results and those of Tanner et al. (30) and of Gates et al.

(11) may be due to species differences, contamination by platelets (in the case of human lymphocytes), or differences in the iodination technique.

Our qualitative and quantitative ultrastructural studies have demonstrated that iodinated plasma membrane proteins of lymphocytes do segregate into caps and are internalized only after exposure to anti-Ig antibody. Iodinated T cells, incubated at 36°C for 30 min, did not display redistribution of iodine (^{125}I) into caps or internalization areas after incubation of B lymphocytes with anti-Ig antibody (Figs. 3-7). These results clearly demonstrate that the phenomena of capping and internalization of surface Ig are indeed induced by the anti-Ig antibody and do not reflect generalized movements of plasma membranes. Our experiments have also demonstrated that about 36% of plasma membrane iodinated proteins do not participate in the segregation of Ig-anti Ig complexes into the cap region. In view of the probable low labeling of surface Ig by ^{125}I , it is unlikely that all grains observed in the cap region or in internalization sites are derived from surface Ig. The observed grains in caps and internalized areas of surface Ig exposed to the bivalent antibody are probably derived from plasma membrane proteins which are closely associated and comigrate with surface Ig, while internalization of surface Ig exposed to monovalent antibody fragments is associated with fewer internal grains; this finding suggests that fewer plasma membrane proteins internalize together with surface Ig exposed to monovalent antibody.

Surface Ig could be interspersed among other plasma membrane proteins in an orderly fashion (top part of Fig. 14, solid line) or randomly (bottom part of Fig. 14, broken lines). Capping and internalization of surface Ig could be associated (a) with similar segregation of all other plasma membrane proteins exposed to iodination (Fig. 14 A), (b) with only certain closely associated proteins (Fig. 14 B) or (c) with selective segregation of Ig alone (S, Fig. 14). These experiments have ruled out the first possibility since about 36% of surface grains of ^{125}I are outside the cap area, and a significant number of grains are at the surface of cells with heavy internalization of Ig-anti-Ig HRP and iodine. In view of the low levels of plasma membrane Ig available to iodination, it is unlikely that all grains in caps are derived from Ig receptors alone which have been concentrated in the cap

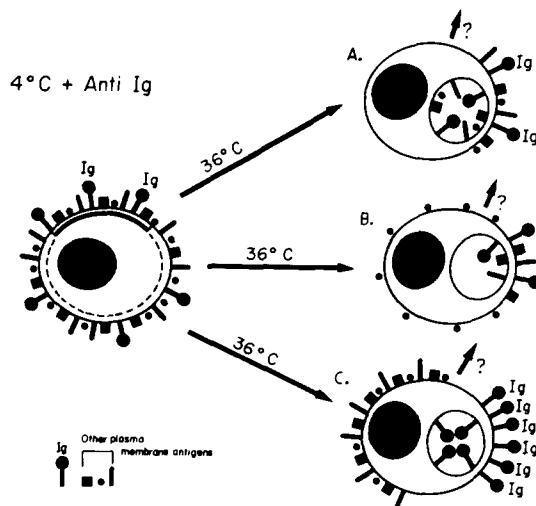


FIGURE 14 Schematic representation of relationship between plasma membrane Ig and other proteins during Ig capping and internalization. Our findings favor possibility B (see Discussion). Arrow: (?) possible shedding off of membrane moieties.

area or in internalization sites (possibility S, Fig. 14); however, this possibility cannot be entirely ruled out. Exposure of the surface Ig to the bivalent antibody results in a larger number of internal grains than does exposure to the monovalent antibody. These findings suggest that Ig segregates into caps and undergoes internalization together with closely associated plasma membrane proteins exposed to iodination (possibility B, Fig. 14).

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