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Expression of IgG1 Germline Transcripts in Germinal Center B Cells of Mouse Popliteal Lymph Nodes

In-Woo Lee, Jin-Ho Kim and Gook-Hyun Chung*

Department of Biology, Chonbuk National University, Chonju 560-756, Korea
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Abstract: Germinal centers (GCs) are formed in peripheral lymphoid tissues in response to protein antigens. In order to see if immunoglobulin isotype switching takes place in GC B-cells, we isolated GC B-cells (PNA positive cells) from mouse popliteal lymph nodes by a flow cytometer after the staining of lymph node cells with PNA-FITC and anti-B220-PE, and determined the expression of γ1 germline transcript and γ1 mRNA by RT-PCR. γ1 germline transcript and γ1 mRNA were amplified specifically in cDNAs from hybridoma expressing IgG1 or splenocytes stimulated LPS plus IL-4. Germinal center B-cells formed in popliteal lymph nodes of mice immunized with chicken ovalbumin were isolated 7 days after immunization. We sorted GC B-cells five times. Immunoglobulin γ1 germline transcripts were expressed in germinal center B-cells in three out of five sorts whereas two out of five sorts did not express γ1 germline transcripts in GC B-cells. The contents of GC B-cells ranged from 5 to 7% of total lymph node cells in most flow cytometric analyses but those of two sorted cells which did not express γ1 germline transcripts were out of normal range. These results imply that isotype switching of immunoglobulins may take place in GCs.
Key words: flow cytometric analysis, germinal center, germline transcript, isotype switching.

Germinal centers (GCs) are histologically defined areas where B-cells develop in response to T cell-dependent antigens (Nieuwenhuis and Opstelten, 1984). GCs are composed of clustered B-cell blasts undergoing antigen-driven activation and proliferation, antigen specific T-cells (5~10%), and follicular dendritic cells (1~2%) (Kroese *et al.*, 1990). B-cells in GCs proliferate rapidly, but a large number of GC B-cells undergo negative selection and die by apoptosis (Liu *et al.*, 1991). GC B-cells are phenotypically distinct from other B-cells. GC B-cells express a high level of peanut agglutinin (PNA) receptors, CD20 and DR, but a low level of sIgD, CD21, CD39, and Leu-8 (Butcher *et al.*, 1981; Rose and Malchiodi, 1981; Kansas *et al.*, 1985; Weinberg *et al.*, 1986). GC B-cells are supposed to have many immunological roles. For instance, GCs are major sites for the generation of memory B-cells and immature plasma cells (Coico *et al.*, 1983; Alt *et al.*, 1987; Kosko *et al.*, 1989). Moreover, Apel and Berec (1990) reported that somatic mutations of antibody genes occur at a high incidence in GC B-cells.

Isotype switching of immunoglobulin heavy chains takes place by the immunization of protein antigens, and this process seems to have taken place in GCs (Kraal *et al.*, 1981). But it was difficult to prove it because GCs are fomred temporarily and the isolation of GC B-cells was very difficult. There are many results which imply isotype switching in GCs. T-cell dependent protein antigens induce murine GCs and preferentially elicit IgG1 antibodies whereas T-cell independent carbohydrate antigens are poor inducers of GCs (Davies *et al.*, 1970) and they primarily produce IgG3 antibodies (Slack *et al.*, 1980). Injection of a mouse with anti-IgD antibodies induces GCs and a large increase in the IgG1 and IgE level in serum (Flote *et al.*, 1984; Finkleman *et al.*, 1987). Neither GC development nor Ig isotype switching occurs in tissues containing $CD5^+$ B-cells (Scher, 1982). All of these results imply that GCs might be the site for isotype switching of immunoglobulins.

Cytokines regulate isotype switching of immunoglobulins; IL-4 induces isotype switching of murine B-cells to IgG1 or IgE; IFN-γ and TGF-β induce isotype switching to IgG2a and IgA, respectively (Berton *et al.*, 1989; Islam *et al.*, 1991). GC T cells produce IL-4 (Butch *et al.*, 1993; Fyfe *et al.*, 1987; Purkerson *et al.*, 1992; Weinstein and Cebra, 1991). Therefore, if GCs are the site for isotype switching, IgM will be switched to IgG1 or IgE by IL-4 produced by GC T cells. But it is hard

*To whom correspondence should be addressed.
Tel: 82-652-70-3360, Fax: 82-652-70-3362.

to believe that isotype switching sustains after the GCs wane because GCs are temporal tissues and the sizes of the GCs is as small as 5% of the lymph node size. We, therefore, hypothesized that isotype switching begins in the GCs and the switched cells migrate to the outside of the GCs. Germline transcripts which contain the I-region instead of the V-region of antibody transcripts are formed temporarily prior to the full expression of antibody genes (Nordgen and Sirlin, 1986; Gerondakis *et al.*, 1991; Guff *et al.*, 1992; Ichiki *et al.*, 1993). The expression of γ1 germline transcripts also preceeds the expression of mature IgG1 mRNAs (Xu and Staveneger, 1990). We, therefore, can identify the initiation of isotype switching by the determination of germline transcript expression. In this study, we isolated GC B-cells using a flow cytometer and determined the expression of γ1 germline transcripts in GC B-cells in order to see if GCs are the major site for IgG1 isotype switching.

Materials and Methods

Materials

Six to eight weeks old BALB/c mice were immunized with 30 μl of chicken ovalbumin (Sigma Chem; 1 mg/ml) dissolved in saline at the hind footpads subcutaneously. Fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA) was purchased from E-Y Lab (San Mateo, USA) and phycoerythrin (PE)-conjugated steptavidine was obtained from Sigma (St. Louis, USA). MMLV reverse transcriptase was the product of Boehringer Mannheim (Indianapolis, USA). Taq DNA polymerase and Vent DNA polymerase were purchased from Perkin Elmer (Norwalk, USA) and New England Biolabs (Beverly, USA), respectively.

Fluorescent Activated Cell Soft (FACS) of germinal center B-cells

Popliteal lymph nodes were resected and single cell suspensions were prepared. For flow cytometric analysis, 1×10^6 cells were incubated with 20 μl of FITC-conjugated PNA for 25 min on ice, and washed 2 times with RPMI-1640. 1×10^4 cells were analyzed using a flow cytometer (FACScan, Becton-Dickinson, USA). For two color fluorescence analysis, 1×10^6 cells were incubated for 25 min on ice with 20 μl of FITC-conjugated PNA that are directed against cell-surface molecules. Cells were then washed with RPMI-1640 twice and incubated again with biotin-conjugated mAb 6B2 (anti-B220, a generous gift of Dr. Hagen of Iowa State Univ.) for 25 min. The cells were washed again with RPMI-1640 and incubated with phycoerythrin-conjugated streptavidin for 25 min. The cells were washed with the same solution and analyzed for their fluorescence intensities by using a flow cytometer. The optimal concentrations of dyes were determined before the flow cytometric analysis.

For the sorting of germinal center B-cells, lymph nodes from 10 mice were pooled and 5×10^7 cells were stained with the same reagents as flow cytometric analyses. Briefly, lymph node cells were stained with PNA-FITC, biotin-conjugated anti-B220 and PE-conjugated streptavidin sequentially and sorted by using a flow cytometer (FACSort, Becton-Dickinson, USA).

Reverse Transcription (RT)

Total RNA was isolated from cell pellets by the method of Chomczynski *et al.* (1987). Briefly, denaturing solution consisting of 4 M guanidium thiocyanate (Sigma Chem., USA), 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, and 0.5% N-laurylsarcosine (Sigma Chem., USA) was added to cell pellets. Carrier rRNA (Sigma Chem., USA) was added to each cell lysate. Fifty microliters of 2 M sodium acetate (pH 4.0) were then added, followed by 500 μl water-saturated phenol (Boehringer Mannheim Biochem., USA), 100 μl of chloroform, and 100 μl of chloroform : isoamyl alcohol (49 : 1 mixture; Fisher Scientific, USA). After a 20 -min incubation at 4℃, the mixtures were centrifuged at 10,000×*g* for 20 min. The aqueous phase was mixed with 1 ml of isopropyl alcohol and placed at −20℃ for 2 h. Precipitated RNA was washed once with absolute ethanol at −70℃, vacuum-dried, and resuspended in 20 μl of diethylpyrocarbonate-treated (Sigma Chem., USA) deionized water.

cDNA synthesis was performed in 40 μl of reverse transcription buffer (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 100 μg/ml BSA) containing 40 U RNasin (Boehringer Mannheim Biochem., USA), 0.5 mM spermidine-HCl (Sigma Chem., USA), 4 μg oligo $(dT)_{15}$ (Sigma Chem., USA), 1 mM deoxynucleotide triphosphate mixture (in equal concentrations), and 200 U of MMLV reverse transcriptase (Boehringer Mannheim Biochem., USA). After a 60 min incubation at 37℃, the samples were boiled briefly and stored at −20℃.

PCR

cDNA was amplified in tubes containing PCR buffer and primers (each primer was used at a final concentration of 0.2 to 0.5 μM) in 50 μl final volume. PCR buffer consisted of 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 1 mM dNTPs, and 1.25 U *Taq* polymerase. PCR tubes were overlayes with mineral oil and amplified for 40 cycles using a Hybaid thermal cycler (National Labnet Co., USA). Samples were

denatured at 92℃ for 1.25 min, annealed at 58℃ for 50 sec, and extended at 72℃ for 1 min.

PCR products (20 μl) and M.W. markers (1 kb DNA ladder) were run on 3% NuSieve GTG agarose (FMC BioProducts, ME) using Tris-borate buffer. Ethidium bromide (0.5 μg/ml) was incorporated into the gel. Electrophoresis of PCR products revealed a dominant band corresponding to the predicted fragment size for each set of primers. The specificity of our PCR was made by demonstrating that restriction enzyme digests of PCR products yielded cDNA fragments of a predicted size. Sense primer sequences for γ1 germline transcript and γ1 mRNA were CAGCCTGGTGTCAACTAG, and AGGT[CG] [AC]A[AG]CTGCAG[GC]AGCT[AT] GC, respectively. The antisense primer sequence for both is GTTAGTTTGGGCAGC and the predicted size of the amplified PCR fragments for germline transcript and mature mRNA were 254 and 397 bp, respectively.

Lymph node kinetics

To determine GC harvesting time, we measured the size of the polited lymph node and the expression of mRNAs by PCR. Cell numbers of popliteal lymph nodes were counted on grided microslides and the formation of germinal center cells were determined by a flow cytometer after fluorescent staining as described above. The expression of γ1 germline transcript and γ1 mRNA was determined by RT-PCR.

Results and Discussion

Flow cytometric analysis

The separation of GC B-cells is a prerequisite step for the present study. In this study, we tried to isoalte GC B-cells by FACS sort. Since B lineage cells within the GCs are characterized by the expression of CD77 binding to PNA (Rose *et al.*, 1980), we stained lymph node cells with PNA-FITC and analysed the fluorescent intensity of cells with a flow cytometer. As shown in Fig. 1A, all naive cells were PNA negative. But even though PNA-positive cells arise after antigenic stimulation, the separation of PNA-positive cells (presumptive GC B-cells) from PNA-negative cells was not effective because many cells with intermediate fluorescent intensity were found between PNA-positive and PNA-negative cells (Fig. 1B). We, therefore, stained the cells with anti-B220-biotin conjugate as well as PNA-FITC to distinguish B-cells from the rest of the cells because all B-cells express a surface antigen, B220 (Schwartz-Albiez *et al.*, 1989). Bound anti-B220 was then reacted with streptavidin-PE. Surprisingly, Fig. 1C shows that the rest of the cells, including T-cells, have a moderate intensity of green color, and B220 negative cells could

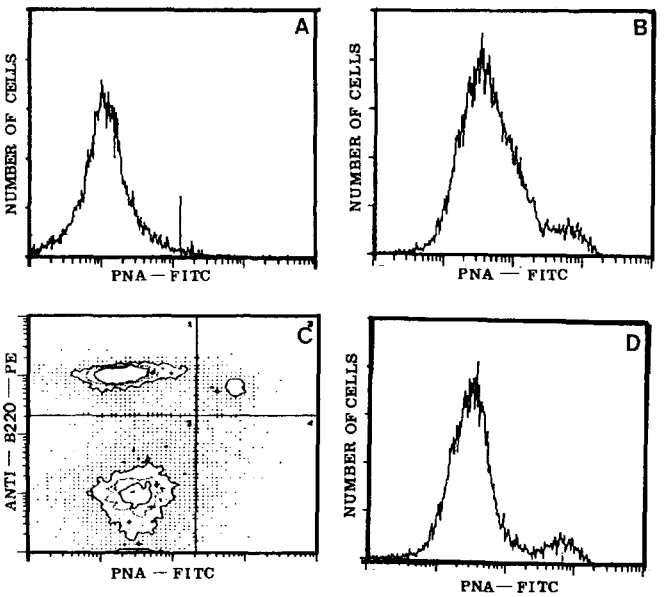

Fig. 1. Immunofluorescence staining of lymph node cells. Popliteal lymph node cells were stained with PNA-FITC (A,B) or with PNA-FITC and anti-B220-PE (B,C). (A) and (B) are histograms of naive lymph node cells and of cells stimulated with ovalbumin (7 days), respectively. (C) is a contour plot of total lymph node cells and (D) is a histogram of cells after the removal of B220 negative cells by gate adjustment.

be removed from other cell groups by gate adjustment (Fig. 1D). We did not expect this result because PNA which binds to CD 77 was known to be expressed only on a part of B-cells or Burkitt's lymphoma cells (Dorken *et al.*, 1989; Wiels and Tursz, 1989). Since cells stained with anti-Thy1.2 were also PNA-FITC positive to some extent (data not shown), we guessed that T cells have a few ligands which bind to PNA. Fig. 1D shows that PNA-positive cells (GC G-cells) can be separated clearly from PNA-negative cells after the removal of T cells by gate adjustment of the flow cytometer. For the finest separation of GC B-cells, we optimized dye concentrations and the optimal concentrations of PNA-FITC, anti-B220-biotin and streptavidin-PE were 1, 0.5 and 1 μg/ml, respectively. Splenocytes which were not immunized had a significant amount of PNA-positive cells (data not shown) whereas intact lymph node cells which were not immunized with antigens did not have any PNA-positive cells. The fact that splenocytes which were not imunized with specific antigen have PNA positive B-cells implies that spleen can be stimulated continuously with various protein antigens in blood.

RT-PCR for the detection of γ1 transcripts

Antibody signal could not be detected at all by ELISA because the number of germinal center B-cells was not enough to make antibodies. We, therefore,

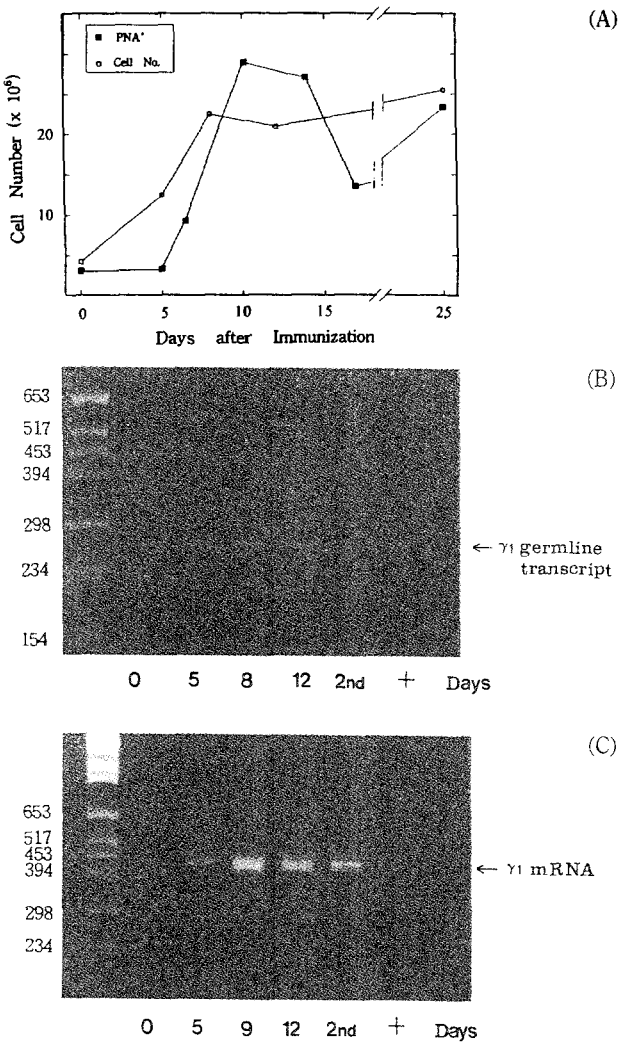

Fig. 2. Lymph node kinetics. Mice were immunized with chicken ovalbumin (30 μg) at hind food pads subcutaneously and then counted total cell number of a poplited lymph node at various day points. The content of PNA(+) cells was determined by flow cytometric analysis. Part of the mice was boosted again at 18 days after first immunization with the same procedure and cell numbers and PNA(+) content were analysed at 7 days after second immunization (2nd). Splenocytes stimulated with IL4+ LPS were used as a positive control(+) of γ1 transcripts. (A) Cell number and PNA(+) content. (B) γ1 Germline transcript expression. (C) γ1 mRNA expression determined by RT-PCR.

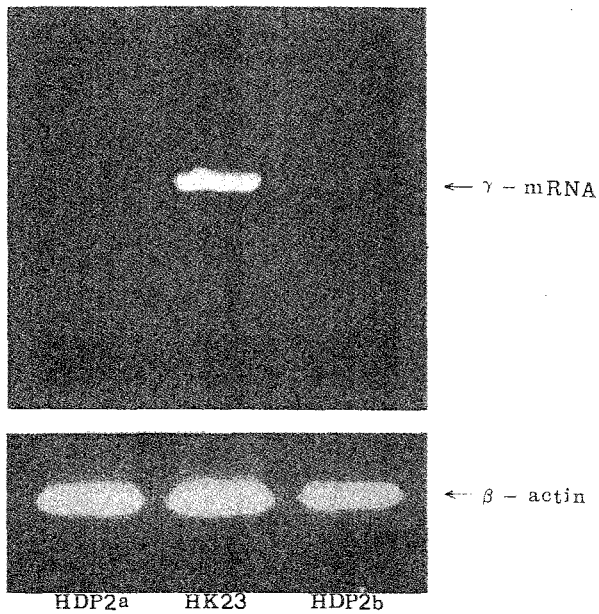

Fig. 3. Specificity of PCR primers of IgG1 Abs. cDNAs were syntehsized frm hybridoma cells and they were amplified with γ1 specific primers. Only HK23 with IgG1 isotype was amplified. HDP2a and HDP2b express IgG2a and IgG2b Abs, respectively.

tried to determine the expression of γ1 mRNA and germline transcript by RT-PCR in this study. We isolated total RNA and synthesized cDNA using oligo-d(T) primer. PCR was carried out using target specific primers by Xu and Staveneger (1990). Antibody PCR is difficult to do because the primary sequences of various antibody isotypes are very close. Primers for the γ1 mRNA were chosen from the framework-1 region (sense primer) of V_H genes and the γ1 C_H1 region (antisense primer). Sense primer for germline transcript was selected from the γ1 region whereas antisense primer for germline transcript was the same as that of γ1 mRNA. We could detect DNA fragments with an estimated size (254 bp for γ1 germline transcript and 397 bp for mature γ1 mRNA), and the fragments produced were confirmed by DNA sequencing. Primers used in this study were not crossreactive to other cDNAs obtained from hybridoma which produce other isotypes (Fig. 3). In order to confirm the γ1 germline transcript again, we cultured lymphocytes with LPS and/or IL-4 because IL-4 induces IgG1 expression (Berton *et al.*, 1989). As shown in Fig. 4, a strong band of 254 bp was detected in cells cultured with LPS and IL4, whereas control cells do not express the γ1 germline transcript.

Lymph node kinetics

GCs wax and wane by antigenic stimulation. Since we wanted to see if the isotype switch of antibodies is initiated in GC B-cells, first we had to check the time when GCs begin forming. The size of popliteal lymph nodes, formation of GCs, and mRNA expression were determined after the immunization of mice with protein antigen. Fig. 2A-C shows that isotype switching and GC formation take place at day 5 after immunization, but we decided to take the lymph nodes at 7 days after immunization because the size of the lymph nodes is not too big to handle at day 5. Fig. 2B shows that the γ1 germline transcript expression reached its maximum at day 5, whereas mature γ1 mRNA expression was maximal at day 7 (Fig. 2C). This result showing that the γ1 germline transcript expression precedes the expression of mature γ1 mRNA coincides with the

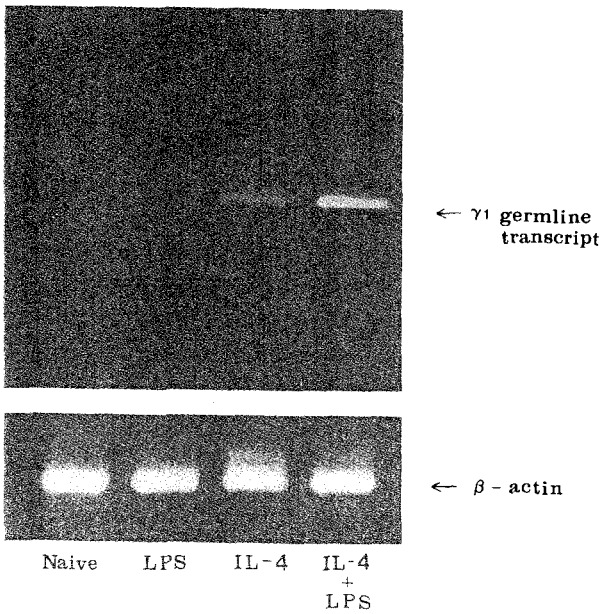

Fig. 4. γ1 germline transcript expression in lymphocytes stimulated with LPS and/or IL4. Naive lymphocytes or cells stimulated with LPS did not express Iγ1 whereas cells stimulated with LPS (50 μg/ml) plus IL4 (100 u/ml) expressed Iγ1 strongly. The expression of Iγ1 in cells stimulated only with IL-4 shows a faint band.

previous results showing that the expression of germline transcript precedes that of mature mRNA in isotype switching (Gerondakis *et al.*, 1991; Guff *et al.*, 1992; Ichiki *et al.*, 1993). GC formation waned around 10 days after immunization but it was restored by boost immunization (Fig. 2A).

RT-PCR of sorted cells

In order to see if isotype switching of antibodies takes place in GC B-cells, GC B-cells were isolated by a flow cytometer 5 times. The percentage of germinal center B-cells over total lymph node cells ranged from 3.8 to 9.3% (Table 1) in sort analyses. We collected only cells in block No. 1 (PNA-negative) or No. 3 (PNA-positive) of Fig. 5A and discarded cells within the overlapped region (block No. 2) for better purity. The purity of PNA-positive cells was over 95% (Fig. 5B). PNA-positive cells died easily after the sort when we did a post-sort analysis. This result is consistent with a previous report which states that centroblasts in GCs die by apoptosis (Liu *et al.*, 1991). We carried out an RT-PCR for PNA-positive and PNA-negative cells. In the first sort, we got a γ1 mRNA signal only in PNA negative cells. This result might be against the hypothesis that the Ig isotype switch takes place in GC B-cells. But the GC B-cell content in the first sort was very high (9.3%) whereas the content in our routine flow cytometric analyses was around 5%. This implies that the first sort seems to have sort problems. Sort No.

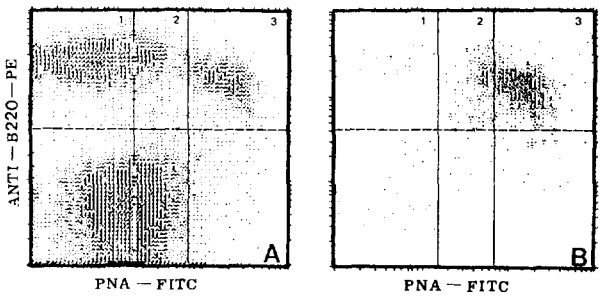

Fig. 5. Post-sort analysis of PNA-positive cells. (A) is dot plot of popliteal lymph node cells before the sort and (B) is a dot plot of PNA-positive cells after the sort. Cells within block 2 were not collected in order to increase purities of PNA-positive and PNA-negative cells.

Table 1. Gamma-1(γ1) germline transcript and γ1 mRNA expression in PNA(+) and PNA(−) cells

Sort No.	PNA positive (%)	Isotype	PNA positive	PNA negative
1	9.3	mγ1[a]	−	+
		GLT γ1[b]	−	−
2	5.3	mγ1	+	+
		GLT γ1	+	−
3	3.8	mγ1	−	−
		GLT γ1	−	−
4	7.1	mγ1	+	+
		GLT γ1	+	+
5	5.8	mγ1	+	+
		GLT γ1	+	−

[a] mγ1 : Mature γ1 mRNA.
[b] GLT γ1 : γ1 germline transcript.

2 and 5 showed that germline transcripts were expressed only in PNA-positive cells. This result implies that GC may be a site where isotype switching is initiated but signals for isotype switching were likely to be transduced to the ouside of GC at day 7 after immunization because mature γ1 mRNA was expressed in both cells (RNA positive and negative cells). In the fourth sort, all cells expressed bouth the γ1 germline transcript and γ1 mRNA. In the third sort, we could not detect any signal. We think that the mice might have been infected with viruses or bacteria. INF-γ produced in infected mice acts as an antagonist against IL-4 (King and Nutman, 1993) so that the isotype switching caused by IL-4 may be inhibited (Butch *et al.*, 1993). Even though these results are not definitive, they show the possibility of initiation of isotype switching in GCs.

We were able to isolate GC B-cells and performed PCR for γ1 germline transcripts in this study. So far, nobody has been able to amplify class specific Ab ge-

nes by PCR because of structural similarities between Ab classes. IL-4 is a cytokine which switches isotype to γ1 or ε (Butch *et al.*, 1993). Nevertheless, we focused on γ1 isotype switching in this study because the ε expression is extremely low and is exclusively influenced by the expression of IFN-γ. Moreover, IFN-γ is readily produced in mice by viral infections. But γ1 can be expressed after the blocking of IL-4 by the binding of anti-IL-4 mAb (Mills *et al.*, 1992). This means that the γ1 expression is not restricted in cells stimulated with IL-4 whereas the IgE expression is dependent on IL-4. The study of isotype switching to IgE must be performed to confirm isotype switching in GCs.

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