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Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes

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Abstract

A mAb J43 has been produced against the product of the mouse PD-1 gene, a member of the Ig gene superfamily, which was previously isolated from an apoptosis-induced T cell hybridoma (2B4.11) by using subtractive hybridization. Analyses by flow cytometry and immunoprecipitation using the J43 mAb revealed that the PD-1 gene product is a 50–55 kDa membrane protein expressed on the cell surface of several PD-1 cDNA transfectants and 2B4.11 cells. Since the molecular weight calculated from the amino acid sequence is 29,310, the PD-1 protein appears to be heavily glycosylated. Normal murine lymphoid tissues such as thymus, spleen, lymph node and bone marrow contained very small numbers of PD-1$^+$ cells. However, a significant PD-1$^+$ population appeared in the thymocytes as well as T cells in spleen and lymph nodes by the *in vivo* anti-CD3 mAb treatment. Furthermore, the PD-1 antigen expression was strongly induced in distinct subsets of thymocytes and spleen T cells by *in vitro* stimulation with either anti-CD3 mAb or concanavalin A (Con A) which could lead T cells to both activation and cell death. Similarly, PD-1 expression was induced on spleen B cells by *in vitro* stimulation with anti-IgM antibody. By contrast, PD-1 was not significantly expressed on lymphocytes by treatment with growth factor deprivation, dexamethasone or lipopolysaccharide. These results suggest that the expression of the PD-1 antigen is tightly regulated and induced by signal transduction through the antigen receptor and do not exclude the possibility that the PD-1 antigen may play a role in clonal selection of lymphocytes although PD-1 expression is not required for the common pathway of apoptosis.

Introduction

The activation of the T cell requires stimulation through TCR by a specific antigen in the context of the appropriate MHC molecule on the antigen-presenting cell (1). The proper TCR engagement results in expression of various cell surface molecules, lymphokine secretion and, consequently, T cell proliferation (2,3). Many previous studies have indicated that T cell surface molecules other than TCR have critical roles for alternative stimulation, co-stimulation and proliferative responses of T cells (4–10) to prevent anergy (11–13) as well as activation-induced apoptotic cell death (14–16).

We previously reported isolation of PD-1 cDNA which encodes a novel member of the Ig superfamily by subtractive hybridization using two murine lymphoid cell lines which can be induced to die by totally different stimulations (17). One is a T cell hybridoma 2B4.11 (18), which dies when stimulated with its cognate antigen (I-E^k plus pigeon cytochrome *c*) or with a combination of phorbol 12-myristate-13-acetate (PMA) and ionomycin (19). Although a considerable amount of PD-1 transcripts was detected in unstimulated 2B4.11 cells, their expression was strongly augmented by stimulation with PMA and ionomycin (17). The other is a lymphoid/myeloid progenitor cell line LyD9 (20,21), which requires IL-3 for its survival and proliferation and dies when IL-3 is deprived from the culture. IL-3-supplemented LyD9 cells do not express PD-1 mRNA at all, but IL-3-deprived LyD9 cells express PD-1 mRNA strongly (17). The expression

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of PD-1 mRNA in murine tissues is restricted to the thymus and PD-1 mRNA in the thymus increases when thymocytes are stimulated by *in vivo* administration of an anti-CD3 mAb (17,22).

Here we describe the generation of a hamster mAb J43 that specifically recognizes the PD-1 gene product, and analyses of the PD-1 antigen by using the J43 mAb in several cell lines and murine lymphocytes. We demonstrate that the PD-1 antigen is a 50–55 kDa membrane protein, expression of which was induced on the surface of murine T and B lymphocytes stimulated through antigen receptors *in vivo* as well as *in vitro*.

Methods

Animals

Armenian female hamsters and C57BL/6 female mice were supplied by Oriental Yeast (Tokyo, Japan) and Shizuoka Laboratory Animal Center (Shizuoka, Japan) respectively.

Cell lines

BHK (23), its PD-1 cDNA transfectant (B12) and 2B4.11 (18) were cultured in RPMI 1640 (Gibco/BRL, Gaithersburg, MD) supplemented with 10% FCS, 2 μM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). LyD9 (20, 21) and its PD-1 cDNA transfectants (L7-9 and LCD5-4) were cultured in the complete medium containing 0.2% culture supernatant of X63Ag8-653 myeloma cells transfected with the murine IL-3 expression vector (24). CHO (25) and its PD-1 cDNA transfectant (C7-4) were cultured in Ham's F-12 (Nissui, Tokyo, Japan) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Transfection of BHK, CHO and LyD9 cells

A PD-1 cDNA expression vector, pMIK-Neo/PD-1 was constructed by inserting the 1990 bp *Eco*RI–*Not*I fragment carrying the entire mouse PD-1 cDNA (17) between the *Eco*RI and *Not*I sites of pMIK Neo expression vector (a generous gift from Dr Maruyama, The University of Tokyo). pMIK Neo/PD-1 CD, an expression vector of the cytoplasmic portion-deleted PD-1 cDNA, was constructed by inserting the 730 bp *Eco*RI–*Alw*NI fragment of the PD-1 cDNA, in which the *Alw*NI site had been blunted, between the *Eco*RI and *Sma*I sites of the pMIK Neo vector. B12 and C7-4 cells were obtained by transfection of BHK and CHO cells respectively with the pMIK Neo/PD-1 plasmid by the calcium phosphate method (26). L7-9 and LCD5-4 cells were obtained by transfection of LyD9 cells with the pMIK Neo/PD-1 and the pMIK Neo/PD-1CD respectively by electroporation (26).

Production of anti-PD-1 mAb J43

Several armenian female hamsters were immunized with 5×10^6 B12 cells by four weekly i.p. injections. Spleen cells from the immunized hamsters were fused with the mouse myeloma P3U1 and selected in HAT medium according to the standard protocol (27). Supernatants of the resulting hybridomas were sequentially screened by flow cytometric analyses as follows; screening first for reactivity with 2B4.11 cells stimulated with PMA and ionomycin for 6 h as described (17), second for reactivity with C7-4 cells and for lack of reactivity with parental CHO cells, and third for reactivity with B12 cells and for lack of reactivity with parental BHK cells. Finally the remaining positive hybridomas were examined in an ELISA for binding to PD-1 Ig chimeric molecules consisting of the putative extracellular portion of PD-1 which was fused with the Fc portion of human IgG1. One hybridoma J43 was subsequently cloned by repeated limiting dilution until a stable clone was obtained.

Antibodies

The J43 mAb was purified from culture supernatants of the J43 hybridoma by affinity-chromatography on Protein G–Sepharose (Pharmacia Biotech, Tokyo, Japan) and biotinylated using Sulfo-NHS-Biotin (Pierce, Rockford, IL) as described (27). An anti-mouse CD3ε mAb was prepared as either culture supernatants of the 145-2C11 hybridoma (28) or ascitic fluids from BALB/c *nu/nu* mice inoculated with the 145-2C11 hybridoma. A polyclonal goat $F(ab')_2$ fragment against mouse IgM was obtained from Cappel (Durham, NC). An anti-mouse Fc receptor mAb was prepared as culture supernatant of the 2.4G2 hybridoma (29). Phycoerythrin (PE)-conjugated anti-CD4 (GK1.5) (30) and FITC-conjugated anti-CD4 (YTS191.1) (31) mAb were purchased from Becton Dickinson (Mountain View, CA) and Caltag (San Francisco, CA) respectively. FITC–anti-CD8 (53-6.7) (32) mAb was purchased from Gibco/BRL. FITC–anti-B220 (RA3-6B2) (33), FITC–anti-CD25 (7D4) (34), and FITC–anti-CD69 (H1.2F3) (9) mAb were purchased from PharMingen (San Diego, CA). FITC–goat anti-hamster IgG was purchased from Jackson Immunoreserch (West Grove, PA). Streptavidin (SA) conjugated with PE and Red670 were purchased from Dako Japan (Kyoto, Japan) and Gibco/BRL respectively.

Immunoprecipitation

The cell surface proteins were biotinylated using Sulfo-NHS-Biotin and $1–2 \times 10^7$ cells were lysed in 1 ml of a lysis buffer containing 1% NP-40, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% NaN_3, 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin. The cell lysates were precleared with Protein G–Sepharose (Pharmacia) preincubated with normal hamster serum and immunoprecipitated with J43 mAb-preloaded Protein G–Sepharose. After washing five times with a washing buffer containing 0.5% NP-40, 50 mM Tris–HCl (pH 8.0), 600 mM NaCl, 0.1% NaN_3, 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin, the immunoprecipitates were heated in the SDS gel-loading buffer (26) containing 100 mM dithiothreitol and subjected to SDS–10% PAGE. The proteins were electroblotted onto an Immobilon-PVDF membrane (Millipore, Bedford, MA) and detected using the enhanced chemiluminescence system (Amersham, Tokyo, Japan) after incubation with horseradish peroxidase-conjugated SA (Amersham).

In vivo stimulation

Normal 4- to 7-week-old C57BL/6 female mice were injected i.p. with 0.2 ml of ascitic fluid containing the anti-CD3 mAb. Cells of the thymus, spleen and lymph nodes (axillal, inguinal

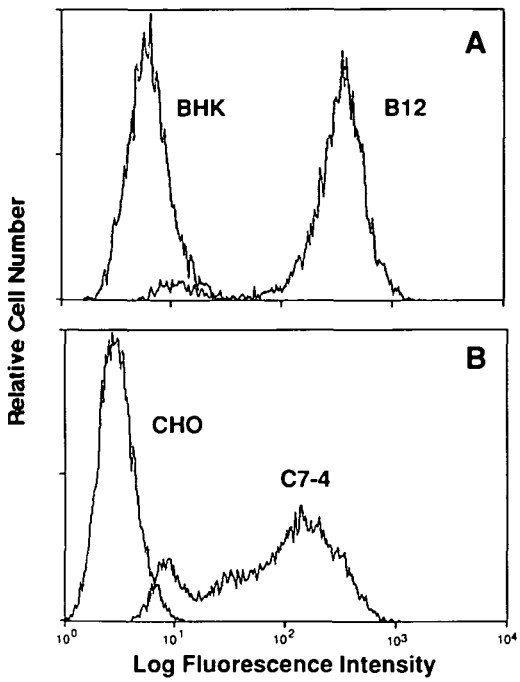

Fig. 1. Staining of the PD-1 cDNA transfectants with the J43 mAb. B12 and parental BHK cells (A) and C7-4 and parental CHO cells (B) were stained with the J43 mAb, followed by FITC–goat anti-hamster IgG. Fluorescence intensity is plotted on a horizontal logarithmic scale. The vertical scale represents the relative number of cells at each fluorescence intensity.

and mesenteric) were prepared from the anti-CD3 mAb-injected mice after 48 h or from the age-matched mice.

In vitro stimulation

Stimulation of 2B4.11 cells and IL-3 deprivation of LyD9 cells were done as described (17). Thymocytes and spleen cells prepared from 4- to 7-week-old C57BL/6 female mice aseptically were cultured at $1–2\times10^6$ cells/ml in the complete medium in the presence of either the immobilized anti-CD3 mAb, 2 μg/ml concanavalin A (Con A) (Pharmacia), 10 μg/ml polyclonal goat $F(ab')_2$ fragment of anti-IgM antibody, 10 ng/ml PMA (Sigma, St Louis, MO) plus 500 ng/ml ionomycin (Calbiochem, San Diego, CA), or 50 μg/ml lipopolysaccharide (LPS) (Sigma). The cultures were incubated at 37°C under 5% CO_2 for indicated periods.

Flow cytometry

Between 1 and 2×10^6 cells of BHK, CHO or their transfectants were washed with a staining buffer (PBS containing 2% FCS and 0.1% NaN_3) and incubated with the culture supernatants of the J43 hybridoma, followed by FITC–goat anti-hamster IgG. Other cells were washed with the staining buffer and incubated with the culture supernatants containing a saturating amount of the 2.4G2 mAb for blockade of non-specific binding to the FcR. The cells were then incubated with the biotinylated J43 mAb together with PE–anti-CD4, FITC–anti-CD4, FITC–anti-CD8, FITC–anti-B220, FITC–anti-CD25 or FITC–anti-CD69 mAb, followed by PE–SA or Red670–SA. A total of 10,000 events were collected and analyzed on a FACScan (Becton Dickinson) using Lysys software. For single- and two-color staining, dead cells and debris were excluded by forward and side scatter gating and propidium iodide staining. For three-color staining of thymocytes, dead cells and debris were excluded by forward and side scatter gating.

Results

Generation of the J43 mAb and biochemical characterization of the PD-1 antigen

We produced a mAb against the PD-1 gene product by immunizing hamsters with a PD-1 cDNA transfectant of BHK cells. The mAb J43 was found to react with PD-1 cDNA transfectants of BHK and CHO cells (named B12 and C7-4 respectively) but not with parental BHK and CHO cells (Fig. 1). This indicates that the J43 mAb specifically recognizes the PD-1 gene product and the PD-1 antigen is expressed on the cell surface as expected from its predicted amino acid sequence (17). The specificity of the mAb was further demonstrated by the following observations: (i) the J43 mAb reacted with a PD-1 Ig fusion protein comprising the PD-1 extracellular domain and the Fc portion of human IgG1, but not with human IgG1 in an ELISA (data not shown) and (ii) the J43 mAb stained lymphocytes of the PD-1 cDNA transgenic mice (data not shown), but not normal murine lymphocytes as described below.

To examine biochemical characteristics of the PD-1 antigen, biotinylated cell surface proteins of the PD-1 transfectants and their parental cells were immunoprecipitated with the J43 mAb (Fig. 2). A 50–55 kDa polypeptide was immunoprecipitated from the B12 and C7-4 cells under reducing conditions (Fig. 2, lanes 2 and 4), but not from their parental BHK and CHO cells (Fig. 2, lanes 1 and 3). The broad immunoprecipitated bands are probably due to glycosylation as predicted from the primary structure of PD-1 (17). In addition, LyD9 cells transfected with the full length PD-1 cDNA (L7-9) expressed the same size antigen as those of the B12 and C7-4 cells, while parental LyD9 cells did not (Fig. 2, lanes 7 and 8). Furthermore, the PD-1 antigen immunoprecipitated by the J43 mAb from LyD9 cells that were transfected with a partially deleted PD-1 cDNA (LCD5-4) had an apparently smaller size than that from the L7-9 cells (Fig. 2, lanes 8 and 9). These results further strengthen the evidence for the specific reactivity of the J43 mAb with the PD-1 antigen.

Expression of the PD-1 antigen on cultured cell lines

We first examined PD-1 antigen expression on apoptosis-induced 2B4.11 and LyD9 cells, which we had used for the isolation of the PD-1 cDNA (17). Flow cytometry analysis showed that unstimulated 2B4.11 cells expressed a considerable amount of the PD-1 antigen, but the expression was significantly enhanced when 2B4.11 cells were induced to die by stimulation with the anti-CD3 mAb or PMA/ionomycin (Fig. 3A and B). A similar result was obtained using another T cell hybridoma, DO11.10 (35) (data not shown). The enhanced expression of the PD-1 antigen upon stimulation was also confirmed by the immunoprecipitation experiments (Fig. 2, lanes 5 and 6). These results are consistent with the previous observation that low level expression of PD-1 transcripts was

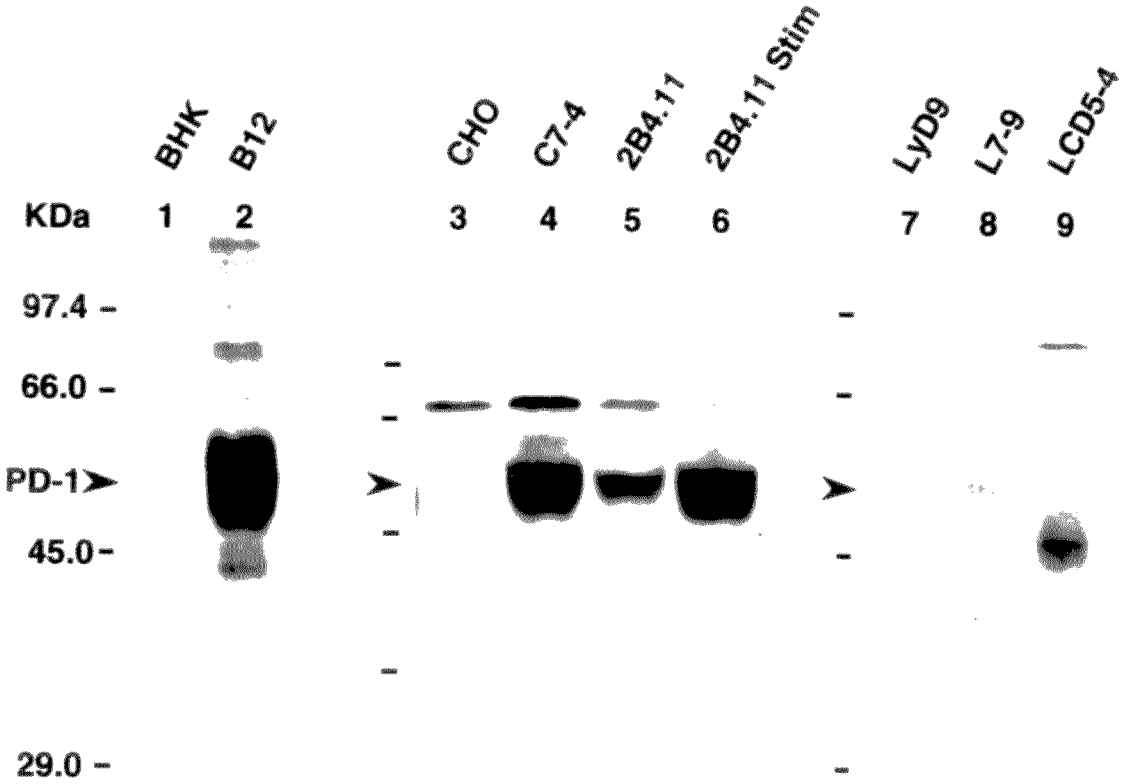

Fig. 2. Immunoprecipitation of PD-1 antigen with the J43 mAb. Cells analyzed were BHK (lane 1), B12 (lane 2), CHO (lane 3), C7-4 (lane 4), 2B4.11 (lane 5), 2B4.11 stimulated with PMA plus ionomycin for 6 h (lane 6), LyD9 (lane 7), L7-9 (lane 8) and LCD5-4 (lane 9).

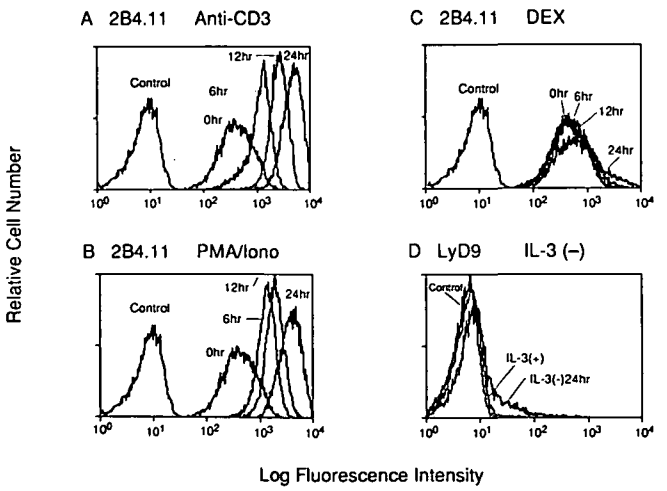

Fig. 3. PD-1 antigen expression on 2B4.11 and LyD9 cells. 2B4.11 cells stimulated for the indicated time with anti-CD3 mAb (A), PMA plus ionomycin (B) or DEX (C) and LyD9 cells (D) cultured in the presence (+) or in the absence (–) of IL-3 for 24 h were stained with the biotinylated J43 mAb, followed by PE–SA. Background fluorescence by PE–SA alone is displayed as control in each histogram.

detected in unstimulated 2B4.11 cells, but their expression was significantly augmented upon stimulation (17). However, normal proliferation of several PD-1 cDNA transfectants and the PD-1 antigen expression on unstimulated 2B4.11 cells indicate that expression of the PD-1 antigen alone does not trigger apoptosis.

On the other hand, normal LyD9 cells did not express any detectable amount of the PD-1 antigen (Fig. 3D). When LyD9 cells were induced to die by IL-3 deprivation, we could detect only a small fraction of weakly stained cells although a significant amount of PD-1 transcripts was detected in IL-3-deprived LyD9 cells (17). We have no explanation to account for this discordant expression between the protein and transcript. Furthermore, the PD-1 expression was not augmented upon apoptosis of 2B4.11 cells by the dexamethasone (DEX) treatment (Fig. 3C). These results suggest that the PD-1 expression is not required for the common pathway of apoptosis.

Expression of the PD-1 antigen on murine T lymphocytes stimulated in vivo

We next examined expression of the PD-1 antigen in normal murine lymphoid organs including the thymus, spleen, lymph node and bone marrow as PD-1 mRNA expression is found almost exclusively in the thymus among murine tissues tested

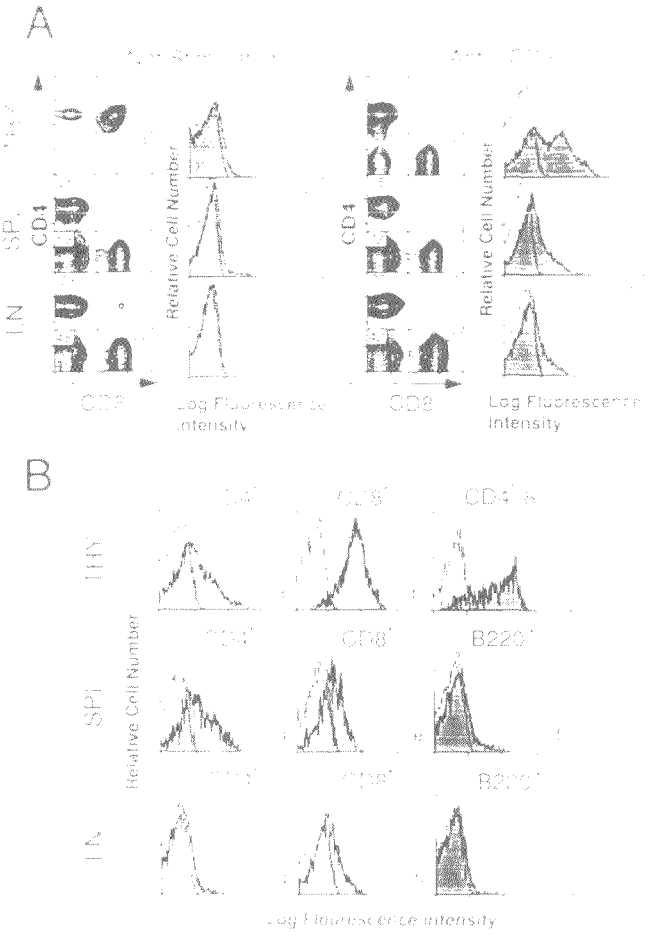

Fig. 4. Induction of PD-1 antigen expression on lymphocytes by *in vivo* administration of the anti-CD3 mAb. (A) Thymocytes (a and d), spleen cells (b and e) and lymph node cells (c and f) prepared from a normal 7-week-old C57BL/6 mouse (a–c) and from an anti-CD3 mAb-injected (48 h after injection) age-matched C57BL/6 mouse (d–f) were stained with the biotinylated J43 mAb, followed by Red670–SA (thymocytes) or PE–SA (spleen and lymph node cells) (stippled area). Background fluorescence by Red670–SA or PE–SA alone is also displayed (open area). Simultaneous CD4 and CD8 staining profile of the same cell preparation is indicated on the left of each PD-1 staining histogram. The percentages of cells present in each quadrant are indicated. (B) Thymocytes (a–c), spleen cells (d–f) and lymph node cells (g–i) were prepared from the same anti-CD3 mAb-injected mouse as in (A). For three-color staining, thymocytes were stained with the biotinylated J43 mAb together with PE-anti-CD4 and FITC-anti-CD8 mAb, followed by Red670–SA. PE-stained CD4 SP cells (a), FITC-stained CD8 SP cells (b) and unstained $CD4^-CD8^-$ DN cells (c) were electronically gated and Red670-stained anti-PD-1 fluorescence (stippled area) and background fluorescence by Red670–SA alone (open area) are displayed. For two-color staining, spleen cells and lymph node cells were stained with the biotinylated J43 mAb together with FITC-conjugated anti-CD4, anti-CD8 or anti-B220 mAb, followed by PE–SA. FITC-stained $CD4^+$ (d and g), $CD8^+$ (e and h), or $B220^+$ (f and i) cells were electronically gated and PE-stained anti-PD-1 fluorescence (stippled area) and background fluorescence by PE–SA alone (open area) are displayed. These results are representative of four experiments.

(17). However, we could detect only a small percentage of the PD-1 antigen positive cells in thymocytes as well as in spleen cells, lymph node cells (Fig. 4A, a–c) and bone marrow cells (data not shown). We then examined the PD-1 expression on thymocytes after the *in vivo* injection of the anti-CD3 mAb which results in massive thymocyte death, T cell activation and a significant augmentation of PD-1 mRNA expression (17,22,36). Although the massive thymocyte death was induced within 24 h after the anti-CD3 mAb injection, we could not observe any significant increase of the $PD\text{-}1^+$ population in such an apoptosis-induced thymocyte population (data not shown).

However, a considerable proportion of thymocytes expressed the PD-1 antigen 24–48 h after the anti-CD3 mAb injection, when 75–95% of thymocytes had been killed and removed (48 h, Fig. 4A, d). These $PD\text{-}1^+$ cells were found to exist in the CD4 single-positive (SP) and CD8 SP thymocyte populations, and also in the $CD4^-CD8^-$ double-negative (DN) population (Fig. 4B, a–c). These populations have been reported to be relatively resistant to induction of apoptosis triggered by the anti-CD3 mAb (22,36). Furthermore, the $PD\text{-}1^+$ cells were found to be blastic (data not shown). Therefore these results indicate that the PD-1 antigen is induced on thymocytes activated by stimulation through the TCR although it is not clear whether dying cells express the PD-1 antigen transiently before they die (see Discussion). Almost similar subsets of thymocytes survived after *in vivo* administration of DEX (37,38), but no $PD\text{-}1^+$ population could be detected (data not shown), suggesting that induction of the PD-1 antigen requires stimulation through TCR. The induced expression of the PD-1 antigen upon T cell stimulation was further supported by the fact that peripheral T lymphocytes such as spleen and lymph node T cells contained a significant $PD\text{-}1^+$ cell population 48 h after the anti-CD3 mAb injection (Fig. 4A, e and f). Two-color flow cytometric analyses of lymphocytes of the anti-CD3 mAb-injected mice revealed that the $PD\text{-}1^+$ cells were found in the CD4 and CD8 T cell populations of spleen cells (Fig. 4B, d and e), and the CD8 T cell population of lymph node cells (Fig. 4B, g and h), but not in $B220^+$ cells of spleen or lymph node (Fig. 4B, f and i). The PD-1 antigen induction on spleen cells is consistent with the previous observation that weak expression of PD-1 mRNA was detected in spleen of the anti-CD3 mAb-injected mice (17).

The PD-1 antigen is induced on in vitro stimulated T and B lymphocytes

We then examined the PD-1 antigen expression on thymocytes and spleen cells stimulated through antigen receptors *in vitro*. When total thymocytes and spleen cells were cultured in the presence of the anti-CD3 mAb, Con A or PMA/ionomycin, the PD-1 antigen expression was markedly induced (Fig. 5) whereas neither *in vitro* incubation alone nor the presence of DEX (data not shown) affected PD-1 expression at all. $PD\text{-}1^+$ T cells recovered 48 h after stimulation of spleen cells with the anti-CD3 mAb co-expressed activation markers such as CD25 and CD69, and were larger in size than $PD\text{-}1^-$ cells (Fig. 6). Since PMA/ionomycin, which can stimulate both T and B cells, induced PD-1 antigen expression on all the spleen cells within 24 h (Fig. 5), we suspected that the PD-1 antigen might be induced on B cells stimulated through surface Ig. Indeed both anti-IgM-stimulated and PMA/ionomycin-stimulated spleen cells contained $PD\text{-}1^+$ lympho-

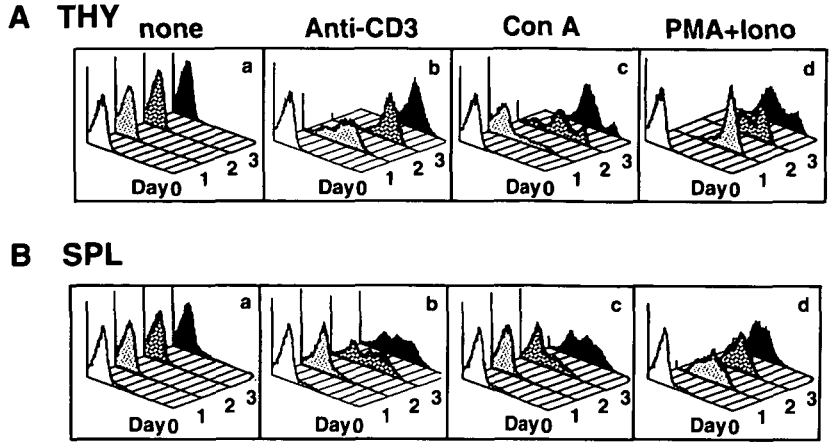

Fig. 5. Induction of PD-1 antigen expression on *in vitro*-stimulated thymocytes and spleen cells. Thymocytes (A) and spleen cells (B) were prepared from normal C57BL/6 mice and cultured in medium alone (a), in the presence of the anti-CD3 mAb (b), Con A (c) or PMA plus ionomycin (d). Cells were collected at indicated time points and stained with the biotinylated J43 mAb, followed by PE–SA. These results are representative of five experiments.

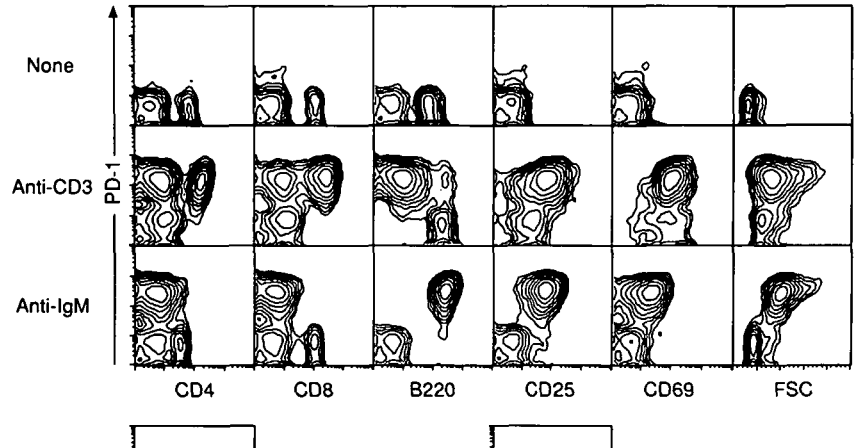

Fig. 6. PD-1 antigen expression on spleen cells stimulated *in vitro*. Spleen cells were freshly prepared from normal C57BL/6 mice (None) and then cultured in the presence of the anti-CD3 mAb, anti-IgM antibody, PMA plus ionomycin, or LPS. Cells were collected at 48 h (anti-CD3 and anti-IgM) or at 24 h (PMA/ionomycin and LPS) after stimulation, and stained with the biotinylated J43 mAb together with FITC-labeled mAb indicated, followed by PE–SA. These results are representative of five experiments. FSC, forward scatter.

cytes co-expressing a B cell marker B220 (Fig. 6). PD-1$^+$ B cells stimulated with anti-IgM antibody also co-expressed activation markers CD25 and CD69, and were larger in size than PD-1$^-$ cells. By contrast, LPS stimulation did not induce expression of the PD-1 antigen. These *in vitro* results indicate that the PD-1 antigen is induced in association with stimulation of T and B lymphocytes through antigen receptors.

Discussion

In this report, we have described the production of the hamster J43 mAb against the PD-1 gene product and characterization of the antigen identified by this mAb. We first provided the evidence for the cell surface expression of the PD-1 antigen on several PD-1 cDNA transfectants as well as cultured cell lines. We could detect only marginal expression of the PD-1 antigen on normal murine lymphocytes. However, we found the induced expression of the PD-1 antigen on T and B lymphocytes activated by stimulation through antigen receptors. A non-physiological stimulation with PMA/ionomycin may mimic the antigen receptor stimulation by inducing protein kinase C activation and Ca^{2+} influx.

The PD-1 antigen immunoprecipitated with the J43 mAb was found to have a molecular weight of 50–55 kDa under reducing conditions, whereas the molecular weight calculated from its amino acid sequence is 29,310, suggesting that the

PD-1 antigen is heavily glycosylated. This is consistent with the notion that PD-1 has four potential *N*-glycosylation sites (17). Furthermore, the apparent size of PD-1 did not change under non-reducing conditions (data not shown), suggesting that PD-1 is expressed as a monomer.

2B4.11 hybridoma, which was used to clone the PD-1 cDNA, expresses a considerable amount of the PD-1 antigen without any stimulation. The several PD-1 cDNA transfectants can grow as vigorously as their parental cells despite the strong expression of the PD-1 antigen. The PD-1 antigen was shown to be induced on the CD4 SP, CD8 SP and $CD4^-CD8^-$ DN thymocytes as well as on distinct T cell populations in spleen and lymph node cells at a later stage after the *in vivo* anti-CD3 mAb injection. These results suggest that the PD-1 antigen expression alone may not trigger a death-inducing signal. However, PD-1 may be involved in regulation of some steps of apoptosis and cell growth when a ligand is provided in a similar way as described for the Fas–Fas ligand system (39). We thus examined the effect of cross-linking of the PD-1 antigen by the J43 mAb on growth of 2B4.11 cells. Cross-linking of the PD-1 antigen alone did not show either inhibition or promotion of cell growth (data not shown). Furthermore, PD-1 cross-linking with the J43 mAb did neither enhance nor abrogate the growth suppression of 2B4.11 induced by CD3 cross-linking (data not shown). However, the failure of the PD-1 antigen to transduce the apoptotic signal might be due to the inability of the J43 mAb to cross-link the PD-1 antigen appropriately because of a distinct epitope or a low binding affinity as described for the anti-EA-1 mAb (40,41).

Apoptosis of the growth factor dependent cell line LyD9 by factor deprivation and of DEX-stimulated thymocytes could be induced without significant expression of the PD-1 antigen, suggesting that PD-1 may not be involved in the common pathway of apoptosis. In the case of thymocytes stimulated *in vivo* with the anti-CD3 mAb, however, it remains possible that the apoptotic thymocytes are cleared by thymic macrophages so rapidly and so efficiently (42) that we could not detect any significant population of PD-1-expressing cells within 24 h. This possibility seems to be intriguing because the PD-1 antigen was indeed induced in a considerable population of *in vitro* anti-CD3 mAb-stimulated CD4/CD8 DP thymocytes (data not shown), which have been reported to be the most apoptosis-sensitive population (43). Although we could not provide the evidence to indicate the direct correlation of the PD-1 antigen expression with induction of apoptosis, the above possibility needs to be further examined.

It is extremely interesting that the PD-1 antigen is induced on both T and B cells by stimulation through their antigen receptors. Antigen receptor cross-linking results in not only activation but also cell death (18,19). The Fas antigen that is similarly induced on stimulated T and B lymphocytes has been shown to be involved in cell death associated with peripheral lymphocyte selection (39). We thus examined the effect of cross-linking of the PD-1 antigen by the J43 mAb on proliferative responses of thymocytes and spleen cells in culture. The J43 mAb alone did not induce proliferation of thymocytes and spleen cells, and the presence of the J43 mAb did neither enhance nor inhibit the proliferative responses of these cells induced by the anti-CD3 mAb, Con A or PMA plus ionomycin (data not shown). However, there still remains the possibility that another mAb against the PD-1 antigen might strongly cross-link the antigen and subsequently modulate activation and proliferation of T and B cells.

PD-1 might be involved in some effector functions of T cells such as a helper or cytotoxic function rather than in a primary T cell proliferative response. PD-1 might also play some roles in the development of thymocytes, since we observed that 30–50% of the $CD4^-CD8^-$ DN thymocytes of adult mice expressed PD-1 and fetal thymocytes expressed PD-1 in the transitional phase from the $CD44^-CD25^+$ DN to the $CD44^-CD25^-$ DN stage (44). The anti-PD-1 mAb which we have described here will facilitate further investigation on possible functions of the PD-1 antigen by combination with transgenic and gene-disrupted mice which we have generated recently.

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Abbreviations

Con A	concanavalin A
DEX	dexamethasone
DN	double negative
DP	double positive
LPS	lipopolysaccharide
PE	phycoerythrin
PMA	phorbol 12-myristate-13-acetate
SA	streptavidin
SP	single positive

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