

DETECTION OF PLASMA MEMBRANE CARBOHYDRATES WITH LECTIN PEROXIDASE CONJUGATES

NICHOLAS K. GONATAS and STRATIS AVRAMEAS

From the Department of Pathology (Division of Neuropathology), University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 and the Laboratory of Immunocytochemistry, Pasteur Institute, Paris, France

ABSTRACT

With the use of the cytochemical stain for horseradish peroxidase of Graham and Karnovsky (1966. *J. Histochem. Cytochem.* **14**:291), conjugates of horseradish peroxidase with ricin, wheat germ agglutinin, and phytohemagglutinin were employed for the morphologic demonstration of *d*-galactose (ricin), *N*-acetylglucosamine (wheat germ), and *N*-acetylgalactosamine (phytohemagglutinin) containing moieties on the surface of unfixed, or paraformaldehyde-fixed rat lymphoid cells. *D*-Galactose, or *d*-galactose containing disaccharides inhibited the interaction between ricin peroxidase and lymphoid cell surface; also, *N*-acetylglucosamine inhibited the wheat germ peroxidase-lymphoid cell interaction, but *N*-acetylgalactosamine failed to inhibit the reaction between phytohemagglutinin peroxidase and the surface of lymphoid cells.

INTRODUCTION

Localization of surface and intracellular antigens and antibodies has been obtained by the use of horseradish peroxidase conjugates (1-3). This procedure is based on the specific interactions between antigen and antibody which are not impaired by the coupling of antigens or antibodies with peroxidase. Similar specific interactions exist in several other systems such as between lectins and carbohydrate moieties, and hormones and their receptors, in which the peroxidase labeling has been applied (4-7). Recently, Nicolson and Singer, and Hirano et al. demonstrated with the use of concanavalin A ferritin, or ricin ferritin conjugates, α -*D*-mannose, α -*D*-glucose, β -*D*-fructose (concanavalin A), and *d*-galactose (ricin) residues on various cytomembranes (8, 9). In the present communication we report the cellular localization of *d*-galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine containing moieties by the use of conjugates of peroxidase with wheat

germ agglutinin (*N*-acetylglucosamine), phytohemagglutinin (*N*-acetylgalactosamine), and ricin agglutinin (*d*-galactose). One advantage of the peroxidase over the ferritin labeling, is the feasibility of the former for both light and electron microscope studies which can be performed in preparations from the same specimen (3).

MATERIALS AND METHODS

Horseradish peroxidase RZ3 was obtained from Worthington Biochemical Corp., Freehold, N. J. *Phaseolus vulgaris* (kidney beans) agglutinin (PHA) was obtained from Difco Laboratories, Detroit, Mich., and was used after dialysis against saline without any further purification. Wheat germ agglutinin was isolated from wheat germ lipase, Type I obtained from Sigma Chemical Co., St. Louis, Mo., following the procedure of Nagata and Burger (11). Ricin agglutinin: a crude extract of *Ricinus communis* beans was prepared by extracting overnight at +4°C, 120 g of finely ground beans with 1 liter of 0.1 M phosphate

buffer, pH 6.8. The suspension was then centrifuged (1 h, +4°C, 90,000 g) and the clear supernate was utilized for the isolation of *Ricinus communis* agglutinin by adsorption of the agglutinin on glutaraldehyde-insolubilized stromata of rabbit erythrocytes; subsequently, the agglutinin was eluted with an acid buffer (13).

Preparation of Peroxidase Containing Active Aldehyde Group, "Activated Peroxidase"

Active aldehyde groups were introduced in the peroxidase by treating the enzyme for 24 h at room temperature with an excess of glutaraldehyde and then by removing the unreacted glutaraldehyde by filtration through a Sephadex G-25 column. The procedure employed has been described in detail elsewhere (12). The activated peroxidase was kept at +4°C and used within 3 h after its preparation.

Preparation of Conjugates of Lectins with Peroxidase

Activated peroxidase was coupled to ricin agglutinin following a procedure similar to that developed for the preparation of peroxidase-labeled antibody (12). To 10 mg of activated peroxidase dissolved in 1

ml of 0.1 M phosphate buffer, pH 7.5, 2.5 mg of agglutinin dissolved in 1 ml of the same buffer were added. After a week at +4°C, 0.1 ml of a 0.2 M solution of lysine was added and the mixture was allowed to stand for 2 h. The preparation was then chromatographed at +4°C on a Sephadex G-200 column (90 × 1.5 cm) equilibrated with 0.1 M phosphate buffer, pH 7.5 (Fig. 1). Fractions (6-ml each) were read at 280 and 403 nm to determine their concentration in proteins and peroxidase, respectively, and were assayed for peroxidase activity and for their capacity to agglutinate rabbit erythrocytes according to previously described methods (13).

Fractions 12-18 showing both peroxidase activity and strong agglutination of rabbit erythrocytes, were pooled and used for the experiments at the cellular level.

The same procedures and the same quantities were employed for the coupling of activated peroxidase with wheat germ agglutinin and phytohemagglutinin.

Morphology

ACTIVATED PEROXIDASE: Lymph node cells were obtained from cervical ganglia of normal adult Lewis rats by teasing the ganglia in Earle's balanced salt solution at +4°C. After three washes in Earle's at +4°C, 0.1 ml of packed cells obtained by mild centrifugation at 1,500 rpm for 10 min, was resuspended

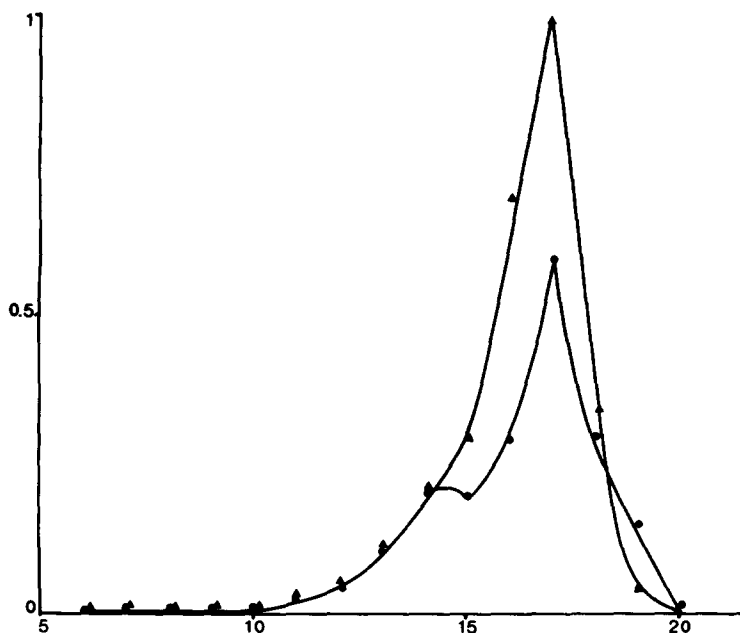


FIGURE 1 Chromatography on Sephadex G-200 column (90 × 1.9 cm), equilibrated with 0.1 M phosphate buffer, of peroxidase-labeled ricin agglutinin: (●—●) optical density (OD) at 280 nm, (▲—▲) OD at 403 nm. Fractions of 6 ml were collected. Abscissa: tube number. Ordinate: OD at 280 nm.

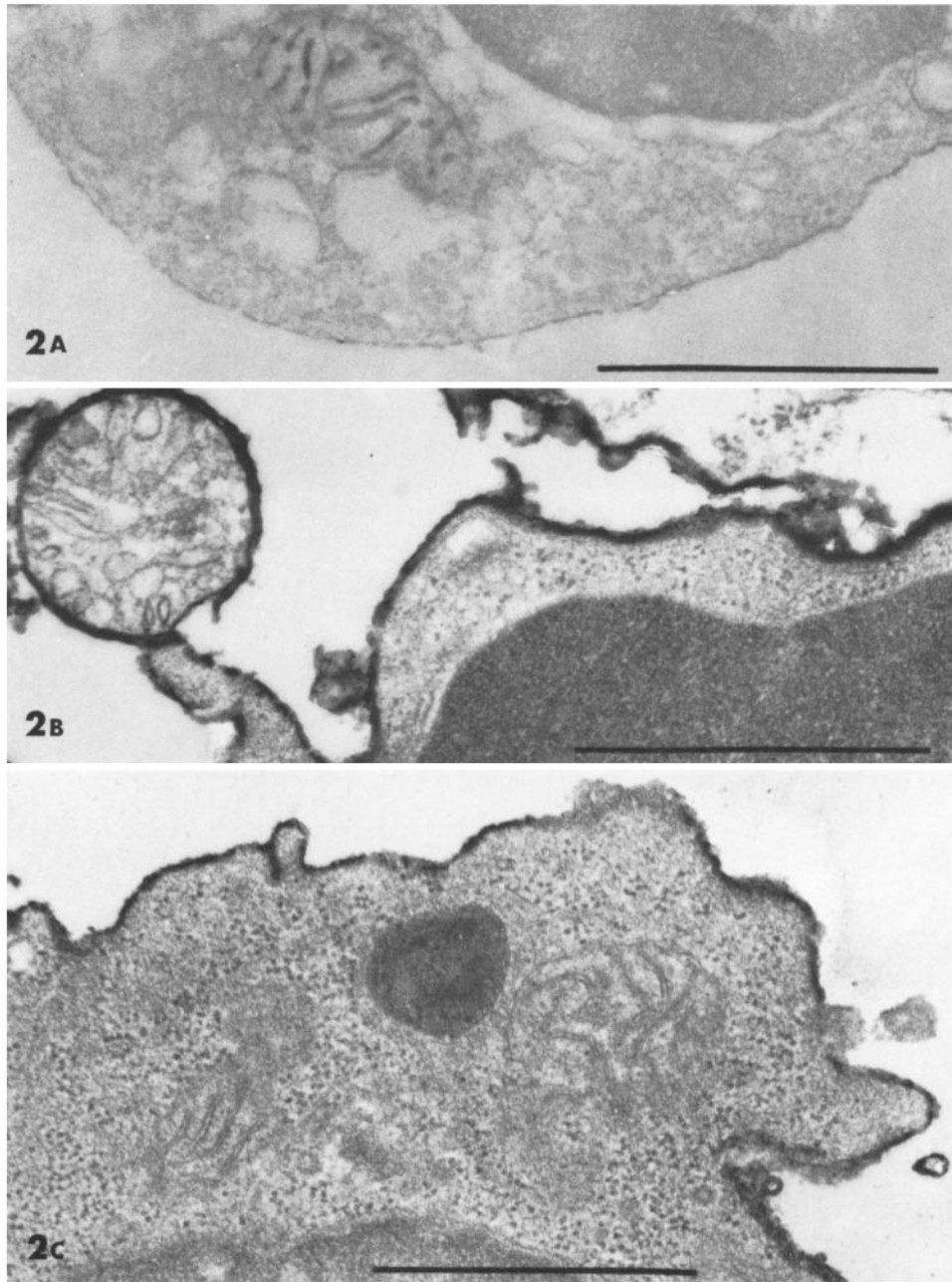


FIGURE 2 A Normal rat cervical lymph node lymphocytes incubated with activated peroxidase and stained with diaminobenzidine. Plasma membrane is unstained. Mitochondrion shows staining of cristae. $\times 45,000$.

FIGURE 2 B Same as Fig. 2 A but incubated with ricin PO and stained with uranyl acetate. Plasma membrane has a heavy deposit of oxidized diaminobenzidine. Scale bar, $1 \mu\text{m}$. $\times 47,000$.

FIGURE 2 C Same as Fig. 2 B but incubation with ricin PO was performed after fixation with paraformaldehyde. Scale bar, $1 \mu\text{m}$. $\times 43,000$.

in 5 ml of activated peroxidase at a concentration of 100 μg of horseradish peroxidase per 1 ml of Earle's or buffered physiologic saline (BPS). Incubations were carried out for 1 h or overnight at 4°C, or for 1 h at room temperature. After termination of incubation, cells were washed thoroughly in BPS, fixed with 4% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.4) for 30 min at room temperature, washed thoroughly in BPS, transferred to clean tubes, and stained for peroxidase with the method of Graham and Karnovsky (5 mg diaminobenzidine

tetrahydrochloride, 10 ml 0.1 Tris buffer, pH 7.4, 1 drop H₂O₂ 30% for 10 min at room temperature) (10). After several washes in BPS, cells were postfixed in 1% osmium tetroxide in 0.2 M cacodylate buffer, pH 7.4, for 30 min at room temperature, dehydrated in ascending ethanol solutions, cleared in propylene oxide, and embedded in Araldite. Sections of gray-interference color were cut with diamond knives in an LKB microtome. Sections were examined unstained, or after staining with lead citrate and uranyl acetate, in an Elmiskop 1A electron microscope.

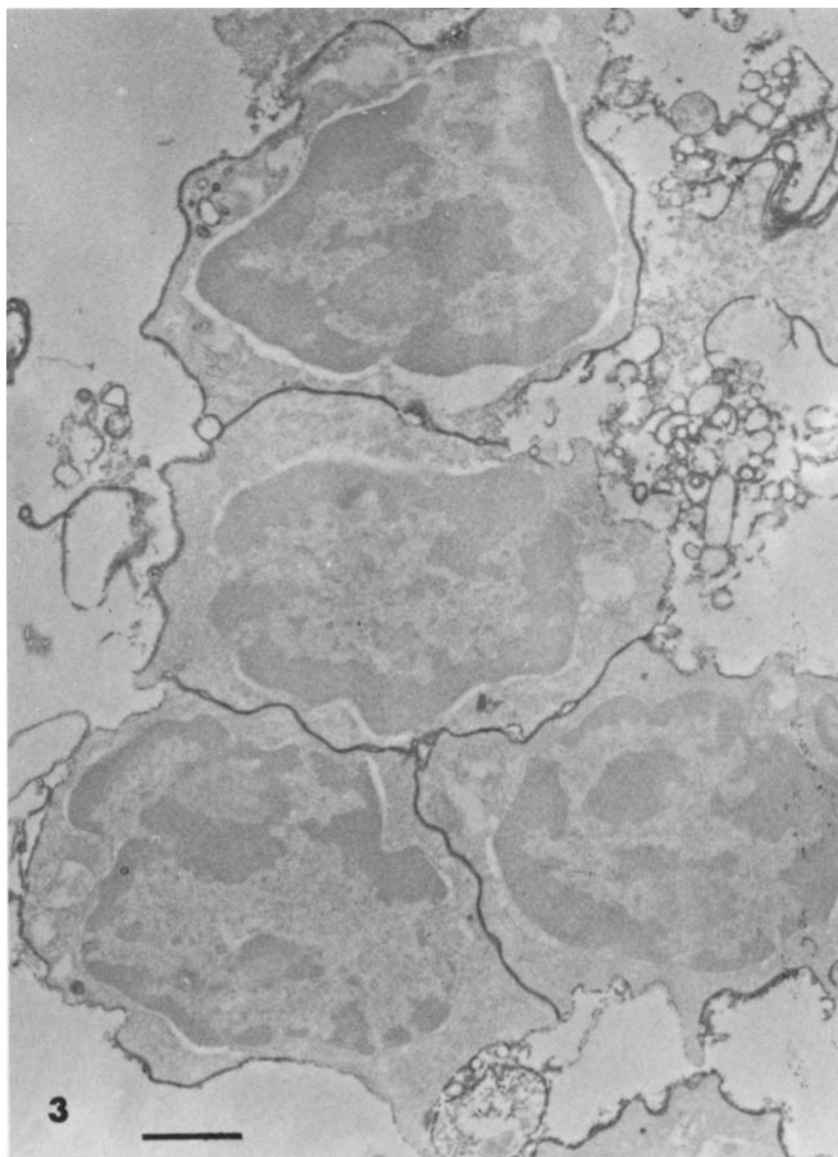


FIGURE 3 Rat lymph node cells incubated with ricin PO stained with diaminobenzidine but not with uranyl or lead. Scale bar, 1 μm . $\times 13,000$.

RICIN PEROXIDASE (PO): Cells from cervical ganglia of normal adult Lewis rats, prepared as described above, were incubated with ricin PO (2–20 μg ricin per 1×10^6 cells) or with excess of ricin PO (5 ml of 100 $\mu\text{g}/\text{ml}$ ricin per 0.1 ml of packed cells) at $+4^\circ\text{C}$ for 1 h. After several washes cells were postfixed in 4% paraformaldehyde and processed as described above (activated PO). For control experiments, lymphoid cells were prepared in Earle's containing 0.1 M of *d*-galactose, *d*-melibiose, *d*-galactosamine, α -methyl-*d*-mannoside, α -lactose, and *N*-acetyl-*d*-galactosamine. Ricin PO conjugates, diluted in Earle's with the corresponding sugar at 0.1 M concentrations, were incubated for 1 h at 37°C before their use with lymphoid cells. Osmolarity of solutions containing sugars varied from 300 (*d*-galactosamine) to 440 mosmol (*d*-glucosamine). Osmolarity was measured in an Advanced Osmometer. All carbohydrates were purchased from Sigma Chemical Co. After termination of incubations, cells were washed in BPS or in BPS containing 0.1 M of corresponding carbohydrate. In another series of experiments, lymph node cells were fixed in 4% paraformaldehyde in 0.2 M cacodylate buffer for 30 min at room temperature, before incubation with ricin PO. Cells incubated unfixed in ricin PO were postfixed in 4% paraformaldehyde in 0.2 M cacodylate buffer at room temperature for 39 min and processed as above.

Lymph node cells were incubated unfixed or after fixation in 4% paraformaldehyde with wheat germ agglutinin or phytohemagglutinin conjugates with horseradish peroxidase (0.1 ml of packed cells in 5 ml of conjugate containing 100 μg agglutinin per 1 ml of Earle's) for 1 h at $+4^\circ\text{C}$. Control media contained 0.2 M *N*-acetylgalactosamine (phytohemagglutinin) or 0.2 M *N*-acetylglucosamine (wheat germ agglutinin). Cells were processed for electron microscopy as described for ricin PO.

RESULTS

Unfixed cells incubated in activated peroxidase or in peroxidase did not show staining of their plasma membrane (Fig. 2 A). Only occasionally, a lymphocyte or plasma cell showed a fine precipitate, about 70 \AA thick, on or within its plasma membrane. Intracytoplasmic staining was seen only in membrane-bound vacuoles of macrophages, or within the limiting membranes and cristae of several mitochondria of plasma cells, lymphocytes, and macrophages (Fig. 2 A).

Ricin PO

Most cells were agglutinated and a 300 \AA thick osmiophilic material was noticed on the cytoplasmic margins of adjacent cells or on the free cytoplasmic surface (Figs. 2 B and 3). Fragmented

cells and membranous debris had reaction product on their surface. The osmiophilic material covered the entire periphery of the cell. The staining was diffuse, but occasionally it appeared in the form of small semicircular patches 300 \AA deep and 380 \AA wide.

Fixation before incubation did not alter the surface staining (Fig. 2 C). Cells incubated with mannose, *d*-galactosamine, *N*-acetylgalactosamine, and *d*-glucosamine in the medium, stained with ricin PO. Stain and agglutination were inhibited in the presence of *d*-galactose, *d*-lactose, or *d*-melibiose (Fig. 4).

Wheat Germ Agglutinin and Phytohemagglutinin, Peroxidase Conjugates

Similar results to those obtained with ricin PO conjugates were obtained with wheat germ agglutinin and phytohemagglutinin. Inhibition of the surface staining by wheat germ agglutinin was achieved with *N*-acetylglucosamine, but *N*-acetylgalactosamine failed to prevent staining of the plasma membrane by phytohemagglutinin PO.

DISCUSSION

The absence of any plasma membrane staining of lymphoid cells incubated with peroxidase or activated peroxidase (Fig. 2 A) contrasts with the definite staining obtained after incubation with ricin PO (Fig. 2 B, C), wheat germ agglutinin, or phytohemagglutinin PO conjugates. Also, the use of peroxidase conjugates with molecules such as normal rabbit IgG or Fab¹ (3), as well as with albumin, and soya bean inhibitor,² which have no affinities with plasma membrane components, have resulted in no staining of plasma membranes of normal rat lymphoid cells.²

Endogenous peroxidase activity, often found in the nuclear envelope and in the endoplasmic reticulum of certain cells (thyroid epithelial cells, serous cells of submaxillary gland) and in mitochondria of most cells (14–18), does not interfere with the interpretation of labeling of the plasma membrane.

¹ Antoine, J. C., N. K. Gonatas, A. Stieber, and S. Avrameas. Surface labeling and redistribution of IgG of rat lymphoid cells obtained by the use of Fab anti-IgG peroxidase conjugates. In preparation.

² Avrameas, S. Unpublished observations.

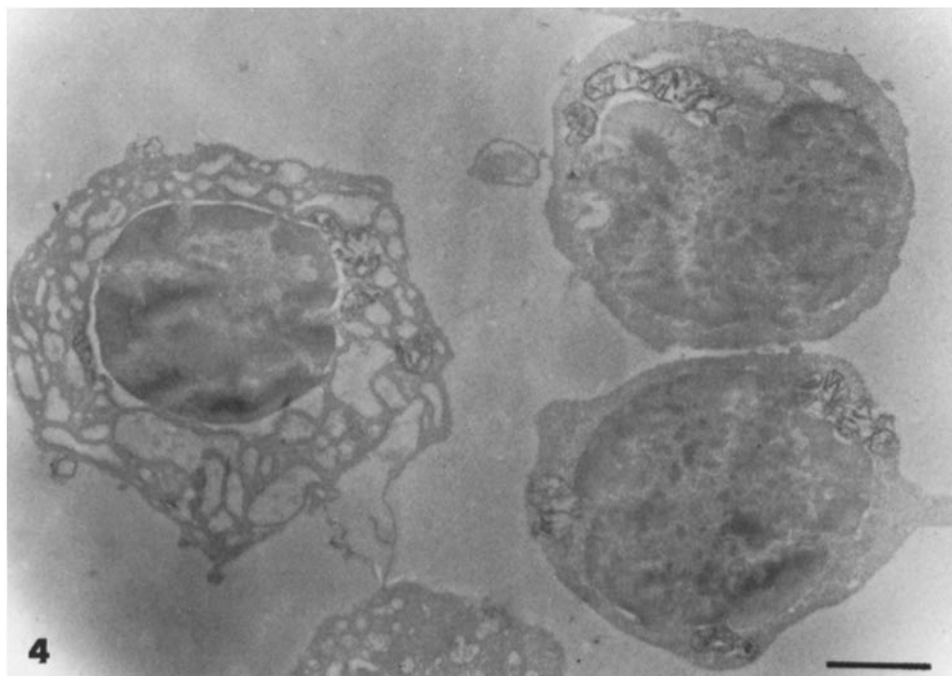


FIGURE 4 Rat lymph node cells incubated with ricin PO and 0.1 M *d*-galactose, stained with diaminobenzidine and uranyl acetate. Note absence of agglutination or peripheral staining. Scale bar, 1 μ m. \times 14,000.

The diffuse nature of the plasma membrane staining observed with ricin PO labeling is similar to the diffuse staining obtained by Hirano et al. with ricin ferritin conjugates (9). However, we made no attempt to investigate the interior face of the plasma membrane which according to Hirano et al. does not bind ricin ferritin. Affinity of ricin PO with subcellular membranes was also observed, but study of the distribution of ricin PO on subcellular fractions has not been performed. Inhibition of the ricin PO conjugation was obtained by *d*-galactose containing disaccharides (19). Other carbohydrates failed to inhibit the binding of ricin PO with plasma membrane. Contrary to our experience with ricin PO conjugates, Hirano et al. could not inhibit the ferritin ricin stain of cytomembranes with *d*-galactose, unless they used 0.5 M *d*-galactose and $\frac{1}{12}$ dilution of the original (2–5 mg/ml) ferritin ricin conjugate. In the case of ricin PO, staining was obtained at low concentrations of the lectin (2–20 μ g per 1×10^6 cells) or in excess of concentrations of 100 μ g of the lectin per ml. According to Nicholson and Blaustein, and Tomita et al., two forms of ricin agglutinin have been isolated with the use of affinity chromatography

through a galactose containing polysaccharide (20, 21). The smaller agglutinin (mol wt 60,000) is inhibited by *N*-acetyl-*D*-galactosamine sugars as well as by *d*-galactose residues (20). Apparently, the contribution of the smaller agglutinin, with *N*-acetyl-*D*-galactosamine specificity, was not significant in our preparations of ricin agglutinin PO, which were not inhibited by *N*-acetyl-*D*-galactosamine.

Wheat germ agglutinin peroxidase conjugates did not interact with the plasma membranes of lymphoid cells in the presence of *N*-acetylglucosamine, but *N*-acetylgalactosamine failed to inhibit the interaction between plasma membrane of lymphoid cells and phytohemagglutinin PO, although this sugar is known to interact specifically with phytohemagglutinin (22). These sugars (*N*-acetylglucosamine, *N*-acetylgalactosamine) are not always effective in inhibiting or dissociating lectin-macromolecular interactions (19, 22, 23); thus *N*-acetylglucosamine could not break ovomucoid-wheat germ agglutinin interactions (23), and *N*-acetylgalactosamine could not inhibit agglutination of erythrocytes by phytohemagglutinin (19).

The present experiments, as well as those already

reported (4-6, 8, 9) indicate that specific interactions between macromolecules can be employed to reveal constituents at the cellular level. Furthermore, the availability of quantitative methods for assay of the activity of peroxidase can be used for simultaneous qualitative and quantitative studies (24, 25).

The authors wish to express their appreciation to Miss Anna Stieber for her excellent technical assistance.

This work was supported by United States Public Health Service grants NS-05572-09 and 1 T01 NS 05712-02, and by research grant D.G.R.S.T. no. 72-70298 (France).

Received for publication 26 February 1973, and in revised form 28 June 1973.

REFERENCES

1. NAKANE, P. K., and G. B. PIERCE. 1967. Enzyme-labeled antibodies for the light and electron microscopic localization of tissue antigens. *J. Cell Biol.* **33**:307.
2. AVRAMEAS, S. 1970. Immunoenzyme techniques. Enzymes as markers for the localization of antigens and antibodies. *Int. Rev. Cytol.* **27**:349.
3. GONATAS, N. K., J. C. ANTOINE, A. STIEBER, and S. AVRAMEAS. 1972. Surface immunoglobulins of thymus and lymph node cells demonstrated by the peroxidase coupling technique. *Lab. Invest.* **26**:253.
4. AVRAMEAS, S. 1970. Emploi de la concanavalin-A pour l'isolement, la detection et la mesure des glycoproteines et glucides extra- ou endo-cellulaires. *C. R. Hebd. Acad. Sci.* **207**:2205.
5. BERNHARD, W., and S. AVRAMEAS. 1971. Ultrastructural visualization of cellular carbohydrate components by means of concanavalin-A. *Exp. Cell Res.* **64**:232.
6. STOBO, J. D., and A. S. ROSENTHAL. 1972. Biologically active concanavalin-A complexes suitable for light electron microscopy. *Exp. Cell Res.* **70**:443.
7. RICHARDSON, J. B., and A. BEAULNES. 1971. The cellular site of action of angiotensin. *J. Cell Biol.* **51**:419.
8. NICOLSON, G. L., and S. J. SINGER. 1971. Ferritin conjugated agglutinins as specific saccharide stains for electron microscopy. Application to saccharides bound to cell membranes. *Proc. Natl. Acad. Sci. U. S. A.* **68**:942.
9. HIRANO, H., B. PARKHOUSE, G. L. NICOLSON, E. S. LENNOX, and S. J. SINGER. 1972. Distribution of saccharide residues on membrane fragments from a myeloma cell homogenate. Its implications for membrane biogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2945.
10. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:201.
11. NAGATA, Y., and M. M. BURGER. 1972. Wheat germ agglutinin. Isolation and crystallization. *J. Biol. Chem.* **267**:2248.
12. AVRAMEAS, S., and T. TERNYNCK. 1971. Peroxidase-labelled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochimistry.* **8**:1175.
13. AVRAMEAS, S., and B. GUILBERT. 1971. Biologically active water-insoluble proteins polymers. Their use for the isolation of specifically interacting proteins. *Biochimie (Paris).* **53**:603.
14. AHLABO, I., and T. BARNARD. 1971. Observations on peroxisomes in brown adipose tissue of the rat. *J. Histochem. Cytochem.* **19**:670.
15. NOVIKOFF, A. B., M. E. BEARD, A. ALBALA, B. SHEID, N. QUINTANA, and L. BIEMPICA. 1971. Localization of endogenous peroxidases in animal tissues. *J. Microsc. (Paris).* **12**:381.
16. NOVIKOFF, P. M., and A. B. NOVIKOFF. 1972. Peroxisomes in absorptive cells of mammalian small intestine. *J. Cell Biol.* **53**:532.
17. HERZOG, V., and F. MILLER. 1972. The localization of endogenous peroxidase in the lacrimal gland of the rat during postnatal development. Electron microscope, cytochemical and biochemical studies. *J. Cell Biol.* **53**:662.
18. WIDMAN, J. J., R. S. COTRAN, and H. D. FAHIMI. 1972. Mononuclear phagocytes (Kupffer cells) and endothelial cells. Identification of two functional cell types in rat liver sinusoids by endogenous peroxidase activity. *J. Cell Biol.* **52**:159.
19. TOMITA, M., T. OSAWA, Y. SAKURAI, and T. UKITA. 1970. On the surface structure of murine ascites tumors. I. Interaction with various phytoagglutinins. *Int. J. Cancer.* **63**:283.
20. NICOLSON, G. L., and J. BLAUSTEIN. 1972. The interaction of *Ricinus communis* agglutinin with normal and tumor cell surfaces. *Biochim. Biophys. Acta.* **266**:543.
21. TOMITA, M., T. KUROKAWA, K. ONOZAKI, N. ICHIKI, T. OSAWA, and T. UKITA. 1972. Purification of galactose-binding phytoagglutinins and phytotoxin by affinity column chromatography using separose. *Experientia (Basel).* **28**:84.
22. SHARON, N., and H. LIS. 1972. Lectins: Cell-agglutinating and sugar-specific proteins. *Science (Wash. D. C.).* **177**:949.

23. BURGER, M. M., and A. R. GOLDBERG. 1967. Identification of a tumor-specific determinant on neoplastic cell surfaces. *Proc. Natl. Acad. Sci. U. S. A.* **57**:359.
24. AVRAMEAS, S., and B. GUILBERT. 1971. A method for quantitative determination of cellular immunoglobulins by enzyme-labeled antibodies. *Eur. J. Immunol.* **1**:394.
25. STEINMAN, R. M., and Z. A. COHN. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. *J. Cell Biol.* **55**:186.