IMMUNOCYTOCHEMICAL DEMONSTRATION OF THE BINDING AND INTERNALIZATION OF GROWTH HORMONE, B-CELL STIMULATING FACTOR-1 AND THY-1.2 IN MURINE LYMPHOCYTES

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The binding and internalization of growth hormone (GH), B-cell growth factor-1/interleukin-4 (BSF-1/IL-4), and Thy-1.2, in the mouse splenic lymphocytes have been described using an electron microscopic immunocytochemical technique. B-cell and T-cell lines in culture are used for the specific binding of BSF-1/IL-4 and Thy-1.2 respectively. A myeloma cell line, X 63/Ag 8.653, showed negative bindings for both BSF-1/IL-4 and Thy-1.2. GH binding is found on the surface and in the cytoplasm of most lymphocytes, but is not internalized by the myeloma cells. BSF-1/IL-4 bindings recognized by a monoclonal rat anti-BSF-1/IL-4 antibody (11B11) are found mainly on B-cells, whereas weak bindings are observed on T-cells. Thy-1.2 bindings are specific for T-cells. BSF-1/IL-4 and Thy-1.2 bindings immunolabelled at freezing temperature and reincubation at 37°C for 20 min are internalized by B- and T-cells respectively. These results indicate that immunocytochemical technique is a sensitive and powerful tool for detection of specific antigens on the cell surface or in the cytoplasm of cells in the immune system. The internalization of GH, growth factors, and specific antigens presents a possible mechanism of the growth promoting functions in the immune system.

Binding the specific receptor on a target cell surface is the first step for the action of a polypeptide hormone or growth factor. Different methods have been used to detect the binding hormones and regulation of their specific receptors on the surface of lymphocytes *in vitro* (4, 5, 10, 12-18, 21). The presence of radioactive-labelled human growth hormone (GH) and its receptor on a human lymphocyte cell line, IM-9, has been demonstrated, and in addition, the concentration of GH-receptors is controlled by the binding of GH to the cell surface (13). Furthermore, internalized I-125-insulin were also detected in this cell line (4). This evidence indicates that GH plays an important role in the complex proliferation system of the immune system (20).

B-cells were stimulated by B-cell growth factors (BCGFs), such as interleukin-2 (IL-2) and BSF-1 (17, 19) to proliferate and differentiate into antibody-forming cells or memory cells. BCGF-1 has also been known as BSF-1 (22) or interleukin-4 (IL-4)

(17, 30). BSF-1/IL-4 is a glycoprotein with a molecular weight of 20,000 Daltons (18). It stimulates the proliferation of B cells (5) as well as T cells (10). The receptors for BSF-1/IL-4 have been detected on IL-2, IL-3 dependent cell lines and B cells, T cells, macrophages and myelomonocytes at different levels (21). The expression of the BSF-1/IL-4 receptors on B cells or T cells indicates the diverse activities stimulated by BSF-1/IL-4. Thus the amount of BSF-1/IL-4 bound on target cell surface indicates a specific activation related to the functions of lymphokines.

The proliferation of T cells can be activated by antigens and lectins, and also depends on a T cell growth factor (TCGF) with a specific receptor on the T cell surface (24). Recently, T-cell receptor complex was found internalized and accumulated intracellularly by immunohistochemistry, and this phenomenon is controlled by the function of cytoplasmic microtubules (27). Evidence indicates that accumulation of the specific antigens in the cell cytoplasm is related to the specific activation of T-cells (26, 27). In the present work, the specific binding of Thy-1.2, a T-cell surface antigen in inbred strains, on the T-cell surface and in the cytoplasm detected by an indirect immunocytochemistry has been identified. This method is available to demonstrate the binding and pathway of internalized ligand-antigen complexes in the cytoplasmic vesicles in immune system as well as in the polypeptide hormone system.

MATERIALS AND METHODS

Reagents and cell lines: Hank's buffer and OPTI-MEM culture medium in 500 ml bottles were purchased from GIBCO Co., Grand Island, NY, USA. 4% fetal bovine serum (FBS, GIBCO), penicilin and streptomycin (10,000 units/ml of each) were added to the OPTI-MEM medium. PRMI-1640 (GIBCO) complemented with 10% FBS and 2 mM L-glutamine and OPTI-MEM were generally used for culture of lymphocytes. Rabbit anti-pig GH was prepared as in a previous experiment (28). Anti-IL-4 (11B11) ascites maintained in PRMI-1640 were a gift from Dr. S. Tonkonogy at the Department of Microbiology, College of Veterinary Medicine, North Carolina State University. Rat anti- Thy-1.2 was obtained from Boehringer Mannheim Biochemicals, USA. CTLL-2 cell line, a clone of cytotoxic T-cell derived from C57BL/6 mice, was obtained from American Type Culture Collection (ATCC) and maintained in RPMI-1640 culture medium containing 10% FBS and 2 mM L-glutamine in a 5% CO₂ incubator at 37°C. A myeloma cell line, X 63/Ag 8.653, was maintained and used as before (28).

Cell culture of EL-4 cells: EL-4 cells, a lymphoma induced from C57BL/6 mouse, were obtained from ATCC and maintained in a serum-free culture medium under regular tissue culture conditions. When cell density reached $5 \times 1,000,000$ cells/ml, three times the culture medium containing 10 ng/ml of 4- β -phorbol-12- α -myristate-12- α -acetate (PMA) (19) was added. Forty-eight hours later, the supernatant was collected by centrifugation at 4000 rpm for 30 min. The IL-4 containing supernatant was used at a dilution of 1:10 as an exogenous BSF-1/IL-4 binding to B-cell surface.

Preparation of splenic lymphocytes: The lymphocytes were collected from the spleen obtained from BALB/c or BDF-1 mice. After washing twice in Hank's buffer, the spleen tissue was trimmed into small pieces by a fine scissors in OPTI-MEM culture medium. The lymphocytes with red blood cells (RBC) were collected by cen-

trifugation at 500 rpm for 1 min. The RBCs were lysed with an osmotic shock technique. The cell samples were centrifuged at 1,500 rpm for 5 min and the pellet was resuspended in 0.5 ml phosphate buffer saline (PBS). The lymphocytes were centrifuged again and ready for immunocytochemistry. The cells were used at a concentration of $5 \times 1,000,000/\text{ml}$.

BSF-1/IL-4 pretreatment: BSF-1/IL-4 containing supernatant from serum-free, PMA stimulated EL-4 cell culture medium was used at a dilution of 1:10 for 1 hr at 10°C. The cell samples were then fixed as before and washed overnight in Dulbecco's phosphate buffer (D-PBS) at 4°C in the refrigerator. Immunolabelling was followed as described.

Enzyme-Linked Immunosorbant Assay (ELISA): Proper antigen-antibody reaction concentrations were determined by ELISA tests before immunolabelling experiments. 96-well plastic plates (Dynatech) were coated overnight at 4°C with each antigen at a protein concentration of 50 ng/50 μl each well in 0.05 M sodium bicarbonate buffer (pH 9.6). The wells were then washed with D-PBS and incubated with 2% bovine serum albumin (BSA) to block the non-specific binding sites. 50 μl of each primary antibody or individual hybrid cell supernatant fluid was added and incubated at room temperature (RT) for 2 hr with stirring. Then the wells were rinsed with 0.1% Tween-20 in D-PBS and incubated with affinity-purified goat antimouse IgG antibody conjugated with peroxidase (PO) (KPL) at a dilution of 1/1000 in D-PBS. After several washes, the plates were treated with 120 μl of substrate mixture containing 0.05% O-phenylenediamine (Sigma) and 0.04% hydrogen peroxide in citrate buffer (pH 5.0). The color reaction was measured by a ELISA spectrophotometer at 450 nm.

Immunocytochemistry: Indirect immunocytochemical labelling technique was used in this study. For post-fixation samples, the lymphocytes were fixed in 2.5% glutaraldehyde in D-PBS, pH 7.4, at RT for 25 min before immunolabelling. The fixative was washed away with the same buffer overnight in refrigerator. The cell samples were rinsed in D-PBS containing 0.3% Tween-20 for 10 min. They were then rinsed in D-PBS and incubated in the 2% BSA in D-PBS or in 1% BSA plus 1:200 dilution of normal rabbit serum for 1 hr in order to block the non-specific binding sites. The samples were incubated in individual primary antibody medium respectively for rabbit anti-GH (1:200–1:500 dilution in D-PBS), for rat anti-Thy-1.2 (1:25) and for 11B11, rat anti-BSF-1 IL-4 (1:10) for 2 hr at RT with stirring. The cells were then washed thoroughly with D-PBS before secondary blocking with 1% BSA with normal goat serum (1:200 dilution) for 1 hr at RT. The antigenic sites were visualized by incubation with a secondary antibody of goat anti-rabbit IgG or goat anti-rat IgG conjugated to PO (KPL, 1:200–1:500 dilution in D-PBS with 0.05% BSA) respectively for 1 hr at RT. The samples were rinsed with 0.05 M Tris-HCl buffer, pH 7.6 for 15 min containing 0.05% diaminobenzidine (DAB). A final concentration of 0.012% hydrogen peroxide was added to the DAB solution and incubated for another 15 min. After washing with distilled water and then post-fixing with 1% aqueous OsO_4 for 1 hr at 4°C, the cells were dehydrated with graded ethanols and embedded in Spurr. Orientation and reembedding were necessary for the samples fixed on the plastic coverslip (Miles) or regular coverslips coated with 1% polylysine. Unstained thin sections were examined under a Zeiss-109 transmission electron microscope (TEM).

For pre-fixation cell samples, all immunolabelling procedures were performed at 4°C before fixation. The cell samples of: (a) without treatment of GH, (b) with or without treatment with BSF-1/IL-4, (c) without treatment with Thy-1.2, were washed with D-PBS three times, and incubated in 2% BSA for 30 min at RT. The cells were subsequently washed and incubated with primary antibody respectively for 20–60 min. After a quick wash of 0.3% Tween-20, the samples were incubated with 2% BSA for another 15 min, then followed with incubation of secondary antibody conjugated with PO. Cells were fixed immediately in 2.5% buffered glutaraldehyde for 30 min at RT after reincubation at 37°C for 5–30 min for promotion of internalization of hormone or antigen-ligand complexes. The cells were washed for 1–2 hr with D-PBS, then reacted with DAB as before. After washing with several changes of distilled water, the cells were post-fixed in cold OsO₄, dehydrated and embedded in Spurr.

Embedding of Cells Stuck on Plastic Coverslips: Post- or Pre-fixed cell samples stuck on coverslips from Miles or coated with 1% polylysine were washed, and immunolabelled as before in a 24-well-plate. The cells were post-fixed with OsO₄ and dehydrated with graded ethanols. The coverslips were passed through changes of Spurr. With cells attached to the top side, the coverslips were inverted and placed on the Spurr filled wells. The resin was polymerized overnight at 70°C. The cured resin block was separated from the coverslip by immersing into liquid nitrogen. The resin blocks with cells were reembedded by inverting the blocks cell-side down, over another mold well filled with fresh pure resin and polymerized again. Unstained thin sections were examined with TEM.

RESULTS

Growth hormone: The binding and internalization of endogenous growth hormone on the lymphocytes freshly prepared from the spleen of BALB/c or BDF-1 mice were localized on part of the membrane or forming a cap close to the microvilli (arrowhead, Fig. 1). The cell samples were incubated at 37°C for 15 min. After being immunolabelled at a freezing temperature. Internalized GH-ligand complex was found as a tadpole-shaped dark granule of HRP-DAB reaction deposit near the Golgi complex (Figs. 1, 2). This granule is composed of a vesicle and a tubule filled with GH-ligand complex. The Golgi cisternae were clean of the reaction deposits. Internalized GH-ligand complex were seen in the vesicles of the cell center or Golgi-En-

FIG. 1. GH bindings on the freshly prepared lymphocytes immunolabelled at freezing temperature with PO-conjugated antibody before fixation. The cells were reincubated at 37°C for 10 min. The GH-ligand complexes were concentrated at the base of microvilli (arrowhead). The cell surface was clean of GH-labelling. A tadpole-shaped vacuole filled with GH-ligands was seen near the Golgi apparatus (G). ×26,450

FIG. 2. Same cell sample as in figure 1. The lymphocytes were reincubated at 37°C for 30 min. The cell surface was clean of GH-ligand binding. Internalized GH-ligands are shown in the lysosome-like vacuoles (V) near the Golgi apparatus (G). N, nucleus. ×26,800

FIG. 3. Same cell sample as in figure 2. The lymphocytes were reincubated at 37°C for 30 min. The cell surface was negative of GH binding. GH-ligands are shown in the lysosome-like vacuoles (V) and multivesicular body (mb) near the GERL area (GE). N, nucleus. ×26,800

FIG. 4. A myeloma cell immunolabelled before fixation for GH bindings was reincubated for 30 min at 37°C. The GH-ligands are concentrated on the cell surface (arrowhead), there was no sign of internalization of these bindings. ×18,000

FIG. 5. Homogenous distribution of BSF-1/IL-4 bindings are seen on the cell surface of the freshly prepared lymphocytes immunolabelled after fixation. About 85% of the splenic lymphocytes showed positive binding. $\times 12,150$

FIG. 6. A freshly prepared lymphocytes was immunolabelled for BSF-1/IL-4 bindings, and then reincubated at 37°C for 10 min. before fixation. Capping of BSF-1/IL-4 bindings are seen on the cell surface (arrowhead). Positive bindings are internalized in a vacuole (V) near the nucleus (N). $\times 30,500$

FIG. 7. Control lymphocytes without primary antibody incubation show negative binding of ligands. $\times 30,500$

doplasmic Reticulum-Lysosome (GERL) area after incubation at 37°C for 30 min in the prefixed lymphocytes from fresh spleen (Fig. 3). The GH-ligand granules were seen mostly in the lysosome-like vesicles of different sizes and shapes (Figs. 2, 3). This pattern of GH-ligand labellings was similar to that in our previous work in the Chang hepatoma ascites cells (25). On the contrary, GH-ligand deposits were seen on the cell membrane of the myeloma cell. No internalization was found after incubation at 37°C for 30 min (Fig. 4).

B-cell stimulating factor-1/interleukin-4 (BSF-1/IL-4): Bindings of exogenous BSF-1/IL-4 on the lymphocytes from fresh spleens were found homogeneously on the postfixed lymphocyte surface (Fig. 5), or accumulated on the cell surface and internalized in the prefixed samples (Fig. 6). A vesicle containing BSF-1/IL-4-ligand granule was seen in the cytoplasm attached to the vesicular membrane (V in, Fig. 6). A cap-forming area of the cell membrane at the root of a microvilli was observed (arrowhead, Fig. 6). The lymphocytes omitting primary antibody incubation were negative of ligand-PO deposits (Fig. 7). On the T-cell samples (CTLL-2) *in vitro*, weak bindings of exogenous BSF-1/IL-4 pretreatment were seen (arrowhead, Fig. 8). Those samples without pretreatment of BSF-1/IL-4 were negative of bindings. Negative binding was observed on the cell surface of the IgG non-secreting myeloma cell (Fig. 9).

Thy-1.2: The binding of Thy-1.2 is specific for T-cell lines (CTLL-2). T-cells *in vitro* showed strong endogenous Thy-1.2 binding on the postfixed cell samples (Fig. 10). About one third of the freshly prepared lymphocytes showed positive Thy-1.2 binding (Fig. 11), which were believed to be T-cells. The Thy-1.2 bindings were seen on part of the cell membranes which is different from the homogenous BSF-1/IL-4 distributions on the B-cells (Fig. 5). Internalization of Thy-1.2-ligand complexes was observed after immunolabelling at freezing temperature and reincubated at 37°C for 20 min (Fig. 12). The Thy-1.2-ligand deposits in small vesicles (50–100 nm in dia.), vacuoles (>0.1 μ m) and tubules close to the Golgi complex and the nucleus were observed.

DISCUSSION

The indirect immunocytochemical tracing techniques of the ligand-mediated internalization of a polypeptide hormone, GH, has been described in Chang hepatoma

FIG. 8. A post-fixed T-cell in tissue culture is slightly labelled (arrowhead) with BSF-1/IL-4 antibody. $\times 15,200$

FIG. 9. An immunoglobulin non-secreting myeloma cell is immunolabelled the same as the lymphocytes in figure 5 and show negative BSF-1/IL-4 bindings (arrowhead). $\times 11,300$

FIG. 10. Thy-1.2 specific binding is detected on the post-fixed T-cell surface (arrowhead). Note the Thy-1.2 bindings are much heavier than that of BSF-1/IL-4 bindings on the same T-cell line in figure 8. $\times 14,200$

FIG. 11. Thy-1.2 bindings is detected on the freshly prepared lymphocytes. About 85% of the lymphocytes show positive Thy-1.2 bindings. Note the specific Thy-1.2 bindings on the T-cell surface are distributed on part of the cell membrane (arrowhead). $\times 16,070$

FIG. 12. The internalization of Thy-1.2 specific bindings into the cytoplasm of a pre-fixed T-lymphocytes from the spleen. Internalized Thy-1.2 bindings are found in the vesicles (v), vacuoles (V), tubules (t) and in the vesicles (arrowhead) close to the Golgi cisternae (G). The cell surface is clean from labelling. $\times 34,400$

ascites cells (28). In the immune system, proliferation and differentiation of activated B-cells into memory cells or plasma cells to produce antibody was modulated by the polypeptides called B-cell stimulating factors (BSFs) secreted by T-cells (19, 30). Using radiolabelled T cell growth factor (TCGF), Robb *et al.* (24) have demonstrated that TCGF interacts with T cells through a specific receptor similar to the reactions of polypeptide hormones. Recently, attention has been focused on the internalization and accumulation of specific antigens and/or receptors located on B-cell or T-cell surfaces (26, 27).

The growing number of polypeptide modulators involved in the immune system (20) and various receptors on lymphocytes has also been demonstrated (8, 10, 15, 16, 21, 29). The present immunocytochemical method provides an indirect visual probe to the evidence that GH and growth factor, or marker antigens located on the cell surface of lymphocytes can induce ligand-mediated internalization of antigen-ligand-complexes. GH receptors on cultured lymphocytes have been demonstrated in Lesniak's laboratory with radioactive labelled human GH (12-14). They indicated that GH binds to the lymphocyte surface, and there is a time/concentration dependent loss of GH receptors. This evidence supports our observations that internalized GH-ligands was found in the lysosome-like vesicles in the GERL region. The internalization of GH-receptor complexes could be the course of induction of cell response or the degradation of extra GH molecules. However, uptake of the capping GH-ligands on the surface of an Ig-non-secreting myeloma cell did not occur.

Exogenous BSF-1/IL-4 pretreatment increased the labelling of Ag-ligand binding. Recently, similar immunochemical methods using anti-IL-2-receptor antibody has been applied to the IL-2 receptor system on the embryonic thymocytes undergoing mitosis (11). The binding detected on the T-cells supports the observation that BSF-1 promotes the growth of T-cells lines (7). The mechanism of a second messenger model by which interleukins regulate gene expression and T-cell proliferation has been described by Farrar *et al.* (6). However, the demonstration of the BSF-1/IL-4 receptor on the lymphocytes (16, 21) and the accumulated BSF-1/IL-4 ligands in the lymphocytes (27) were parallel to our immunocytochemical observations that BSF-1/IL-4 bound and then internalized in the splenic lymphocytes.

Ligand-induced internalization and reexpression of receptors labeled with radioactive iodine has been describe for IgE receptors (23). Internalization of interleukin-1 receptor complexes radioactively labeled with iodine was found in T cells, bearing IL-1 receptors with two different affinities (15). The mechanism of the T-cell activation induced by the ligand-receptor complexes of T3-T-cell antigen (29) looks quite similar to that of hormone-induced internalization of hormone-receptor complexes (28). However, there was no proof that internalization of ligand-receptor complexes happens in the T-cell activation system for cell responses until the report of Tse *et al.* (27). After capping of the ligand compexes on cell surface, endocytosis or internalization could vary in different cell populations by forming endosomes, lysosomes, and vesicles fusing with GERL complex for further processes of ligand-receptor sorting, degradation and/or recycling. The internalized ligand-receptor complexes in the lymphocytes of the immune system are similar to the internalization of human growth hormone through the mechanism of receptor-mediated endocytosis visualized by autoradiography (9). Earlier study by labelling Con-A on the cell surface of lymphocytes (2) indicates that the internalization of Con-A-receptor complexes occurs in

about 80% of the lymphocytes is increased according to the time of incubation. Lectin-labelling (3) can block the association of GH to its receptor, which is a glycoprotein. However, neither Con-A or WGL labelling prevents the recognition of anti-GH antibody to GH, since there is no glycan present on the GH molecule (28). According to our observations, the polyclonal antiserum to porcine GH is not species specific, however, the GH receptor-mediated pinocytosis is a cell-specific reaction (1, 16).

In summary, the pattern of the internalization of the growth factors and specific antigens in the immune system is similar to the reaction of the polypeptide hormone with its target cell. Several possibilities for growth factor interactions with its binding site in the immune system could be predicted: (a) The growth factor remains on the cell surface by binding to the receptor and initiating cell growth as in the non-Ig-secreting myeloma cells. (b) The receptor-ligand-antibody complexes are internalized and concentrated in the lysosome-like vesicles or endosomes for storage and possibly delay the activity of growth factor. (c) The complexes move to the lysosomes, thereby undergoing a sorting of growth factor and receptor, or degradation of the growth factor and/or the receptor, or possibly the recycling of receptors to the surface. (d) Further movement of growth factor to their growth-promotion sites, such as the nucleus.

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