J Clin Exp Hematopathol
Vol. 46, No. 2, Nov 2006

Original Article

Immunoelectron Microscopic Examination of Cord Blood $CD5^+$ B cells

Shigeyuki Asano[1], Haruki Wakasa[2]

In this study, we have categorized cord blood $CD5^+$ B cells, which were examined using the horseradish peroxidase-colloidal gold double labeling immunoelectron microscopy, into three subtypes based on their morphology and immunohistochemical characteristics. Type *1a* cells and type *1b* cells (9% and 17% of the $CD5^+$ B cells, respectively) had few cytoplasmic organelles, a high nuclear/cytoplasmic (N/C) ratio (0.66 ± 0.03 and 0.58 ± 0.04, respectively), and a low nuclear contour index (NCI) value (1.56 ± 0.30 and 1.50 ± 0.27, respectively), whereas type *2* cells (74% of the $CD5^+$ B cells) had a low N/C ratio (0.44 ± 0.11) and a high NCI value (2.05 ± 0.68). Type *2* cells, which had many cytoplasmic organelles, frequently had several uropod-like processes that bound to the gold particles. The N/C ratios clearly showed that there were significant differences among the three types of $CD5^+$ B cells ($p < 0.01$), and between $CD5^+$ T cells and the three types of $CD5^+$ B cells ($p < 0.05$). For the NCI values, only type *1b* and type *2* cells showed a significant difference ($p < 0.05$). These findings suggest that type *1a* cells are transformed into type *1b* cells, and then into type *2* cells. 〔*J Clin Exp Hematopathol 46(2) : 83-88, 2006*〕

Keywords: $CD5^+$ B cell, double labeling immunoelectron microscopy, nuclear cytoplasmic ratio, nuclear contour index

INTRODUCTION

$CD5^+$ B cells, which have been identified in cord blood[1-12], have a unique developmental course during which they don't migrate into germinal centers[1]. The detailed morphological characteristics of these cells, however, have not yet been described.

In the present investigation, we have studied $CD5^+$ B cells using a double immunolabeling method[13-17], and described three types of $CD5^+$ B cells and their maturation.

MATERIALS AND METHODS

Cell preparation and prefixation

Cord blood samples containing $CD5^+$ B cells were obtained from placental cord vessels soon after delivery using a 21-gage syringe. The samples were centrifuged (Lymphoprep™, Nycomed Pharma, Oslo, Norway) and washed twice in ice-cold Dulbecco's phosphate-buffered saline (PBS), pH 7.4[16,17]. The washed cells were prefixed on ice for 15 min in Zamboni's solution (pH 7.3) containing 2% paraformaldehyde and 0.5% glutaraldehyde. The cells were then rinsed twice in PBS for 30 min at 4℃.

Immunohistochemical procedure

Double immunolabeling was performed using a modified version of a previously described method[13,18]. All lymphocytes (5×10^6 cells/ml) were first incubated with anti-CD20 monoclonal antibody (MoAb) (1 : 10, Dakopatts, Denmark) (5 μl for 1×10^6 cells) overnight at 4℃. The cells were then washed twice for 5 min in ice-cold PBS and incubated with goat anti-mouse IgG antibody coupled to 20-nm colloidal gold particles (1 : 10, Zymette, USA) for 60 min at 4℃. After two more washes in PBS, the cells were incubated with biotinylated anti-CD5 MoAb (1 : 10, Dakopatts) for 120 min at room temperature, washed twice in PBS, and stained for 60 min at 4℃ using the avidin-biotin-horseradish peroxidase complex (1 : 100, Dakopatts). After two more washes, the cells were preincubated with 0.025% 3-3'-diaminobenzidine tetrahydrochloride (DAB ; Sigma, USA) for 30 min at 4℃ before a final incubation with 0.025% DAB and hydrogen peroxide for 5 min at 37℃. After washing the samples twice in PBS buffer, the cells were fixed in the previously described Zamboni's mixture on ice for 30 min and then washed with ice-cold PBS.

Received : Oct 18, 2005
Revised : Feb 24, 2006
Accepted : Mar 27, 2006

[1] Division of Pathology, Iwaki Kyoritsu, General Hospital, Iwaki, Japan
[2] Faculty of Medical Science & Welfare, Tohoku Bunka Gakuen University, Sendai, Japan
Address correspondence and reprint request to Shigeyuki Asano, Division of Pathology, Iwaki Kyoritsu, General Hospital, 16 Kusehara, Mimaya-machi Uchigo, Iwaki, 973-8555, Japan

Control samples to test for labeling specificity were treated by substituting mouse purified IgG and IgM for the anti-CD20 and anti-CD5 MoAbs, respectively.

Postfixation and cell embedding

After the double immunolabeling procedure, cells were mixed with 14% calf serum and incubated for 20 min at 4℃. Glutaraldehyde was gently added to the cells for more than 30 min at 4℃ to create cell blocks. The cell blocks were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 60 min at 4℃, dehydrated using a graded series of alcohols, and embedded in Araldite.

Ultrastructural and statistical evaluation

Ultrathin sections that were prepared with a diamond knife on a RMC MT-7 ultramicrotome were left unstained or were lightly stained for 5 min with uranyl acetate. A JEOL 100 CX electron microscope was used to examine and photograph the samples. For gold labeling, only those cells with at least three gold particles on the cell surface were counted as $CD20^+$ cells, whereas only cells showing complete peripheral HRP-staining of the cell surface were considered to be $CD5^+$ cells.

Cells that were double labeled with gold particles and HRP deposits were identified as $CD5^+$ B cells[13-19]. At least three samples from each experiment were examined. A total of 150 to 200 stained and unstained cells from each sample were counted for statistical evaluation. The nuclear/cytoplasmic (N/C) ratio and the nuclear contour index (NCI) of the $CD5^+$ cells, $CD20^+$ cells, and $CD5^+$ B cells were evaluated with a color image processor (SPICCA II, Nippon Avionics Co. Ltd., Tokyo, Japan) and three-dimensional TRI reconstruction software (Ratoc System Engineering, Tokyo, Japan). Statistical analysis was carried out using Student's tests[13,17]. A probability value of less than 0.05 ($p < 0.05$) was considered significant.

RESULTS

Cord blood lymphocytes expressing CD5, CD20, or the combination of CD5 and CD20 were easily identified by labeling with the respective antibodies.

$CD5^+$ cells

Lymphocytes labeled with anti-CD5 antibody had a rough cell membrane with few microvilli on their cell surface. The nuclei were reniform and the nucleoli were often distinct. The N/C ratio and NCI values are shown in Fig. 1. A small numbers of organelles including the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, and small vesicles were observed at the nuclear indentation. Additionally, there were a few clustered dense bodies. HRP deposits were only seen on the cell surface.

$CD20^+$ cells

A number of short microvilli were observed on the surface of lymphocytes labeled with anti-CD20 antibody. The N/C ratio and NCI values are shown in Fig. 1. As in the $CD5^+$ cells, the nuclei with heterochromatin were reniform and the nucleoli were often distinct. Organelles, such as ER, Golgi apparatus, mitochondria and small vesicles, were observed at the nuclear indentation. The $CD20^+$ cells labeled with gold particles were similar to B lymphocytes. Gold particles were observed on the microvilli and on a few flat cell surfaces.

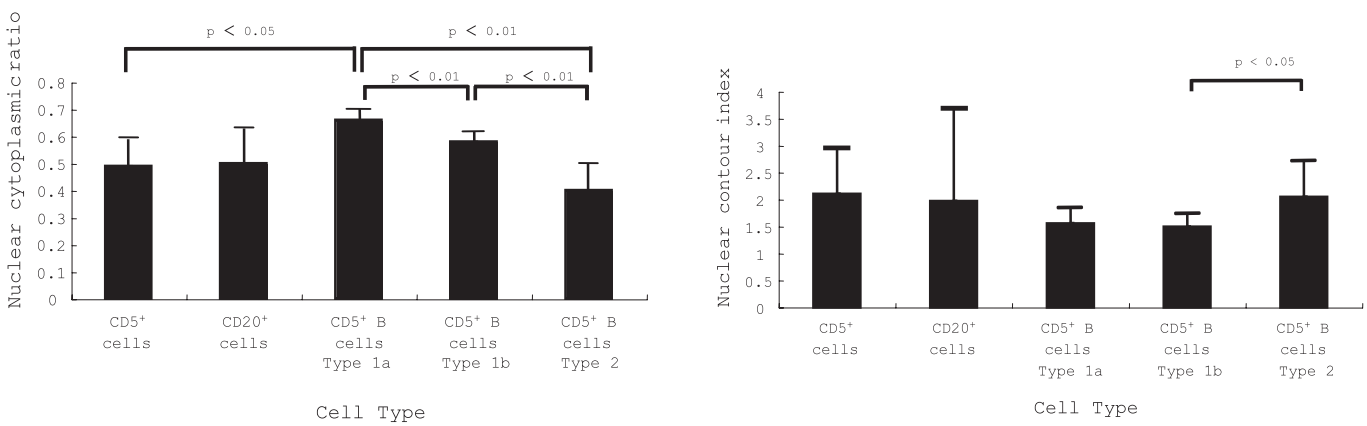

Fig. 1. Comparison of the N/C ratios and NCI values of cord blood lymphocytes. (*Left*) The nuclear cytoplasmic (N/C) ratio. (*Right*) The nuclear contour index (NCI). The results are the mean values and the error bars represent the standard deviations. Among the $CD5^+$ B cells, there were no significant differences in the NCI values except between the type *1b* and type *2* cells.

CD5+ B cells

The cell surfaces of the CD5+ B cells were discontinuously stained by HRP deposits with a weaker intensity than was observed for other CD5+ cells. The gold particles were mainly observed along the microvilli. Each CD5+ B cell was classified into one of three subtypes based on its morphology and the observed immunolabeling pattern (Fig. 1).

Type *1a* cells : There were a small number (9%) of type *1a* cells among the CD5+ B cells. These cells had a few microvilli and several long, slender cytoplasmic processes on the cell surface. The nuclei with heterochromatin were relatively irregular and were indented in the direction of the long cytoplasmic processes. A few organelles, including mitochondria, vacuoles, Golgi apparatus, and ER, were observed at the nuclear indentations. The N/C ratio and NCI values are shown in Fig. 1. The HRP deposits, which were weaker than those of other CD5+ cells, were evenly distributed on the cell surface. A small number of gold particles were confined to the cell surface (Figs. 2A, 2B).

Type *1b* cells : Type *1b* cells were also relatively rare (17% of the CD5+ B cells). There were several microvilli-like processes on the cell surface of these cells, whereas the long processes observed in the type *1a* cells were not seen. The nuclei with heterochromatin showed a slightly indented morphology. Several cytoplasmic organelles were observed at the nuclear indentations. The N/C ratios and NCI values are shown in Fig. 1. As was observed for type *1a* cells, weak HRP staining was continuously distributed on the cell surface. The number of gold particles on the cell membranes of the type *1b* cells was equal to or slightly greater than the number on the type *1a* cells (Figs. 3A, 3B).

Type *2* cells : Seventy-four percent of the CD5+ B cells were type *2* cells. These cells often had several uropod-like processes and microvilli on the cell surface. The nuclei with heterochromatin were irregular and indented on the side clos-

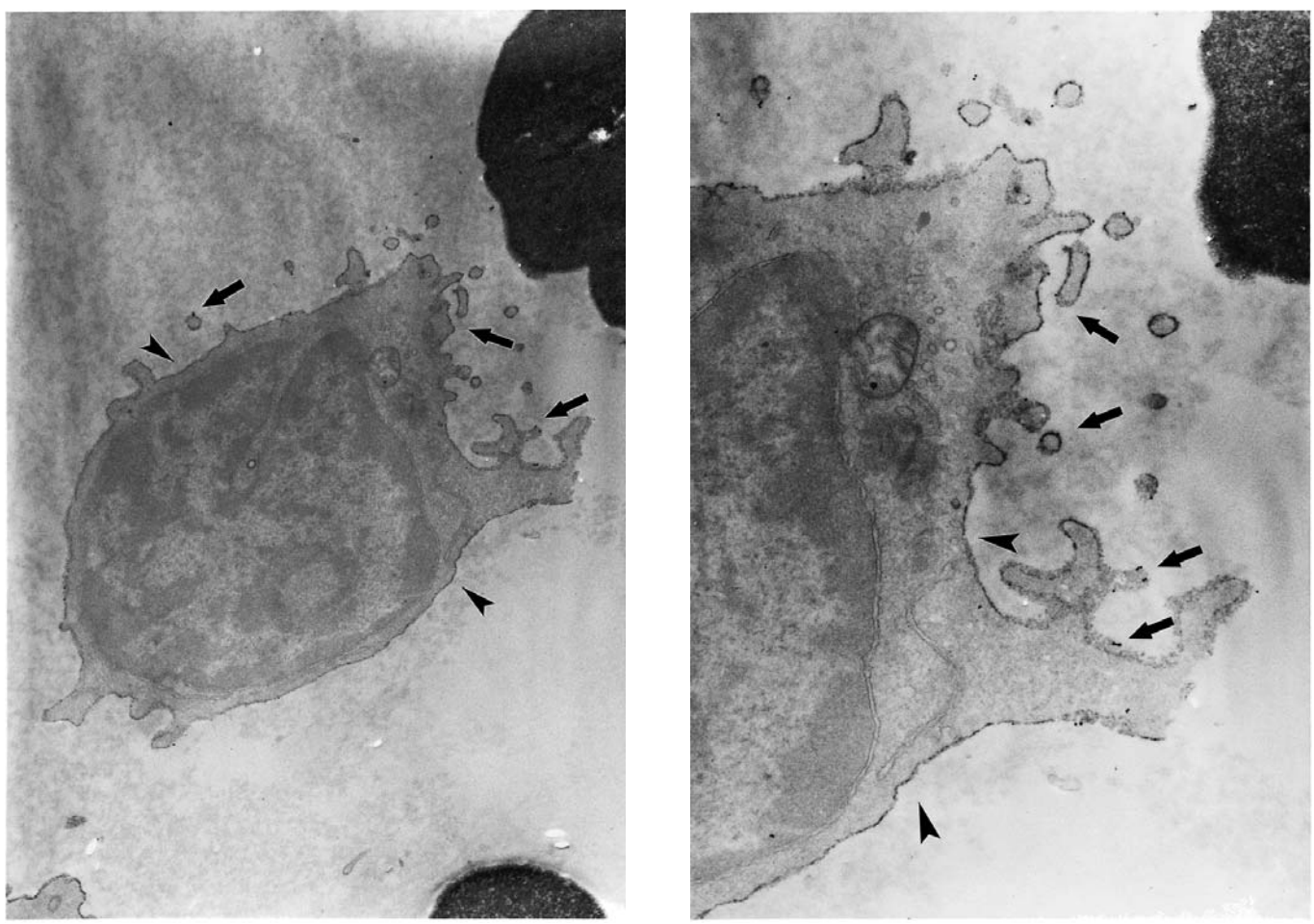

Fig. 2. (*2A*) A type *1a* CD5+ B cell is labeled by both gold particles and HRP deposits on the cell membrane. The cell has a high nuclear/ cytoplasmic (N/ C) ratio, long cytoplasmic processes, a small number of organelles, and a relatively less indented nucleus. Gold particles are scattered on the cell membrane. N/C ratio = 0.70, NCI = 1.43. *Arrows* indicate gold particles. *Arrowheads* show HRP deposits. (No electron staining, × 15,000). (*2B*) A higher magnification picture of *2A*. (No electron staining, × 36,000).

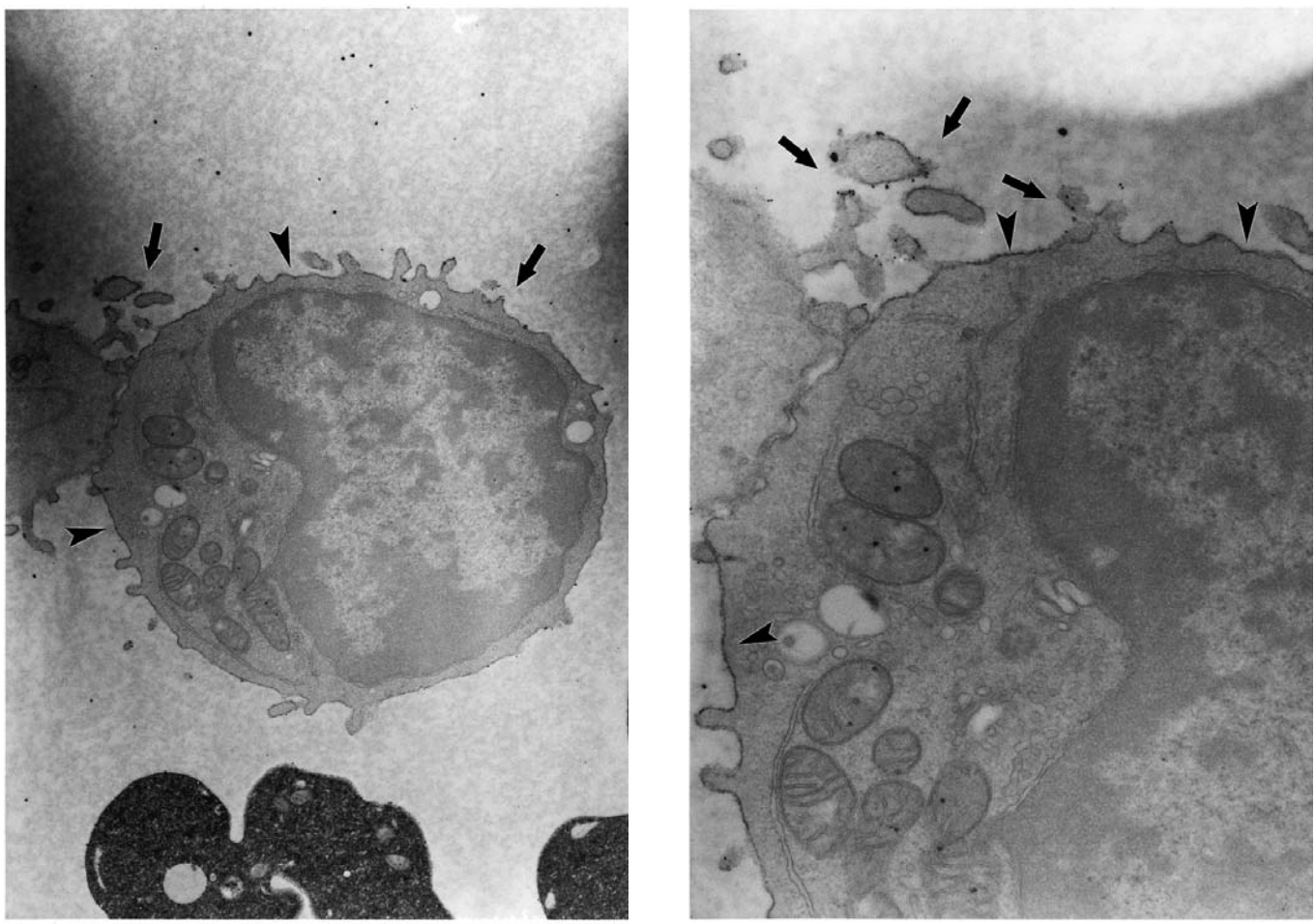

Fig. 3. (*3A*) A type *1b* $CD5^+$ B cell has several microvilli and a cytoplasm with more organelles than type *1a* cells have. N/C ratio = 0.59, NCI = 1.71. The number of gold particles (*arrow*) located on the microvilli is equal to or a little greater than in the type *1a* cells. *Arrowheads* show HRP deposits. (No electron staining. × 15,00). (*3B*) A higher magnification picture of *3A*. (No electron staining, × 36,000).

est to the uropod-like processes. Organelles, such as mitochondria, vacuoles, Golgi apparatus, ER, and centrioles, were observed at the nuclear indentations. The N/C ratio and NCI values are shown in Fig. 1. HRP deposits and gold particles were observed on the surfaces of the cells (Figs. 4A, 4B). The HRP deposits, which were of a weaker intensity than those of other $CD5^+$ cells, were continuously distributed on the cell surfaces and numerous gold particles were distributed on the microvilli. The majority of the gold particles were observed on the uropod-like processes.

There were statistically significant differences between the N/C ratios of the three types of $CD5^+$ B cells, and between the N/C ratios of the $CD5^+$ B and T cells. Among the $CD5^+$ B cells, there were no significant differences in the NCI values except between the type *1b* and type *2* cells.

DISCUSSION

Immunofluorescence analysis has shown that $CD5^+$ B cells are morphologically similar to conventional B lymphocytes[6,9], whereas the fluorescence intensity of $CD5^+$ B cells labeled with anti-CD5 antibody is weaker than that observed for labeled T cells, suggesting a lower epitope density in the B cell subpopulation. Although conventional May-Grünwald-Giemsa staining has shown that these cells have basophilic cytoplasm with indented nuclei, and rarely have nucleoli[6], no immunoelectron microscopic studies of $CD5^+$ B cells have been reported.

The present study is the first in which cell surface double labeling of cord blood $CD5^+$ B cells was performed together with immunoelectron microscopy (IEM). $CD5^+$ B cells, a special subtype of B cells, showed faint discontinuous HRP deposits and various depositions of gold particles on the cell surface. This finding agrees with the immunofluorescence

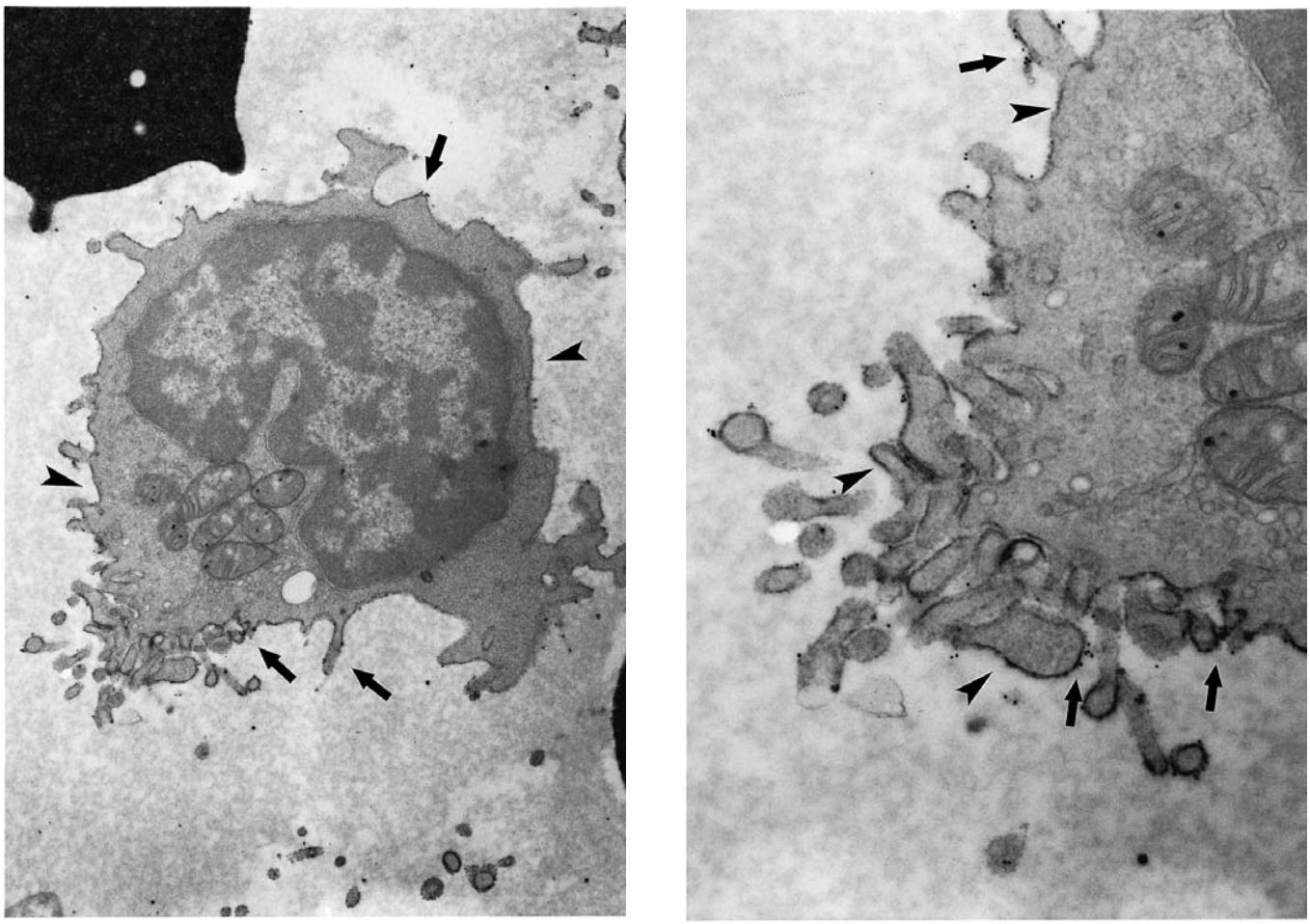

Fig. 4. (*4A*) The cytoplasm of this type *2* cell shows many organelles, uropod-like processes, and significant nuclear indentation. N/C ratio = 0.46, NCI = 1.89. Gold particles are mainly located on the microvilli of the uropod-like process (*arrows*). (No electron staining, × 18,000). (*4B*) A higher magnification picture of *4A*. *Arrowheads* show HRP deposits. *Arrows* indicate gold particles. (No electron staining, × 47,000).

findings described above[9]. In addition, three types of $CD5^+$ B cells were identified based on the cellular morphology (organelles, chromatin patterns, microvilli, the N/C ratio, and the NCI)[20] and the labeling patterns of HRP deposits and/or gold particles[13,15].

The N/C ratio of type *1a* cells exceeded 0.6, which was the highest among the three types of $CD5^+$ B cells. Therefore, the type *1a* cells appear to be immature cord blood cells based on morphological and immunoelectron microscopic studies. Compared to type *1a* cells, type *1b* cells had more microvilli and organelles, but a lower N/C ratio (approximately 0.6). There was no significant difference between the NCI values of the type *1a* and *1b* cells. Type *1b* cells appeared to be more mature than type *1a* cells because of the increased number of organelles, the lower N/C ratio, the irregular nuclei, and the larger number of gold particles on the cell surfaces. Statistical analysis showed a significant difference between the N/C ratios of type *1a* and type *1b* cells, although there was no significant difference in the NCI values. Type *2* cells, which made up 74% of the $CD5^+$ B cells, had a number of microvilli and many organelles in the cytoplasm. Gold particles were frequently observed on the microvilli. The N/C ratio of these cells was the lowest (approximately 0.5) among the $CD5^+$ B cells. These cells were likely mature cells with higher NCI values than the type *1b* cells[13,15].

These findings suggest that type *1a* cells (immature cells) are transformed into type *1b* cells, and then into type *2* cells (mature cells), most likely due to the influence of agents such as lymphokines[21]. Although cord blood lymphocytes include a large number of mature type *2* cells (74% of the $CD5^+$ B cells), the primary follicles of the fetal lymph nodes and spleen should have more immature type *1a* and *1b* cells than cord blood due to non-antigenic stimulation[6,21].

In this investigation, we were able to identify two distinct surfaces antigens using IEM. Immunogold-immunoperoxidase double labeling confirmed and extended

the findings of previous immunofluorescence analysis[9]. This method combined with IEM improves the analysis of membrane phenotypes because two different membrane antigens can be simultaneously detected[13,15-18,22]. It is worth noting that several different membrane antigens may be identified using HRP staining and differently sized gold particles together with the previously described method. Further studies, however, are required to detail the origin and function of the $CD5^+$ B cells.

ACKNOWLEDGMENT

We would like to express our thanks to the staff of the Department of Pathology of the Fukushima Medical University School of Medicine for their assistance in the statistical analyses.

REFERENCES

1 Nakamura N, Abe M : Histogenesis of CD5-positive and CD5-negative B-cells neoplasms on the aspect of somatic mutation of immunoglobulin heavy chain gene variable region. Fukushima Med J Sci 49 : 55-67, 2003

2 Antin JH, Emerson SG, Martin P, Gadol N, Ault KA : Leu-1^+ ($CD5^+$) B cells. A major lymphoid subpopulation in human fetal spleen : Phenotypic and functional studies. J Immunol 136 : 505-510, 1986

3 Ault KA, Antin JH, Ginsburg D, Orkin SH, Rappaport JM, Keoham ML, Martin P, Smith BR : The phenotype of recovering lymphoid cell population after marrow transplantation. J Exp Med 161 : 1483-1502, 1985

4 Bofill M, Janossy G, Janossa M, Burford GD, Seymour GJ, Wernet P, Kelemen E : Human B cell development. II. Subpopulations in the human fetus. J Immunol 134 : 1531-1538, 1985

5 Caligaris-Cappio F, Gobbi M, Bofill M, Janossy G : Infrequent normal B Iymphocytes express features of B-chronic lymphocytic leukemia. J Exp Med 155 : 623-628, 1982

6 Caligaris-Cappio F, Rivaa M, Tesio L, Schena M, Gaidano G, Bergui L : Human normal $CD5^+$ B lymphocytes can be induced to differentiate to CD5-B Iymphocytes with germinal center cell features. Blood 73 : 1259-1263, 1989

7 Gobbi M, Caligaris-Cappio F, Janossy G : Normal equivalent cells of B cell malignancies. Analysis with monoclonal antibodies. Br J Haematol 54 : 393-403, 1983

8 Hardy RR, Hayakawa K, Shimizu M, Yamasaki K, Kishimoto : Rheumatoid factor secretion from human Leu-1^+ B ceIIs. Science 236 : 81-83, 1987

9 Plater-Zyberk C, Maini RN, Lam K, Kennedy TD, Janossy G : A rheumatoid arthritis B cell subset expresses a phenotype similar to that in chronic lymphocytic leukemia. Arthritis Rheum 28 : 971-976, 1985

10 Van den Oord JJ, de Wolf-Peeters C, Desmet VJ : The marginal zone in the human reactive lymph node. Am J Clin PathoI 86 : 474-479, 1986

11 Wang JJ : Immunocytochemical demonstration of the binding of growth-related polypeptide hormones on chick embryonic tissues. Histochemistry 93 : 133-141, 1989

12 Weisenburger DD, Linder J, Daley DT, Armitag JD : Intermediate lymphocytic lymphoma. An immunohistologic study with comparison to other lymphocytic lymphomas. Hum PathoI 18 : 781-790, 1987

13 Kang YH, Carl M, Grimley PM, Serrate S, Yaffe L : Immunoultrastructural studies of human NK cells. 1. Ultracytochemistry and comparison with T cell subsets. Anat Rec 217 : 274-289, 1987

14 Kang YH, Carl M, Watson LP, Yaffe L : Immunoelectron microscopic identification of human NK cells by FITC-conjugated anti-Leu-11a and biotinylated anti-Leu-7 antibodies. J Immunol Methods 84 : 177-196, 1985

15 Manara GC, De Panfilis G, Ferrari C : Ultrastructural characterization of human large granular lymphocyte subsets defined by the expression of HNK-1 (Leu-7), Leu-11, or both HNK-1 and Leu-11 antigens. J Histochem Cytochem 33 : 1129-1133, 1985

16 Manara GC, De Panfilis G, Ferrari C, Scandroglio R : Immunoperoxidase-immunogold double labeling in immunoelectron microscopy of large granular lymphocytes. J Immunol Methods 75 : 189-192, 1984

17 Manara GC, Sansoni P, Ferrari C, De Panfils G : Natural killer cells expressing the Leu-11 antigen display phagocytic activity for 2-amino ethyl-isothiouronium bromide hydrobromide-treated sheep red blood cells. Lab Invest 55 : 412-418, 1986

18 Morris RE, Saelinger CB : Combined use of immunoferritin and immunocolloidal gold for simultaneous demonstration of two antigens by immunoelectron microscopy. Stain TechnoI 57 : 225-237, 1982

19 Lafferty MD, Ackerman RD, Dunn CDR, Lange RD : UItrastructuraI, immunocytochemicaI localization of presumptive erythropoietin binding sites on developing erythrocytic cells of normal human bone marrow. J Histochem Cytochem 29 : 49-56, 1981

20 Feroze N. Ghadially ; Ultrastructural pathology of the cell. A text and atlas of physiological and pathological alterations in cell fine structure. London, Butterworths, 1975

21 Timens W, Boes A, Rozenboom-Uiterwijk T, Poppemma S : Immuno-architecture of human fetal lymphoid tissues. Virchows Arch A PathoI Anat 413 : 563-571, 1988

22 Romano EL, Romano M : Staphylococcal protein A bound to colloidal gold : a useful reagent to label antigen-antibody sites in electron microscopy. Immunochemistry 114 : 711-715, 1977