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High CD99 Expression in Memory T and B Cells in Reactive Lymph Nodes

We investigated the expression of CD99 in 35 hyperplastic perigastric lymph nodes, which were resected for gastric carcinoma or chronic peptic ulcer. Essentially, all lymphocytes in lymph nodes expressed CD99, but there were two populations with respect to the intensity of CD99 expression - $CD99^{high}$ and $CD99^{low}$ cells. We showed $CD99^{high}$ cells were distributed in paracortical and medullary cords by immunohistochemical study while germinal center cells were $CD99^{low}$. Using three-color flow cytometric analysis with CD3, CD4, CD8, CD19, CD23, CD45RA, CD45RO, CD69, CD138, IgM, IgD, and IgG, most of $CD99^{high}$ cells were shown to be activated/memory T cells. $CD4^+CD45RO^+$ T cells were the subset revealing the highest intensity of CD99 expression while $CD4^+CD45RA^+$ T cells were $CD99^{low}$. Among B cells, IgG^+ B cells revealed a higher level of CD99 molecules than IgM^+ B cells. These results suggest that CD99 is one of activation-related molecules which are upregulated in recently activated lymphocytes.

Key Words: *Immunohistochemistry; Lymphocyte transformation; Memory*

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INTRODUCTION

The CD99 (MIC2) molecule is a 32 kDa transmembrane glycoprotein, which is expressed on a variety of cell types (1-5). The expression level of CD99 was shown to be high in cortical thymocytes, pancreatic islet cells, granulosa cells of ovary, and Sertoli cells of testis (2). Sequence analysis of CD99 cDNA suggests the CD99 protein contains an extracellular domain heavily-glycosylated with O-linked sugars, a putative transmembrane domain, and a short cytoplasmic domain (3, 4). Although its function is not understood clearly, CD99 has been implicated in a few cellular processes; homotypic adhesion, protein transport, and cell death. First of all, anti-CD99 mAb was shown to induce homotypic aggregation of double positive ($CD4^+$ $CD8^+$) thymocytes and IM9 B cells (6, 7) and phosphatidylserine exposure at the thymocyte surface (8), an event most likely linked to the adhesion process. Secondly, facilitated protein transport was appreciated upon CD99 engagement in double positive thymocytes (9). Finally, the role of CD99 in apoptosis of thymocytes and Ewing's sarcoma cell line was recently described (10, 11). Thus, CD99 appears to also be important in peripheral immune reaction such as cytokine secretion or antigen presentation in addition to developmental processes like hematopoietic and neural precursor cell differentiation.

In this paper, we addressed the question concerning which populations of lymphocytes express CD99 in lymph nodes to get an insight into the role of CD99 in peripheral immune responses. We investigated 35 cases of perigastric lymph nodes which were removed during gastrectomy for gastric carcinoma or ulcer by immunohistochemical study and three-color flow cytometric analysis

MATERIALS AND METHODS

Tissue and cells

Human perigastric reactive lymph nodes of 30 patients with gastric cancer (9 early and 21 advanced gastric cancer) and of five patients with chronic peptic ulcer were obtained during surgery ranging in age from 30 to 70 years and from 42 to 63 years, respectively. For flow cytometric analysis, fresh tissues were minced with sharp scissors in RPMI 1640 media after blood clots and fibrous

capsules had been carefully removed, followed by straining through a stainless steel mesh. Viable lymphocytes were isolated by Ficoll-Hypaque density centrifugation and washed twice in RPMI 1640 media. Fresh lymphocytes were resuspended in phosphate buffered saline (PBS) containing 0.05% sodium azide and 2% fetal calf serum (FCS; GIBCO) before flow cytometric analysis.

Monoclonal antibodies

The mAbs used were: CD99 (TU-12-FITC), CD3 (UCHT1-PE), CD4 (RPA-T4-PE), CD8 (RPA-T8-PE), CD19 (HIB19-PE), CD23 (M-L233-PE), CD45RA (HI100-PE), CD45RO (UCHL1-PE), IgD (IA6-2-PE), IgG (G18-145-PE), IgM (G20-127-PE) and CD4 (RPA-T4-Cy-Chrome) from Pharmingen (San Diego, CA, U.S.A.); CD69 (Leu^{TM}-23-PE) from Becton-Dickinson (San Jose, CA, U.S.A.); CD138 (MCA681-PE) from Serotec (Oxford, England). DN16 anti-CD99 mAb was described in the previous report (7).

Immunohistochemistry

Paraffin and frozen sections from the same perigastric lymph nodes as in flow cytometric analysis were processed for CD99 immunohistochemistry. Sections were deparaffinized in xylene and hydrated with ethanol to phosphate buffer (0.1 mol/L, pH 7.3); this was followed by rinsing in H_2O_2 (0.5%) to remove endogenous peroxidase activity, and in 3% (vol/vol) normal goat serum to reduce nonspecific binding of immunoglobulins. Sections were incubated in mouse anti-CD99 mAb (DN16) in a humid chamber for 2 hr at room temperature. After rinsing with phosphate-buffered saline (PBS), the sections were incubated for 30 min at room temperature with affinity-purified biotinylated goat anti-mouse immunoglobulin G (DAKO, Carpinteria, U.S.A.), and then in a 1:400 dilution of streptavidin-horseradish peroxidase conjugate (DAKO, Carpinteria, U.S.A.). The chromogen reaction was developed for 2 min in 0.5 mg/mL 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO, U.S.A.) in the presence of H_2O_2. A brown precipitate indicated the presence of antigen/antibody/horseradish peroxidase complexes. Counterstaining with Harris' hematoxylin was done after immunoperoxidase staining.

Flow cytometric analysis

One million lymphocytes were simultaneously incubated with anti-CD99-FITC and PE- and/or Cy-Chrome-labeled mAbs in PBS containing 0.1% sodium azide and 1% BSA at 4℃ for 30 min, and washed three times. Negative control samples were incubated with isotype-matched control fluorescent antibodies (Pharmingen). Two-color or three-color flow cytometric analysis was performed and the fluorescence intensity of 10^4 cells was determined (FACScan, Becton-Dickinson).

RESULTS

CD99-positive cells were scattered throughout the lymph nodes, but most germinal center cells were negative in immunohistochemical staining

Strong and intermediate reactivity in the hyperplastic lymph nodes of 30 patients with gastric cancer was shown by an intense membranous staining pattern throughout the cortex to medulla. Strong CD99-positive cells were found mainly in the superficial cortex, paracortex and medullary cord, while only occasional positive cells were found in germinal centers of the follicles and medullary sinus (Fig. 1). There were no significant differences between CD99 expression level in the lymph nodes from different stages or types of gastric carcinoma when we collected and analyzed different lymph nodes taken from cases of early and advanced gastric carcinomas (data not shown). The distribution pattern of CD99-positive cells in hyperplastic lymph nodes of patients with chronic peptic ulcer was similar to that in gastric cancer, but the number of CD99 positive cells tended to be lower than

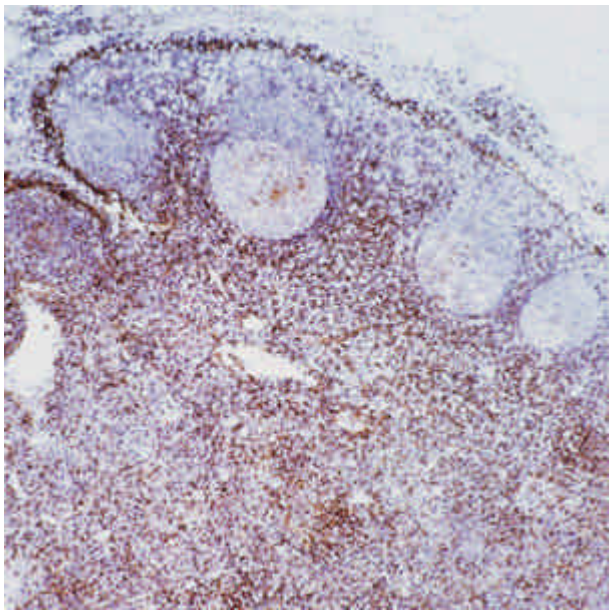

Fig. 1. Immunohistochemical staining for CD99 in the perigastric lymph node of advanced gastric cancer. Strong reactivity is found in superficial cortex, paracortex, and medullary cord, whereas CD99-positive cells are rare in germinal center. Occasional positive cells are detected in medullary sinus.

that in gastric cancer. It appeared that the intensity of CD99 expression correlated with the degree of the reactive response of lymph nodes since there was a tendency that the enlarged lymph nodes contained more CD99-positive cells than small lymph nodes (data not shown). This finding led us to interpret CD99-positive cells as recently activated cells. The scarcity of strong CD99-positive cells in the germinal center was an interesting finding since most germinal center cells are activated B cells, which are undergoing hypermutation and selection process.

Most of CD99 high cells are T cells

Single cell suspension of fresh lymph node cells from the 10 patients with gastric cancer or chronic peptic ulcer were prepared for CD99 expression by flow cytometric analysis. Essentially all lymph node cells were clearly positive for CD99, which indicate that apparently CD99-negative cells in immunohistochemical study were in fact expressing CD99 at a low level. A bimodal anti-CD99 reactivity pattern was observed in all samples, reflecting the presence of subpopulations of lymphocytes; one with low CD99 expression and the other with high CD99 expression (Fig. 2). To determine the identity of $CD99^{high}$ cells, we performed two- or three-color flow cytometric analysis. Lymphocytes were stained with anti-CD99 mAb in combination with antibodies against either CD3, CD4, CD8, CD19 or CD23. $CD3^+$ cells tended to be CD99-high while $CD19^+$ cells were $CD99^{low}$ (Fig. 2). $CD23^+$ cells showed a similar pattern of CD99 expression to that of $CD19^+$ cells. These data indicated that most $CD99^{high}$

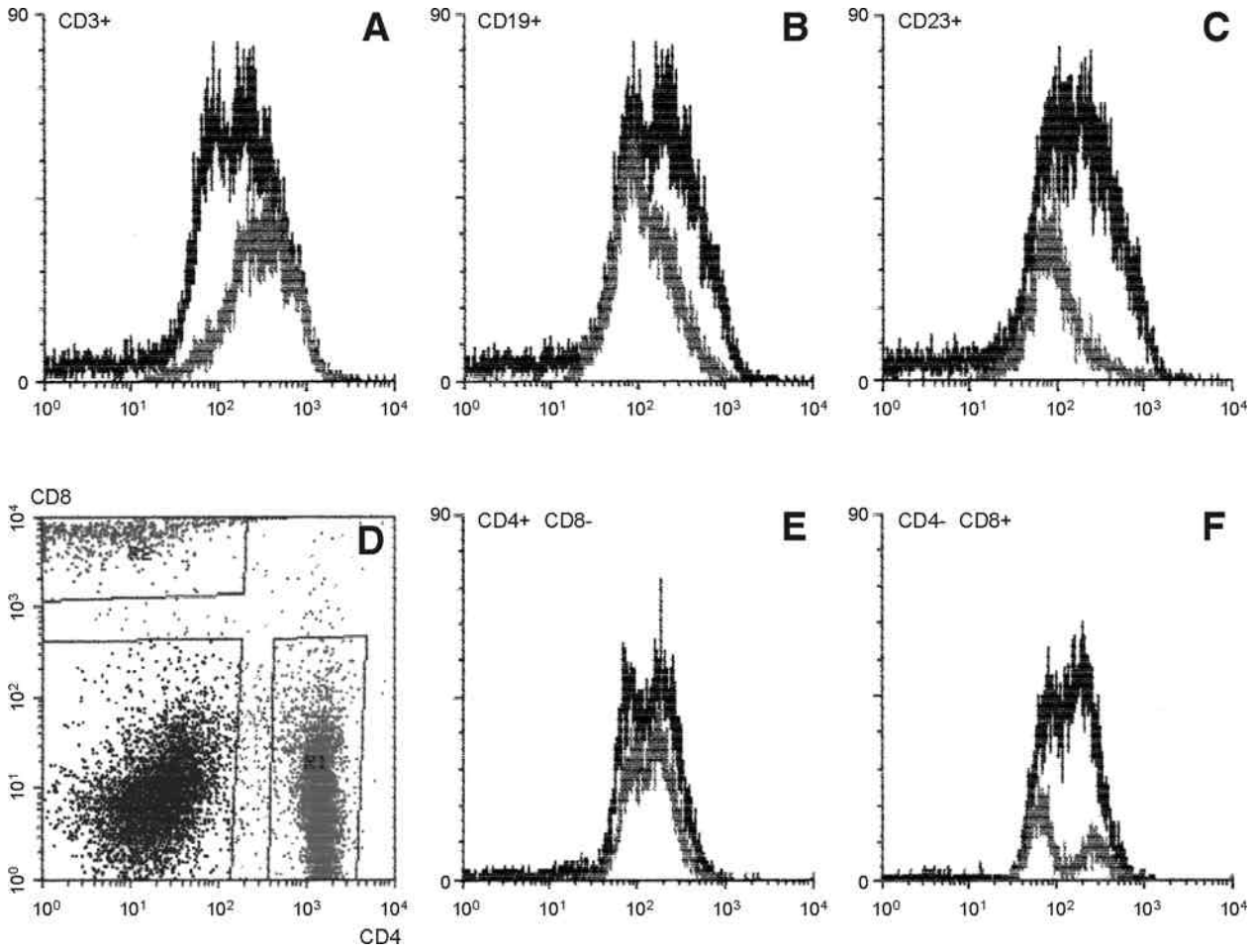

Fig. 2. Flow cytometric analysis of CD99 expression of lymph node cells. Lymphoid cells were stained with anti-CD99 antibody in combination with anti-CD3, CD4, CD8, CD19 or CD23 antibodies. Ten thousand cells each were acquired on a FACScan. Histograms for the anti-CD99 antibody staining are shown: Horizontal axes illustrate log-fluorescence, and the vertical axes indicate cell numbers. Gray line histograms reflect the distribution of CD99 expression on gated $CD3^+$ (A), $CD19^+$ (B), $CD23^+$ (C), $CD4^+CD8^-$ (E), and $CD4^-CD8^+$ (F) cells. Black line histograms represent the corresponding CD99 expression patterns of total lymphoid cells in each analysis. $CD4^+$ or $CD8^+$ cells are subdivided into low or high CD99 expression, but $CD19^+$ or $CD23^+$ cells show low or intermediate CD99 expression. In (D), dotplot of $CD99^+$ cells is shown with respect to CD4 and CD8 antibodies.

cells were T cells. Next, we checked whether $CD99^{high}$ cells were $CD4^+$ and $CD8^+$ T cells. As shown in Fig. 2, $CD4^+$ and $CD8^+$ T cells showed no difference in CD99 expression. Both populations of T cells were divided into $CD99^{high}$ and $CD99^{low}$ cells.

Memory T and B cells expressed a higher level of CD99 than naive cells

Even though most B cells were $CD99^{low}$, there was a correlationship between immunoglobulin isotype and CD99 expression. IgG^+ B cells revealed a higher expression of CD99 than IgM^+ or IgD^+ B cells (Fig. 3), although the intensity of CD99 expression in IgG^+ B cells was lower than that in T cells. On the other hand, $CD138^+$ plasma cells showed a broad range of CD99 expression level (Fig. 3). This data suggested that isotype-switched memory B cells contained a larger amount of CD99 than IgM^+IgD^+ B cells.

Next, we examined whether $CD99^{high}$ T cells are recently activated or memory T cells within the $CD4^+$ T cell subpopulation similarly to B cells. We performed three-color experiments using anti-CD99 mAb and anti-CD4 mAb, along with either anti-CD45RA, anti-CD45RO or anti-CD69 mAb. Clearly, $CD45RO^+CD4^+$ T cells were high in CD99 expression, while $CD45RO^-CD4^+$ T cells were $CD99^{low}$ (Fig. 4). Consistently, $CD69^+CD4^+$ T cells showed a higher expression of CD99 than $CD69^-CD4^+$ T cells (Fig. 4). These data suggest that memory or recently activated $CD4^+$ cells are $CD99^{high}$ while naive resting $CD4^+$ T cells contain a low amount of CD99.

DISCUSSION

In this study, we investigated the expression pattern of CD99 in peripheral lymph nodes. We showed that CD99 expression is upregulated on activated or memory T and B cells. According to differential expression of CD45 isoforms, $CD4^+$T lymphocytes have been subdivided into two major subsets with distinct activation requirements and functional programs, i.e. naive T cells ($CD45RA^+RO^-$) and activated/memory T cells ($CD45RA^-RO^+$) (12, 13). As revealed by our results, CD99 is highly expressed in the $CD45RO^+CD4^+$ T cell subsets, intriguingly similar to adhesion molecules such as CD2, CD29, CD44, CDw49d, LFA-1 and LFA-3 (14, 15). These data

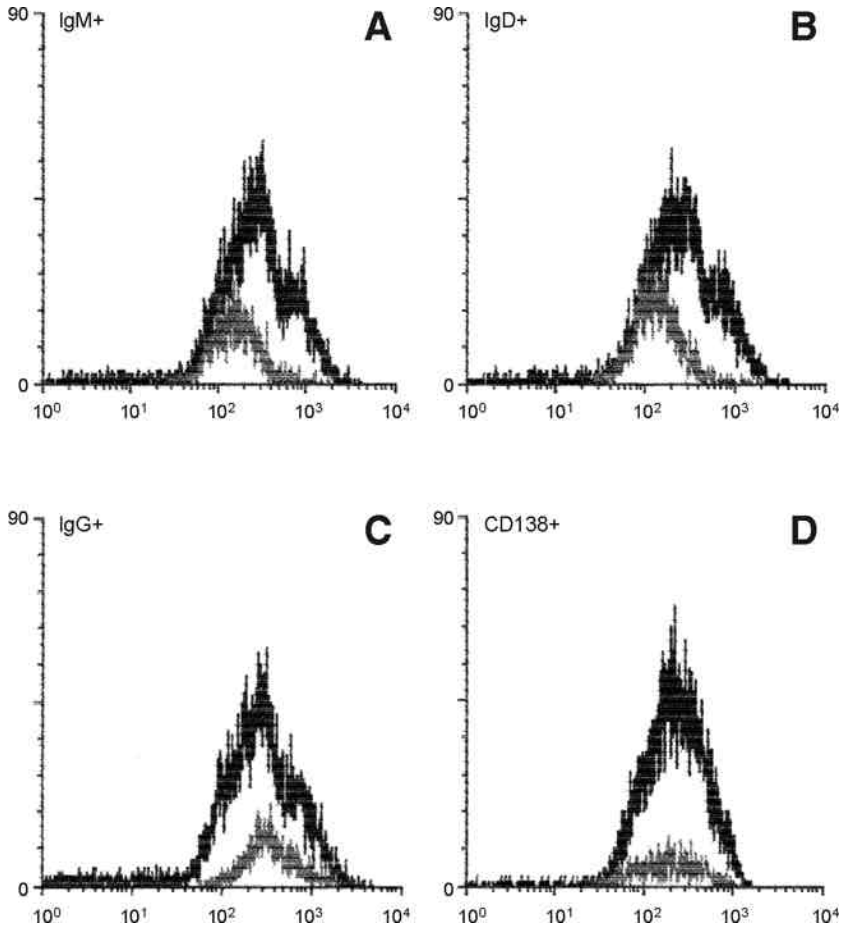

Fig. 3. Histograms of lymph node cells stained with anti-CD99 antibody in combination with anti-IgM, IgD, IgG, and CD138 antibodies, respectively. Horizontal axes illustrate log-fluorescence, and the vertical axes indicate cell numbers. Gray line histograms reflect the distribution of CD99 expression on gated IgM^+ (A), IgD^+ (B), IgG^+ (C), and $CD138^+$ (D) cells. Black line histograms represent the corresponding CD99 expression patterns of total lymphoid cells in each analysis. IgG^+ cells display higher CD99 expression than IgM^+ or IgD^+ cells. Note broad range of CD99 expression level in $CD138^+$ cells.

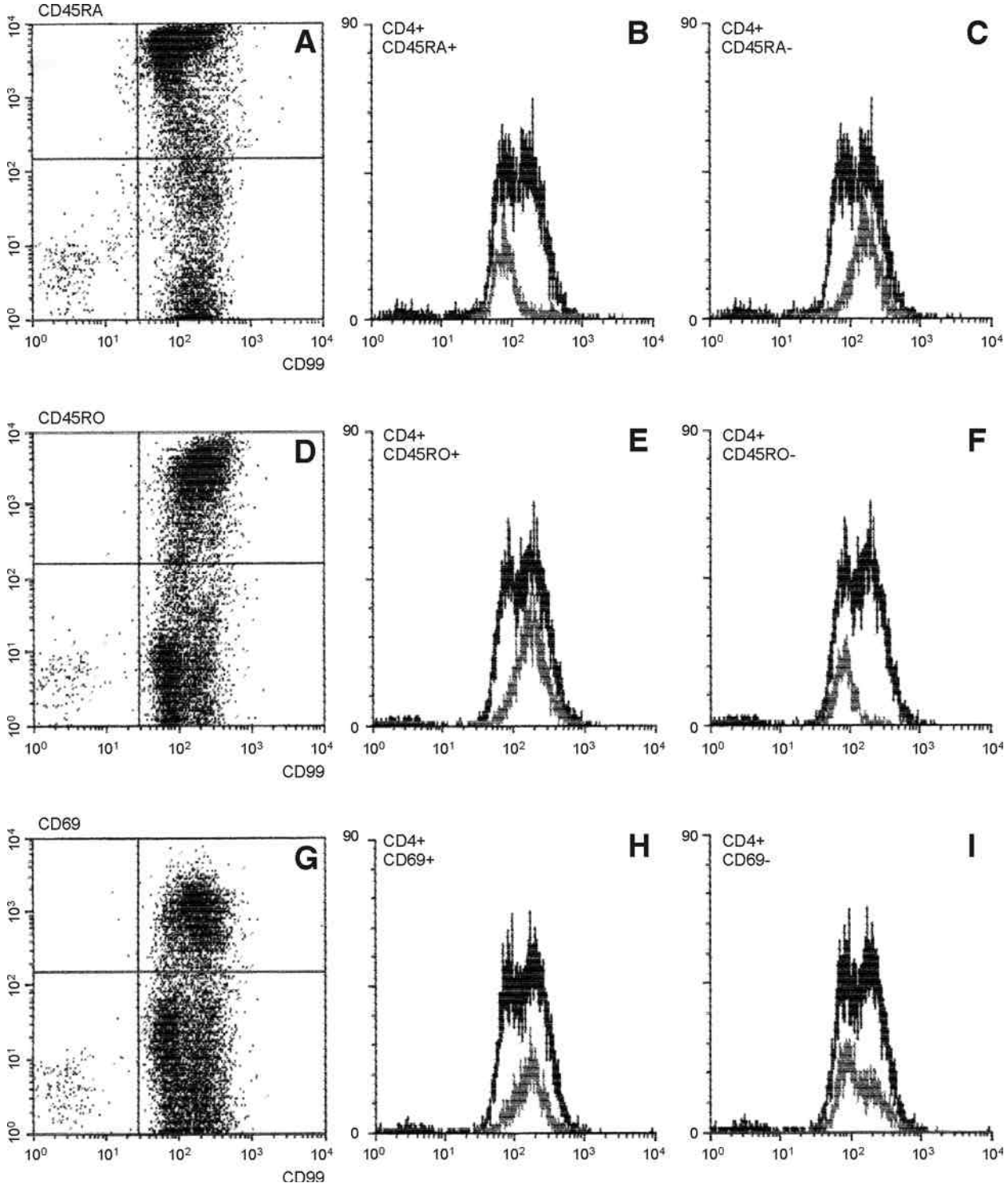

Fig. 4. Flow cytometric analysis of CD99 expression of lymph node cells using anti-CD99 antibody and anti-CD4 antibody, in combination with anti-CD45RA, CD45RO or CD69 antibody. Horizontal axes illustrate log-fluorescence, and the vertical axes indicate cell numbers. Gray line histograms reflect the distribution of CD99 expression on gated $CD4^+CD45RA^+$ (B), $CD4^+CD45RA^-$ (C), $CD4^+CD45RO^+$ (E), $CD4^+CD45RO^-$ (F), $CD4^+CD69^+$ (H), and $CD4^+CD69^-$ (I) cells. Black line histograms represent the corresponding CD99 expression patterns of total lymphoid cells in each analysis. $CD4^+CD45RO^+$ cells and $CD4^+CD69^+$ cells express high levels of CD99, whereas $CD4^+CD45RA^+$ cells express low levels of CD99 expression. In (A, D, G), dotplots of $CD4^+$ cells are shown with respect to anti-CD99 antibody in combination with anti-CD45RA, CD45RO, and CD69 antibody, respectively.

imply upregulation of CD99 expression upon T cell priming, along with isoform switching of CD45 (16). And the similar distribution of CD99 and various adhesion molecules on T cell subsets could have a functional significance and supports the idea of CD99 involvement in the network of antigen-independent adhesion pathways, which play a crucial role in lymphocyte development and function. According to the observation of marked proliferative effects of anti-CD99 mAb (HBA-71) on human thymocytes as well as to a lesser extent on PBL in general (17), it becomes most likely that CD99 represents a new independent activation pathway for T cells.

It was clear in flow cytometric analysis that most cells expressed CD99 at the intensity of one to two log scale higher than background staining. Based on previous reports, it seemed that CD99 is involved in some essential roles for maintenance of cell shape, division, aggregation, and apoptosis (6-10, 18). However, there were clearly two populations of lymphocytes which express CD99 at low and high densities, respectively. In immunohistochemical staining, only a proportion of lymph node cells were positive for CD99. We interpreted that only $CD99^{high}$ cells by flow cytometric analysis were stained in immunohistochemical study due to the low sensitivity of immunohistochemistry. Most $CD99^{high}$ cells were distributed in paracortical T zones and medullary cords. Although a few germinal centers contain occasional CD99 positive cells, most germinal centers did not show CD99 positivity in immunohistochemical study. In conjugation with flow cytometric data, CD99 seems to be a pervasively expressed molecule in lymphocytes.

To solve the question which subpopulation $CD99^{high}$ cells belong to, we did two or three color flow cytometric study with CD3, CD4, CD8, CD19, CD23, CD69, CD45RA, CD45RO, IgM, IgD, IgG, and CD138. We found that most $CD99^{high}$ cells resided in $CD4^+CD69^+$ $CD45RO^+$ population. This result is consistent with the previous study by Dworzak et al., showing that the highest expression of CD99 was found in most immature lymphocytic and granulocytic cells and also activated/memory T cells (2). It seems that with antigenic stimulation, $CD99^{high}$ cells accumulated in paracortical T zones, but it is not clear that the proportion of $CD99^{high}$ cells are reduced in completely resting lymph nodes. In immunohistochemical study, all cases had more or less $CD99^{high}$ cells in T zones. We presumed that in most lymph nodes, $CD99^{high}$ cells are present, and they represent memory T or recently activated T cells.

Activation of T cells changes the expression of several cell surface molecules. Resting naive T cells express L-selectin, through which they home on to lymph nodes, with relatively low expression levels of other adhesion molecules CD2 and LFA-1. Activated T cells express higher densities of the adhesion molecules CD2 and LFA-1, increasing the avidity for interaction of activated T cells with potential target cells. Engagement of various cell surface molecules, including CD2, TCR, surface Ig, MHC class II, CD11a, CD19, CD20, CD39, CD40, CD43, CD44, CDw49, Leu-13, TAPA-1 as well as CD99, with ligands and mAbs generates proadhesive signals that activate LFA-1 molecules at the surface, resulting in LFA-1-mediated adhesion events (19-29). Upon antigenic encounter, an expression of L-selectin homing receptor is lost and instead, increased amounts of the integrin VLA-4 are expressed. VLA-4 acts as a homing receptor for vascular endothelium in sites of inflammation and ensures that activated T cells recirculate through peripheral tissues where they may encounter infection sites. In addition, the isoform of CD45 molecule expressed by activated cells changes, by alternative splicing of the RNA transcript of CD45 gene, resulting in activated T cells now expressing the CD45RO isoform which associates with TCR and CD4. The consequences of this change in CD45 make the T cells more sensitive to stimulation by low concentration of peptide/MHC complexes. The roles of CD99 in activated or memory T cells should be defined. Based on the previous studies, we hypothesized that CD99 may be essential for cytokine secretion and T-B interaction (30). From this study, it appeared that the high expression of CD99 in IgG^+ B cells may reflect its function in T-B cell collaboration and adhesion.

In summary, we showed the distribution of CD99 in lymph nodes. By combining immunohistochemical and flow cytometric studies, $CD99^{high}$ cells were shown to be mainly $CD4^+$ T cells whereas germinal center B cells were mostly $CD99^{low}$. Ligation of CD99 increases surface expression of various receptors including T cell receptor, LFA-1, and induces cell-cell aggregation, which may affect the outcome of immune responses by regulation of intercellular aggregation and cytoskeleton.

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