Immunoglobulin A (IgA) Polymerization Sites in Human Immunocytes: Immunoelectron Microscopic Study

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ABSTRACT. The cytoplasmic affinity of polymeric IgA for secretory component (SC) and the expression of joining (J) chain were examined in pokeweed mitogen (PWM)-stimulated human peripheral blood lymphocytes (PBL) to determine, on the ultrastructural level, the polymerization sites of human IgA. SC-binding was found in 5.7% of transformed PBL on day 7 of culture; SC-binding was observed in a high proportion of IgA-producing cells. A low proportion of IgM-producing cells also bound to SC, while there was virtually no SC-binding by IgG-producing cells. A high proportion of IgA- and IgM-producing cells expressed intracellular J chain, while approximately half of the IgG-producing cells were positive for J chain.

The number of J chain-positive cells exceeded the number of SC-binding cells among transformed PBL on day 7 of culture. Immunoelectron microscopic study of the sites of SC-binding, and of IgA and J chain expression, revealed that polymerization of human IgA and the addition of J chain occur in the perinuclear space and endoplasmic reticulum, prior to immunoglobulin secretion.

Secretory immunoglobulin A (S-IgA), which plays important roles in the defense of mucosal surfaces, is composed of a secretory component (SC), joining (J) chain and two to four covalently linked molecules of monomeric IgA (for view, see ref. 22). SC is a glycoprotein produced in the endoplasmic reticulum and perinuclear space of the epithelial cells of glandular tissues (5). SC is then directed to the basolateral membranes of epithelial cells and functions as a receptor for polymeric immunoglobulin (5, 22, 26). J chain-containing polymeric IgA, synthesized and secreted by plasma cells, binds to SC on epithelial cell membranes, and is then internalized by pinocytosis and finally secreted into the glandular lumen in the form of S-IgA (5, 22).

It was proposed in the early reports (31, 35) that monomeric IgA produced by plasma cells is polymerized in the glandular epithelial cells by complexing with SC to form S-IgA. However, subsequent immunochemical studies of rabbit (19) and human S-IgA molecules (2), and of IgA released into the culture media from explants of secretory tissues or isolated cells from such tissues (for review, see ref. 22) convincingly demonstrated that polymers are formed within IgA-producing cells before their selective transport into external secretions. Although the site of polymerization was not determined by morphological studies, biosynthetic studies revealed that IgA and IgM were polymerized shortly before, or at the time of secretion, and that J chain was

required for polymerization in mouse myeloma cells secreting IgA and IgM (1, 8, 15, 22, 27–28). However, the role of J chain in the polymerization of IgA and IgM has been questioned (9, 11, 16, 22); J chain was found not only in polymer-producing cells, but also in the cells that produce monomeric IgA, IgG, or IgD (4, 13–15, 22–23, 36). Thus the actual function of J chain remains controversial.

Free SC isolated from external secretions can combine in vitro with polymeric immunoglobulins (20, 22, 29). Applying this principle to tissue sections, Brandtzaeg (3, 6) and Radl *et al.* (30) found that J chain-producing IgA and IgM immunocytes in various mucosa-associated tissues and glands can express cytoplasmic affinity for SC (SC-binding test), and showed that immunocytes producing monomer or polymer forms can be distinguished in such tissues by the paired immunofluorescence method. Although it has been described that polymerization of IgM occurs in the endoplasmic reticulum of IgM-secreting murine plasmablasts examined by a combination of pulsechase protocols, subcellular fractionation and electron microscopic autoradiography (34), the polymerization site of human IgA has not been conclusively determined at the ultrastructural level.

Thus, in this study we have examined the relationship between J chain expression and polymer production, using the SC-binding test in transformed human

peripheral blood lymphocytes (PBL) stimulated with pokeweed mitogen (PWM). Furthermore, the sites where the polymerization of human IgA and the addition of J chain occur were determined by means of immunoelectron microscopy.

MATERIALS AND METHODS

Tissue culture and cell processing. The peripheral blood used in these studies was obtained from healthy young university students of both sexes. Mononuclear cells were isolated by centrifugation on a Ficoll-Hypaque density gradient. After the removal of monocytes by adherence to plastic dishes, the remaining cells were cultured at a concentration of 1.0×10^6 /ml of RPMI 1640 medium, supplemented with 20% fetal bovine serum (Gibco Co., Grand Island, NY) and 50 μ g/ml gentamicin (Schering Corp., Kenilworth, NJ), in the presence of 0.1 ml PWM/ml culture medium. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in air. Cyto centrifuge slides of cultured cells were fixed for 10 min in 95% ethanol containing 5% acetic acid, and after extensive washing in phosphate (0.01 M)-buffered saline (0.15 M) pH 7.2 (PBS), the slides were used for immunofluorescent staining. The remaining cells were processed for immunoelectron microscopic staining.

SC purification and SC-binding test. SC was purified from human colostrum according to previously published methods (21). Detection of cytoplasmic affinity for SC (SC-binding test) in transformed PBL was performed according to the method of Brandtzaeg (3). Briefly, transformed PBL was incubated with SC diluted in 1% normal rabbit serum to a concentration of 200 μ g/ml for 2 hr at room temperature. After washing with PBS, smears were incubated for 45 min with fluorescein isothiocyanate (FITC)-conjugated anti-human SC (Dakopatts, Copenhagen, Denmark), washed, then mounted with Elvanol (Monsanto, St. Louis, MO). To determine the proportion of SC-binding cells of each immunoglobulin isotype, cells were incubated further with tetramethylrhodamine isothiocyanate (TRITC)-conjugated F(ab')₂ fragments of anti-human IgA, IgM, or IgG (Nordic Immunol. Lab., Tilburg, The Netherlands). Similarly, to determine the proportion of J chain-positive cells among IgG-, IgA-, or IgM-containing cells, transformed PBL were incubated for 45 min with TRITC-conjugated anti-human J chain prepared in this laboratory (13, 18, 23) and, after washing with PBS, cells were further incubated with FITC-conjugated heavy chain-specific anti-human IgA, IgM, or IgG reagents. At least 1000 cells were counted with a fluorescence microscope.

Immunoelectron microscopic procedures. To determine the localization of SC-binding sites in transformed PBL, cultured cells were initially fixed in periodate-lysine-2% paraformaldehyde (PLP) for 30 min at room temperature and then resuspended in PBS. Fifty μ l of fixed cells at a concentration of 4×10^6 cells/ml were transferred in a cyto centrifuge onto glass slides coated with 0.01% poly-L-lysine and were

dried for 2 hr at 4°C. After extensive washing with PBS, the cells were incubated with normal rabbit serum for 20 min and then with SC for 6 hr. After washing, horseradish peroxidase (HRP)-labeled F(ab')₂ fragments of anti-human SC were added for 6 hr. Specimens were immersed for 25 min in 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution without H₂O₂, and subsequently in DAB solution containing 0.005% H₂O₂ for 5 min (12). The cells were extensively washed, fixed in 2.5% glutaraldehyde and 1% O₃O₄ fixatives, dehydrated in graded ethanol and embedded in Spurr's resin (32). Localization of SC-binding sites in the human small intestine was determined using the method described by Nagura *et al.* (26). In brief, a histochemically normal small intestine obtained from a patient with stomach carcinoma was divided into small pieces by means of a razor blade. Such samples were fixed in 4% PLP for 6 hr at 4°C, washed successively with PBS containing 10, 15, and 20% sucrose, and then embedded in OCT compound (Miles Scientific, Naperville, IL). Frozen sections were made with a cryostat and processed for the same SC-binding test described above. Omission of SC or HRP-labeled anti-human SC reagent were used as controls in the immunoelectron microscopic SC-binding test.

To examine the localization of both SC-binding sites and IgA in a single cell, a double immunoelectron microscopic procedure was applied to transformed PBL. After immunoelectron microscopic staining for SC-binding and DAB reaction, cells on the glass slides were subsequently incubated overnight with biotinylated anti-human IgA (Vector Lab., Burlingame, CA) at 4°C. Cells were washed extensively with PBS and further incubated overnight with avidin-labeled ferritin (Vector) under the same conditions. Localization of both SC-binding sites and J chain in a single cell was examined similarly; after immunoelectron microscopic staining for the SC-binding test and DAB reaction, cells were incubated with rabbit anti-human J chain followed by biotinylated anti-rabbit IgG (Vector) and then with avidin-labeled ferritin (Vector). Omission of biotinylated anti-human IgA, avidin-labeled ferritin, anti-human J chain, or biotinylated anti-rabbit IgG, were used as controls. In addition, non-immune human IgG was used as a control for all experiments.

RESULTS

SC-binding in PWM-stimulated blood lymphocytes. There were no cells that displayed cytoplasmic affinity for SC among unstimulated peripheral blood lymphocytes in the 8 cases examined. However, after PWM stimulation, two patterns of cytoplasmic affinity for SC were observed by immunofluorescence: one exhibited diffusely stained cytoplasm, and the other showed a spotlike staining which probably corresponded to the Golgi area. A few SC-binding cells appeared on days 3–4 of culture after PWM stimulation. The number of positive cells increased gradually day by day and reached a plateau at days 6–7 of culture. The in-

Table I. CYTOPLASMIC SC-BINDING AND J CHAIN POSITIVITY IN PWM-STIMULATED PERIPHERAL BLOOD LYMPHOCYTES (7 DAY CULTURES).

Subject	Total Ig	Total SC-B	Total J	Percentage of Positive Cells								
				IgA Cells			IgM Cells			IgG Cells		
				IgA	SC-B	J	IgM	SC-B	J	IgG	SC-B	J
1	27.7	8.9	77.8	11.0	74.1	93.2	7.4	10.0	74.3	7.5	0	61.0
2	14.0	4.2	82.1	3.9	71.4	91.9	3.1	27.8	86.1	5.3	7.1	72.9
3	20.4	4.8	72.8	4.5	86.7	86.4	5.9	5.0	72.5	8.3	0	57.5
4	10.1	3.3	72.4	3.7	60.0	83.8	2.4	12.5	71.4	2.3	0	56.3
5	16.8	4.8	71.1	3.8	93.8	90.4	7.3	16.6	89.7	5.0	0	28.6
6	24.2	8.2	74.5	7.4	85.7	93.7	8.3	29.4	91.1	8.5	0	47.5
7	18.8	7.3	74.7	5.0	87.5	94.8	5.4	54.2	82.9	8.4	0	52.5
8	7.4	4.2	75.7	3.4	69.0	87.9	2.4	42.9	80.0	2.1	0	42.9

PWM, Pokeweed mitogen; SC, Secretory component; B, Binding; Ig, Immunoglobulin; J, J chain.

crease in SC-binding cells paralleled the increase in immunoglobulin-producing cells, and on day 7 approximately 5.7% of total cells showed cytoplasmic affinity for SC. Only IgA- and IgM-producing cells had a cytoplasmic affinity for SC; SC-binding was detected in approximately 78.5% of IgA- and 24.8% of IgM-producing cells. SC-binding was not detected in IgG-producing cells at this time except for in one of the 8 samples examined. In this case, SC-binding was found in approximately 7.1% of IgG-producing cells (Table I).

J chain expression in PWM-stimulated peripheral blood lymphocytes. J chain expression was detected

in all of the immunoglobulin (IgA, IgM and IgG)-producing cells with an average of 75.1%. A high percentage of IgA- and IgM-producing cells expressed J chain with averages of 90.2% and 81.0%, respectively, while approximately 52.4% of IgG-producing cells were positive for J chain. Table I summarizes the relationships between SC-binding positivity and J chain expression in IgA-, IgM- and IgG-producing cells in PWM-stimulated peripheral blood lymphocytes on day 7.

Immunoelectron microscopy. By immunoelectron microscopy, SC-binding sites were observed in the perinuclear space and the endoplasmic reticulum of PWM-

Fig. 1. Immunoelectron microscopy of peripheral blood lymphocytes stimulated with PWM for 7 days in culture. To reveal the SC-binding sites, fixed cells were incubated first with SC followed by HRP-labeled anti-SC. The binding sites are localized in the endoplasmic reticulum (arrows) and perinuclear space (arrow heads). Original magnification $\times 6,030$. Bar = 1 μ m

Fig. 2. Immunoelectron microscopy of plasma cells in the lamina propria of the human small intestine. SC-binding sites are found in the endoplasmic reticulum and perinuclear space of such plasma cells. Original magnification $\times 3,190$. Bar = $2 \mu\text{m}$

Fig. 3. The coincidental localization of SC-binding sites (revealed by incubation with SC and HRP-labeled anti-SC) and IgA (ferritin-labeled anti-IgA) in the endoplasmic reticulum of PWM-stimulated (7 day culture) human peripheral blood lymphocytes. Original magnification $\times 73,000$. Bar = $0.3 \mu\text{m}$

Insert: high magnification of the dashed area. Arrows indicated the SC-binding sites and arrow heads indicate IgA-containing sites. Original magnification $\times 103,300$. Bar = $0.1 \mu\text{m}$

Fig. 4. Immunoelectron microscopic localization of J chain (revealed by HRP-labeled anti-J chain) in PWM-stimulated (7 day culture) human peripheral blood lymphocytes. Original magnification $\times 6,900$. Bar = $1 \mu\text{m}$

stimulated PBL (Fig. 1). SC-binding sites, including the perinuclear space and endoplasmic reticulum, were detected in plasma cells infiltrating in the lamina propria of a human small intestine (Fig. 2). No endogenous SC was detected in plasma cells of any control sections, while it was found in the perinuclear space, endoplasmic reticulum, small vesicles and basolateral membrane of epithelial cells when HRP-labeled anti-human SC was used.

By double immunoelectron microscopy with SC and HRP-labeled anti-SC, and ferritin-labeled anti-IgA,

coincidental localizations of SC and IgA were observed. SC-binding sites and IgA were found in the perinuclear space and endoplasmic reticulum of transformed peripheral blood lymphocytes (Fig. 3). Some ferritin particles indicating the presence of IgA were observed in electron-lucent areas of the endoplasmic reticulum and also in the SC-binding areas (Fig. 3, insert). These findings suggest that both polymeric as well as monomeric IgA may have been present in this area. J chain was found in the perinuclear space and endoplasmic reticulum of PWM-stimulated PBL (Fig. 4). Coincidental

Fig. 5. The coincidental localization of SC-binding sites (revealed as described in Fig. 3.) and J chain (ferritin particles; see Materials and Methods) in the PWM-stimulated cells. Ferritin particles (arrows) are localized on the electron-lucent or dense areas in the endoplasmic reticulum. Original magnification $\times 99,000$. Bar = $0.1 \mu\text{m}$

localizations of SC-binding sites and J chain were observed in the perinuclear space and endoplasmic reticulum within a single transformed peripheral blood lymphocyte by double immunoelectron microscopy (Fig. 5). HRP and ferritin that were not linked with antibody did not bind to transformed PBL used as controls.

DISCUSSION

These results indicate that virtually all of the SC-binding is restricted to IgA- and IgM-producing cells; only a few IgG-containing cells bound SC as reported previously (3, 10, 18). These findings suggest that most of the IgA- and IgM-containing cells in PWM-stimulated cultures of PBL are involved in the synthesis of polymeric IgA. Immunochemical analysis of culture supernatants of such cells indicated that most of secreted IgA is indeed polymeric (17).

As reported previously, J chain was detected in PWM-stimulated PBL that produced IgA, IgM or IgG (24, 36). However, a high proportion of IgA- and IgM-positive cells expressed J chain while only approximately half of the IgG cells contained this polypeptide. Thus, the proportion of J chain-positive cells in stimulated PBL was higher than that of SC-binding cells.

Although the expression of J chain and the binding of SC by immunoglobulin-producing cells has been used to detect cells that produce polymeric immunoglobulins (3, 6, 10, 18, 24, 25, 30), the immunofluorescence technique did not permit the precise identification of intracellular sites and structures where the polymerization occurs. The immunoelectron microscopic studies presented here revealed that this event takes place in the perinuclear space and endoplasmic reticulum in PWM-stimulated PBL, and in plasma cells found in the lamina propria of the human small intestine. A previous study by Tartakoff *et al.* (34) suggested that the polymerization of IgM in murine plasmablasts also occurs in the endoplasmic reticulum.

The role of J chain in the process of intracellular polymerization of IgA and IgM has not been unequivocally established. Although secreted polymeric IgA and IgM molecules contain J chain, it remains unclear whether this glycoprotein initiates the polymerization (for review, see refs. 15, 22, 33). More recently Cattaneo and Neuberger (9) reported that J chain was not necessary for the polymerization of IgM in a unique cell system explored in their experiments. However, J chain in polymeric immunoglobulins plays a crucial role in the binding of SC, as demonstrated by extensive studies of the interaction of free SC with polymeric IgA or IgM, and of SC-bearing epithelial cell lines with polymeric IgA and IgM that contained variable quantities of J chain (for review, see refs. 5, 7, 22). The presence of J chain

in cells that produce monomeric IgG or IgD has been considered as a sign of an early maturation stage of such cells (5, 22). Our results obtained by double immunoelectron microscopy of the coincidental localization of J chain and SC-binding sites suggest that polymerization occurs in the perinuclear space and endoplasmic reticulum of PWM-stimulated PBL.

In contrast to these morphological studies, which clearly indicated the presence of intracellular polymers, detailed biosynthetic investigations of polymerization of IgA and IgM in murine and human cells revealed only small amounts of intracellular polymers, even in cells that secreted predominantly polymers (1, 8, 18, 25, 27). Based on such studies, Parkhouse and Della Corte (27) suggested that polymerization occurs shortly before or at the time of immunoglobulin secretion. This apparent variance in the results obtained by morphological, as compared to biosynthetic approaches, may be reconciled by considering the possibility that polymers assembled in the perinuclear space and endoplasmic reticulum are promptly exported from the cell.

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