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Distribution of Cellular Isoform of Prion Protein in T Lymphocytes and Bone Marrow, Analyzed by Wild-Type and Prion Protein Gene-Deficient Mice

Atsutaka Kubosaki,* Seiichi Yusa,* Yukiko Nasu,* Takuya Nishimura,* Yuko Nakamura,* Keiichi Saeki,* Yoshitsugu Matsumoto,* Shigeyoshi Itohara,† and Takashi Onodera*,[1]

**Department of Molecular Immunology, School of Agricultural and Life Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan; and †Laboratory for Behavioral Genetics, Brain Science Institute, RIKEN, Saitama 351-0198, Japan*

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In this study, the authors investigated normal cellular prion protein (PrP^C) expression on murine immune systems using prion protein gene-deficient mouse as negative control. Immunocytes expressing PrP^C in adult and fetal mice were detected by flow cytometry with the monoclonal antibody against PrP^C, 6H4. Cells from thymus and bone marrow reacted positively with 6H4, while spleen cells, peritoneal cells, peripheral blood leukocytes, and intestinal intraepithelial lymphocytes were nonreactive. In thymus, PrP^C was observed in $CD4^-CD8^-$ double-negative thymocytes. PrP^{C+} cells of double-negative thymocytes belonged to the $CD3^-$ subset, but not to the $CD3^+$ subset. Triple-negative PrP^{C+} thymocytes expressed CD44 or CD25 antigens. Furthermore, PrP^C was observed in c-kit^+ bone marrow cells. In fetuses, PrP^{C+} cells were observed in the liver and thymus at day 16.0 and 15.0 of gestation, respectively. These results demonstrated that PrP^C is expressed on immature immunocytes.

Key Words: **prion protein; thymus; bone marrow; fetus; flow cytometry.**

The prion protein (PrP) is a glycoprotein implicated in the pathogenesis of several neurodegenerative diseases such as scrapie in sheep and goats; bovine spongiform encephalopathy in cattle; and kuru, Creutzfeldt-Jakob disease, fatal familial insomnia, and Gerstmann-Straussler-Scheinker syndrome in humans (1, 2). The classic neuropathological features of prion disease include spongiform degeneration, gliosis, and neuronal loss in the absence of inflammatory reaction. These diseases are marked by accumulation of the scrapie isoform of prion protein (PrP^{Sc}) that is thought to be identical in amino acid sequence to the normal cellular isoform of prion protein (PrP^C) (3).

According to the "protein-only hypothesis," PrP replication results from conversion of endogenous PrP^C into infectious PrP^{Sc}. Models of prion propagation and PrP^{Sc} synthesis propose a direct, homophilic interaction between the "substrate" molecule PrP^C and the "template" PrP^{Sc} (1). Prion protein gene-deficient ($PrP^{0/0}$) mice are devoid of developmental and behavioral abnormalities, and do not develop scrapie after inoculation with PrP^{Sc}. Expression of PrP^C is required for prion replication and neurodegenerative changes to develop and spread within the central nervous system (4). These results demonstrate that PrP^C is necessary for prion diseases, both for development of the disease and propagation of the agent.

Cells of the immune system play an important role during PrP^{Sc} neuroinvasion in mice after peripheral inoculation (5, 6). The follicular dendritic cell is a major antigen-presenting cell in lymphoid tissues, and is the site for PrP^{Sc} accumulation in the lymphoreticular system of mice (7). Mature B lymphocytes are essential in scrapie neuroinvasion because *uMT* mice (lacking all B lymphocyte differentiation stages beyond the large pre-B lymphocyte stage) and Rag-$1^{-/-}$ mice (lacking both mature T and B lymphocytes) are resistant to scrapie infection applied to the peritoneum in mice. However, these mice eventually develop the disease with the same timing and histopathological characteristics as wild-type mice after intracerebral PrP^{Sc} inoculation. Since replication of PrP^{Sc} and transport of PrP^{Sc} from the periphery to the central nervous system depend on PrP^C expression (8), it is important to determine the distribution of PrP^C in the immune system.

In this study, the subsets expressing PrP^C in the lymphoid system of mice were determined in detail using $PrP^{0/0}$ mice as negative control.

[1] To whom correspondence and reprint requests should be addressed. Fax: 0081-3-5841-8020. E-mail: aonoder@mail.ecc.u-tokyo.ac.jp.

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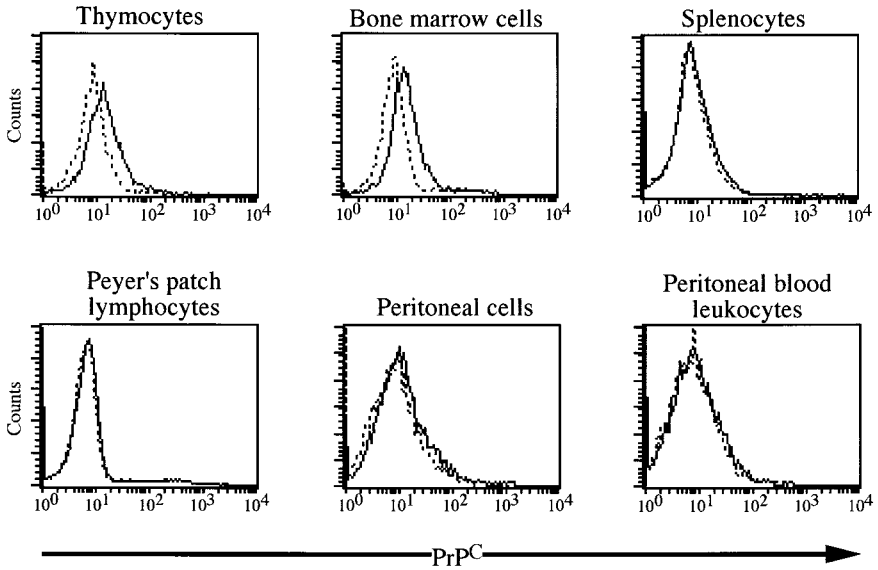

FIG. 1. Expression of PrP^C in lymphoid organs and tissues. Immunocytes from lymphoid organs and tissues were stained with mouse anti-PrP^C monoclonal antibody 6H4. Mouse monoclonal antibody was detected by FITC-goat F(ab′)2 anti-mouse IgG1 antibody. The histogram of WT murine cells and PrP$^{0/0}$ murine cells are indicated by the solid and broken lines, respectively. Staining and analyses were performed two to four times with similar results.

MATERIALS AND METHODS

Animals. C57BL/6CrSlc (WT) mice (Nippon SLC, Shizuoka, Japan) were employed at 7–10 weeks old. For the analysis of fetal thymocytes and liver cells, mature females were caged with breeding males. Fetuses were obtained from time-mated females. The day of the vaginal plug observation was considered as day 0.5 (day post-coitus). As a control PrP$^{0/0}$ mice (9) were used.

Antibodies and reagents. The mAb 2.4G2 (anti-FcgRII/III, CD16/CD32), PE-RM4-4 (anti-CD4), PE or Cy-Chrome (Cy)-53.6.7 (anti-CD8a), Cy-145-2C11 (anti-CD3ϵ), PE-3C7 (anti-IL-2R/CD25), biotin-2B8 (anti-c-kit, CD117), biotin-1M7 (anti-CD44), and isotype-matched rat or hamster immunoglobulins were purchased from PharMingen (San Diego, CA). PE- and Cy-streptavidin (PharMingen, CA) were used for the detection of biotinylated antibodies. The mAb 6H4 for PrP (IgG1) was a commercial product of Prionics (Zurich, Switzerland). FITC-goat F(ab′)2 anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) was used for the detection of 6H4. Propidium iodide (PI) (Molecular Probes, Eugene, OR) was mixed with PBS (100 mg/ml) to produce a stock solution.

Cell preparation. Thymocytes, spleen cells, bone marrow (BM) cells, peritoneal cells, peripheral blood leukocytes, and intestinal intraepithelial lymphocytes (IEL) were prepared from adult WT mice and PrP$^{0/0}$ mice, as described previously (10, 11). Fetal thymocytes and liver cells were obtained from day-matched WT or PrP$^{0/0}$ mouse fetuses, as previously described (12). Viable cells were counted by trypan blue exclusion.

Cell enrichment by magnetic cell sorting (MACS) Superparamagnetic microbeads conjugated with monoclonal rat anti-mouse CD4 (IgG2b) and CD8a (IgG2a) Abs were purchased from Miltenyi Biotec GmbH (Germany). CD4$^-$CD8$^-$ thymocytes were purified by magnetic separation with a MACS column according to the manufacturer's instructions.

Flow cytometry. Flow cytometric analysis and most of the mAbs techniques used in this study have been described previously (10). Briefly, single-cell suspensions from spleen, thymus, BM, gut-associated lymphoreticular tissues (GALT), fetal thymus, and fetal liver were prepared. Peripheral blood leukocytes and peritoneal cells were collected. Each single-cell suspension was washed with ice-cold Ca^{2+}- and Mg^{2+}-free PBS (pH 7.4) containing 1% FCS and 0.1% NaN_3 (washing buffer). In order to block the free binding sites of antibodies and FcgRII/III on the cells, murine immunocytes were first incubated with 2.4G2 on ice for 30 min. For indirect staining, cells were incubated with 6H4 on ice for 60 min, washed thrice with washing buffer, and incubated with FITC-goat F(ab′)2 anti-mouse IgG1 antibody (as the second antibody) on ice for 60 min. For direct or indirect three-color staining, cells were incubated with 6H4 and/or biotin-conjugated Abs on ice for 60 min and washed thrice with washing buffer. Cells were then incubated with direct-labeled Abs or FITC-goat F(ab′)2 anti-mouse IgG1 antibody and/or labeled streptavidin (as the second antibody) on ice for 60 min. After the final washing, samples were analyzed with FACScan (Becton Dickinson, San Jose, CA) and data were analyzed by Cellquest software (Becton Dickinson). Selective gating on forward light scatter versus side scatter was used to eliminate red blood cells and dead cells from the analysis. Viable cells determined by the forward and side scatter were gated. For single-color samples, uptake of PI (500 ng/ml for 10^6 cells) was used to exclude dead cells.

RESULTS AND DISCUSSION

Spleen, thymus, and lymphatic organs of PrP$^{0/0}$ mice developed normally. No difference was observed in the number of cells in BM, thymus and spleen between adult PrP$^{0/0}$ mice and WT mice (data not shown). By flow cytometry, differences were not observed in the populations of T cells with CD4 and CD8a in the thymus (data not shown). The mature B and T lymphocyte populations, defined by CD4 and CD45R/B220 in the PrP$^{0/0}$ mice spleen, were also normal (data not shown). Hereafter, to investigate PrP^C expression on various immunocytes, cells were stained by 6H4 or isotype-matched Ab. In this study, the data from PrP$^{0/0}$ mice and WT mice were illustrated.

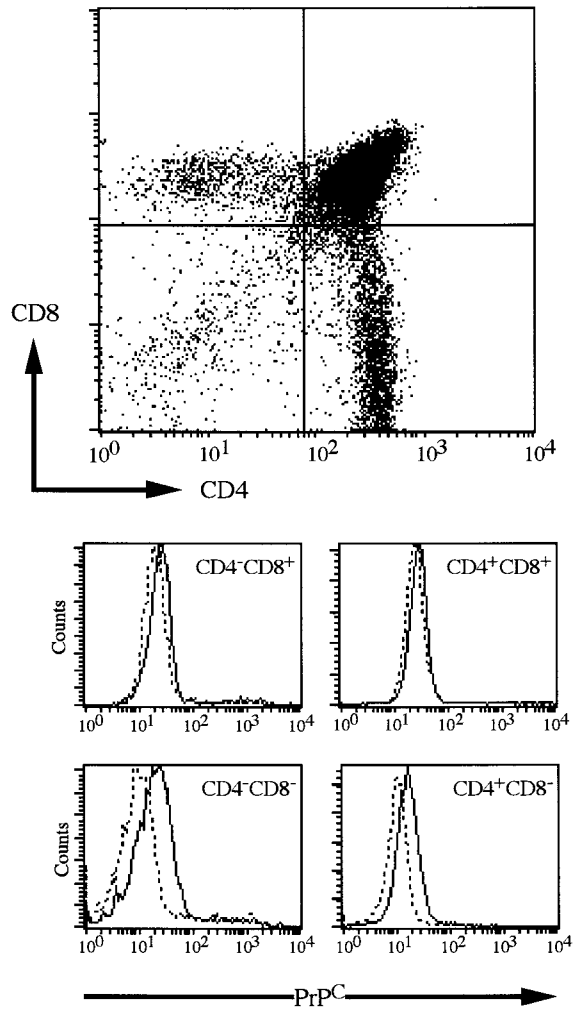

FIG. 2. Expression of PrP^C in the thymus. Thymocytes were stained as described in a methods employing mouse anti-PrPC monoclonal antibody (6H4), PE-RM4-4 (anti-CD4), and Cy-53.6.7 (anti-CD8a). 6H4-positive cells were detected by FITC-goat F(ab′)2 anti-mouse IgG1 antibody. The dotted plot (upper panel) indicates the fractions defined by CD4 and CD8a expression. The histogram of WT murine cells and $PrP^{0/0}$ murine cells are indicated by the solid and broken lines, respectively. Staining and analysis were repeated four times with similar results.

To investigate the subsets of immunocytes expressing PrP^C, immunocytes were isolated from various organs. Thymocytes, spleen cells, BM cells, peritoneal cells, peripheral blood leukocytes, and intestinal intraepithelial lymphocytes were used for analysis. The reactivity of 6H4 mAb was analyzed by flow cytometry. Although some of these cells showed relatively weak staining, the results were obvious. Cells from thymus and BM (as primary lymphoid tissues) were stained positively with 6H4, while spleen cells, peritoneal cells, peripheral blood leukocytes, and intestinal intraepithelial lymphocytes were non-reactive (Fig. 1). Since splenic lymphocytes and IEL were functionally and phenotypically more mature than thymocytes and BM cells, these data suggest that immature immunocytes expressed PrPC.

Since staining reactivity of 6H4 mAb was detected by thymocyte analysis, further fractionated thymocytes were prepared on the basis of the expression of CD4 and CD8a prior to investigation on PrPC expression. PrP^C was expressed on $CD4^-CD8^-$ double-negative (DN) thymocytes (Fig. 2). This does not necessarily mean that T progenitor cells expressed PrP^C. There

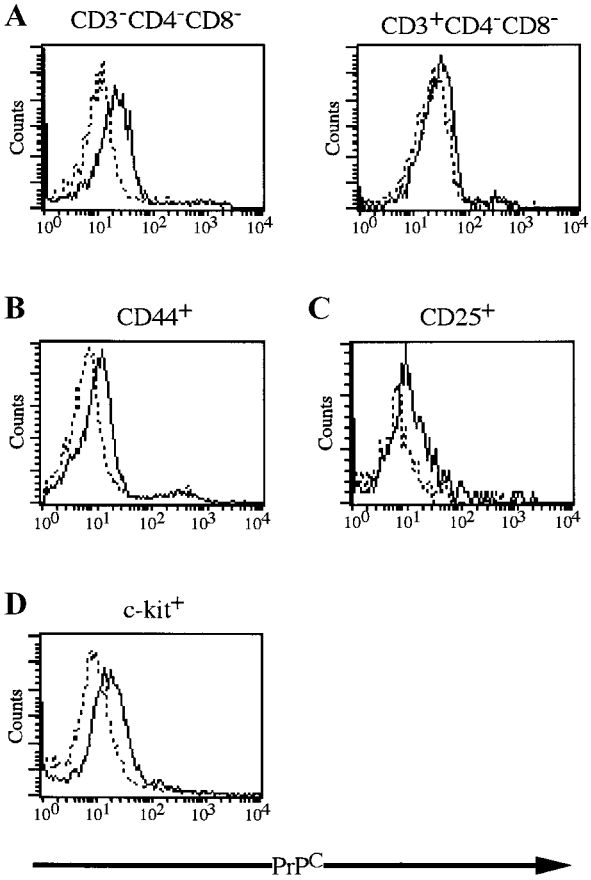

FIG. 3. PrP^C expression in immature immunocytes in adult thymus and BM. (A) Thymocytes were stained with mouse anti-PrP^C monoclonal antibody, PE-RM4-4 (anti-CD4), PE-53.6.7 (anti-CD8a), and Cy-145-2C11 (anti-CD3ϵ). The mouse monoclonal antibody was detected by FITC-goat F(ab′)2 anti-mouse IgG1 antibody. $CD4^-CD8^-$ DN thymocytes were gated, and expressions of PrP^C in $CD3^-$ DN cells and $CD3^+$ DN cells are shown. Sorted thymocytes ($CD4^-CD8^-$ DN cells), as described under "Materials and Methods," were stained with mouse anti-PrP^C monoclonal antibody and either biotin-1M7 (anti-CD44) (B) or PE-3C7 (anti-IL-2R/CD25) (C). The mouse monoclonal and biotinylated antibodies were detected by FITC-goat F(ab′)2 anti-mouse IgG1 antibody and PE-streptavidin, respectively. PrP^C expression on $CD44^+$ thymocytes and $CD25^+$ thymocytes are shown, accordingly. The histogram of WT murine cells and $PrP^{0/0}$ murine cells are indicated by the solid and broken lines, respectively. BM cells were stained with mouse anti-PrP^C monoclonal antibody (6H4) and biotin-2B8 (anti-c-kit, CD117). The mouse monoclonal and biotinylated antibodies were detected by FITC-goat F(ab′)2 anti-mouse IgG1 antibody and PE-streptavidin, respectively. PrP^C expressions on c-kit^+ BM cells (D) are shown. Staining and analysis were repeated four times with similar results.

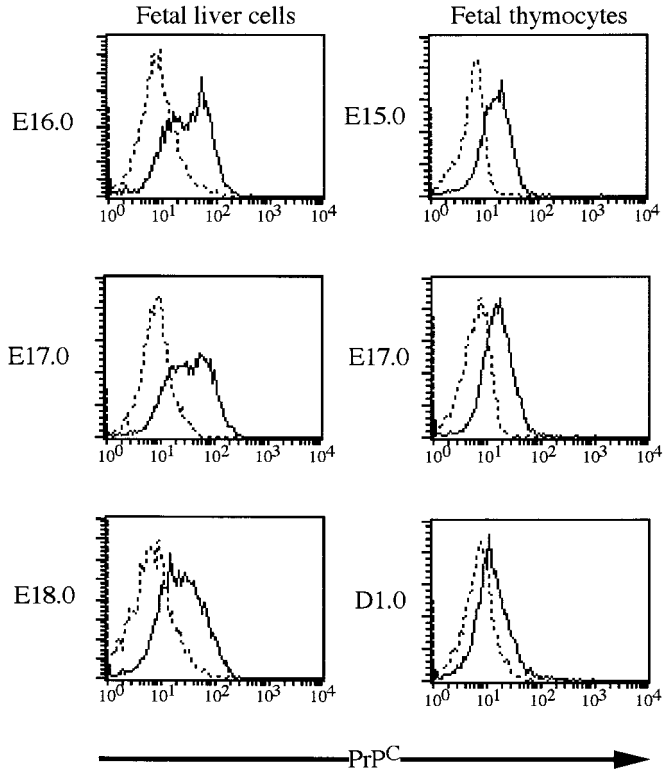

FIG. 4. Expression of PrP^C on fetal liver cells and thymocytes. Fetal liver cells and thymocytes at the indicated gestational day were stained with mouse anti-PrP^C monoclonal antibody, which was detected by FITC-goat F(ab′)2 anti-mouse IgG1 antibody. Dead cells were excluded by staining with PI. The histogram of WT murine cells and $PrP^{0/0}$ murine cells are indicated by the solid and broken lines, respectively.

were non-T lineage cells and $CD3^+$ T cell subsets among DN thymocytes. Therefore, thymocytes were then stained with anti-PrP, PE-anti-CD4, PE-anti-CD8a and biotinylated anti-CD3ϵ, and examined for PrP^C expression in DN $CD3^-$ [triple-negative (TN)] and DN $CD3^+$ fractions. The result revealed that PrP^{C+} cells belonged to the $CD3^-$ fraction and not the $CD3^+$ fraction (Fig. 3A). $CD4^+CD8^-$ cells have higher levels of PrP^C than $CD4^+CD8^+$ and $CD4^-CD8^+$ cells (Fig. 2). To examine if PrP^{C+} cells in $CD4^+CD8^-$ fraction were helper T cells, thymocytes were stained with PE-CD4, PE-CD8a, and PE-CD8e antibodies. Most of thymocytes belonged to PE^+ subset, however PE^+ fraction does not contain PrP^{C+} (data not shown). Since $CD4^+CD8^-$ cells include CD4 low TN cells, it is possible that CD4 low TN cells may also express PrP^C.

By this staining approach, most PrP^{C+} cells were found to be triple-negative. The TN fraction also contained B lymphocytes, macrophages, dendritic cells, and granulocytes. To examine if PrP^{C+} cells in the TN fraction were T progenitor cells, DN thymocytes were enriched by MACS before staining with CD25 and CD44 antibodies. Figures 3B and 3C show that PrP^C was expressed on $CD25^+$ and $CD44^+$ cells. BM cells were fractionated with 2B8 (anti-c-kit, CD117). Figure 3D shows that c-kit+ cells in BM express PrP^C. These results demonstrated that PrP^C is expressed on immature immunocytes in adult thymus and BM.

In addition, PrP^C expression on fetal thymocytes and liver cells was analyzed. Fetal liver cells and thymocytes from individual mice from day 15 of the embryo stage through birth (D 1.0) were compared for levels of PrP^C. The fetal liver cells expressed PrP^C on day 16.0 (E 16.0), while PrP^{C+} cells coped well on day 17.0 (E 17.0) and decreased on day 18.0 (E 18.0) (Fig. 4, left). Analysis on PrP^C expression in fetal thymocytes on gestation day 15.0 (E 15.0) indicated remarkable PrP^C expression. The PrP^C expression level gradually decreased until birth (Fig. 4, right). After gestation day 11, the liver becomes the main organ of fetal hematopoiesis where all hematopoietic precursors proliferate and differentiate (13, 14). The fetal liver provides cells that migrate to the fetal thymus where T lymphocytes mature. In short, fetal thymocytes and liver cells contain immature immunocytes. Thus, these findings support the proposal that PrP^C is expressed on immature immunocytes.

In prion diseases, cells of the lymphoreticular system are involved in PrP^{Sc} and PrP^{Sc} replication sites (15, 16). Klein *et al.* have reported a crucial role for B lymphocytes in PrP^{Sc} neuroinvasion (17). Furthermore, studies have shown that prion infectivity in the spleen is associated with B and T lymphocytes (18–20). Over the past few years, several studies have been made on expression of PrP^C in human peripheral blood lymphocytes to understand PrP^{Sc} infectivity (21–23). Our study demonstrated the subsets expressing PrP^C in the lymphoid system of mice in detail. These results will be helpful for a clearer understanding of the normal function of PrP^C and transport of PrP^{Sc} infectivity in blood.

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