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Expression Analysis of the Ligand to Ly-6E.1 Mouse Hematopoietic Stem Cell Antigen

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Ly-6E.1 antigen was proposed as a regulatory molecule of T lymphocyte activation, a hematopoietic stem cell marker, a memory cell marker, and an adhesion molecule. Though there were several reports suggesting the presence of Ly-6 ligand, the characterization of the ligand was not yet performed. As an attempt to screen the expression of Ly-6E.1 ligand, we prepared a probe for detecting Ly-6E.1 ligand by producing a fusion protein between Ly-6E.1 and hIgC$_{\gamma 1}$. A mammalian cell expression vector with Ly-6E.1/hIgC$_{\gamma 1}$ chimeric cDNA was transfected in SP2/0-Ag14 myeloma cells, and stable transfectants were selected. The fusion protein was produced as a dimer and maintained the epitopes for monoclonal antibodies specific for Ly-6E.1 and for anti-human IgG antibody. The purified fusion protein through Gammabind G column was used for FACS analyses for the expression of Ly-6E.1 ligand. The fusion protein interacted with several cell lines originating from B cells, T cells, or monocytes. The fusion protein also strongly stained bone marrow, lymph node, and spleen cells, but thymic cells weakly, if any. The staining was more obvious in C57BL/6 (Ly-6^b) than Balb/c (Ly-6^a) mice. These results suggest that the interaction of Ly-6E.1 with Ly-6E.1 ligand may function both in the stem cell environment and in the activation of mature lymphocytes. The fusion protein may be a valuable tool in characterization of biochemical properties of the Ly-6E.1 ligand and, further, in isolating its cDNA.

The Ly-6 locus, a large multigene family on chromosome 15 of mice, encodes at least 18 closely linked homologous genes and pseudogenes (LeClair et al., 1987; Kamiura et al., 1992). Ly-6 antigen was first identified as an alloantigen differentially expressed on the surface of hematopoietic stem cells, lymphocytes, monocytes, granulocytes, and of other non-hematopoietic tissues (Kimura et al., 1984; Reiser et al., 1988; Spangrude et al., 1988a; van der Rijn et al., 1989; Codias and Malek, 1993). The genes belonging to the Ly-6 family, Ly-6A.2 (Palfree et al., 1987; Reiser et al., 1988), C.1 (Bothwell et al., 1988), C.2 (Palfree et al., 1988), E.1 (LeClair et al., 1986), F.1 (Fleming et al., 1993), G.1 (Fleming et al., 1993), ThB (Gumley et al., 1992), and Sca-2 (TSA-1) (MacNeil et al., 1993) were cloned, but functions of these genes were largely unknown.

Ly-6E.1 gene is an alternate allele of Ly-6A.2, resulting in two amino acid differences in the 15 to 20 kDa glycosyl phosphatidylinositol-linked glycoprotein products (Palfree and Hammering, 1986; Reiser et al., 1988; Fleming et al., 1993). Ly-6E.1 is expressed on pluripotent hematopoietic stem cells so that it is regarded as an earliest stem cell marker (Spangrude et al., 1988b; van der Rijn et al., 1989). Ly-6E.1 antigen continues to be expressed on pro-thymocytes and double negative ($CD4^-CD8^-$) progenitor thymocytes, but is absent on double positive thymocytes (Yeh et al., 1986; Spangrude et al., 1988b). The expression of Ly-6E.1 is recovered on mature single positive cells later and were induced more in activated T lymphocytes (Spangrude et al., 1988a; Yeh et al., 1986). Interferons are strong inducers for the expression of Ly-6E.1 antigen on T, B lymphocytes and monocyte/macrophages (Dumont et al., 1986, Dumont and Boltz, 1987; LeClair et al., 1989; Malek et al., 1989).

Several studies indicate that Ly-6A/E is involved in the regulation of T cell activation (Malek et al., 1986; Ortega et al., 1986; Rock et al., 1986). Cross-linking Ly-6A/E with specific monoclonal antibody usually stimulates T cells, whereas under some conditions it inhibits reponses (Flood et al., 1985; Codias et al., 1990; Fleming and Malek, 1994). Antisense oligonucleotide suppressing Ly-6A.2 expression blocks T cell activation (Flood et al., 1990). Such signaling of T cell activation is dependent on T cell receptor

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(TcR) expression (Bamezai et al., 1988; Sussman et al., 1988; Yeh et al., 1988).

During thymocyte development, the interactions of accessory molecules with their ligands as well as the T cell receptor with peptide antigen/MHC molecules on antigen presenting cells may function as pivotal roles in positive and negative selection of thymocytes. The incoming thymocytes from bone marrows to thymic medulla migrate to the cortex area during maturation then back to medulla as mature T lymphocytes. The functionally mature T cells then travel to secondary lymphoid organ and constitute immunological integration of the organs. We do not fully understand which accessory molecules are involved in the thymocyte development. Since the enriched population of Thy-1^{low}Lin$^-$Sca-1^+ cells from bone marrow was found to be able to reconstitute all blood cells (Spangrude et al., 1988b), the interaction of accessory molecules with putative corresponding ligands may be critical for maintaining the self renewal and the differentiation properties.

Ly-6A.2 antigen plays a role in thymocyte development, possibly by interaction with an unknown ligand (Bamezai et al., 1995). The constitutive overexpression of Ly-6A.2 *in vivo* leads to a marked impairment in the generation of mature thymocytes. Thymocyte development was arrested at $CD4^-$ $CD8^-$ stage when Ly-6A.2 expression is normally turned off. Thymocytes from the transgenic mice aggregate in culture showing that a putative Ly-6A.2 ligand is expressed on thymocytes (Bamezai and Rock, 1995). The presence of Ly-6C ligand on fibroblast cells was shown by Johnson et al. (1993), which may be recognized by Ly-6C molecules on cytotoxic T lymphocyte (CTL). However, there was no direct report identifying the Ly-6 ligand.

Recently, numerous studies have demonstrated the usefulness of recombinant fusion protein of lymphocyte accessory molecule with Fc portion of Ig in identifying the corresponding ligand, or in studying receptor-ligand interactions (Aruffo et al., 1990; Byrn et al., 1990; Linsley et al., 1991a, b). We report here that the fusion protein of Ly-6E.1 and human $IgC_{\gamma 1}$ was successfully generated, and used to analyze the expression of Ly-6E.1 ligand on the cells of various lymphoid organs.

Materials and Methods

Monoclonal antibodies and cell culture

Murine monoclonal antibodies, Sca-1 (rat IgG2a), D7 (rat IgG) and SK70.94 (mouse IgG), have been described previously (Kimura et al., 1984; Malek et al., 1986; Spangrude et al, 1988a), and were purified from ascites before use. FITC- or AP-conjugated secondary antibody was purchased from Sigma. All cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Cansera), 50 μM 2-mercaptoethanol, 50 U/ml penicillin, and 50 U/ml streptomycin (Gibco-BRL).

Polymerase chain reaction (PCR)

cDNA fragments were amplified by PCR, using primer pairs described below. PCR reactions (100 μl volume) were run in *Taq* polymerase buffer (Stratagene), containing 20 μmol each dNTP; 50-100 pmol of primers; 1 ng plasmid template encoding cDNAs in pUC19; and *Taq* polymerase. Reactions were run on a FTC2000 thermocycler (Daehan Medical Co.) for 35 cycles (a typical cycle consisted of steps of 5 sec at 94℃, 10 sec at 55℃, and 15 sec at 72℃).

Construction of expression vector

cDNAs encoding Ly-6E.1 (LeClair et al., 1986) and hIgG1 heavy chain (Ellison et al., 1982) have been described previously. A genetic fusion encoding Ly-6E.1 and $hIgC_{\gamma 1}$ cDNAs was made in pNeoSR αII vector. For Ly-6E.1 cDNA amplification, the oligonucleotide 5'-CGCGTCGACTGAGGATGGACACT-3' (corresponding to *Sal*I restriction sequence and NH_2-terminal four amino acids of Ly-6E. 1 leader) was used as a forward primer, and 5'-CGCGGATCCACG CGGAACCAGATTGCAGAGGTCTTC-3' (corresponding to C-terminal four amino acids of mature form Ly-6E.1, thrombin recognition amino acid sequence, and *Bam*HI restriction sequence) was used as a reverse primer. The amplified fragment was phosphorylated and subcloned into *Hind*III/*Sma*I-digested pBluescript II KS(+) (pBLS) by blunt end ligation. For $hIgC_{\gamma 1}$ amplification, the oligonucleotide 5'-CGCGGAT CCGAGCCCAAATCTTGT-3' (corresponding to *Bam* HI restriction site and four amino acids of hinge region) was used as a forward primer, and 5'-CG CTCTAGATCATTTACCCGGAGA-3' (corresponding to C-terminal four amino acids of $hIgC_{\gamma 1}$ and *Xba*I site) was used as a reverse primer. The amplified $hIgC_{\gamma 1}$ cDNA fragment was subcloned into *EcoR*V- digested pBLS vector. The *Sal*I/*Bam*HI fragment from pBLS-Ly-6E.1 plasmid and the *Bam*HI/*Apa*I fragment of pBLS-$hIgC_{\gamma 1}$ plasmid were ligated into *Sal*I/*Apa*I digested pBLS (see Fig. 1). Each PCR-amplified cDNA was sequenced before chimeric cDNA construction. A point mutation was found at 882 nucleotide in $hIgC_{\gamma 1}$ cDNA, but was silent. The *Sal*I/*Xho*I fragment from resultant chimeric cDNA in pBLS was subcloned into *Xho*I site of pNeoSRαII expression vector. The resulting construct (Ly-6E.1/$hIgC_{\gamma 1}$) encodes a protein comprising residues 1-79 of Ly-6E.1, 6 amino acids of thrombin target sequence, and followed by the hinge region/CH2/CH3 of $hIgC_{\gamma 1}$.

Transfection, selection, and screening

SP2/O-Ag14 cells were transfected with pNeoSRαII

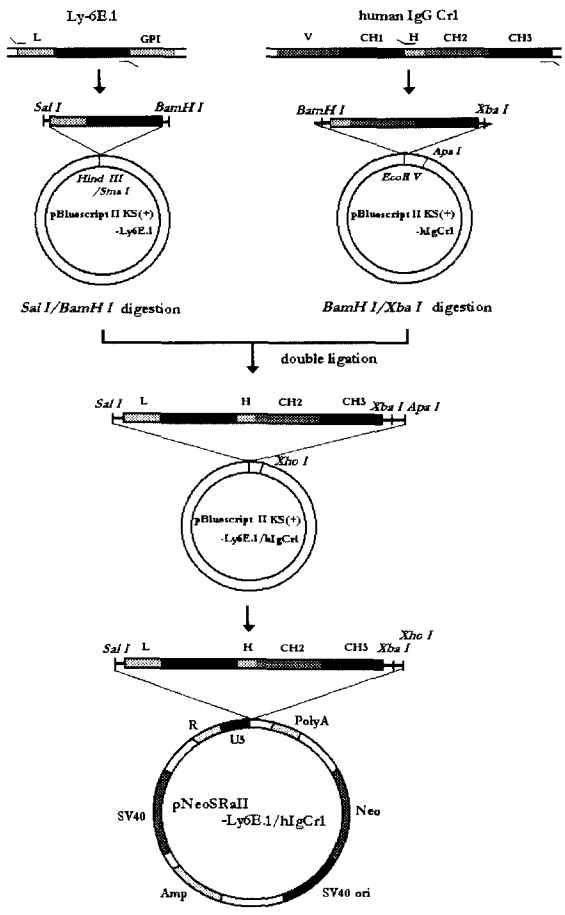

Fig. 1. Diagrammatic representation of the construction of expression vector of Ly-6E.1 and hIgC$_{\gamma 1}$ chimeric cDNA.

vector only or pNeoSRαII subcloned with Ly-6E.1/hIgG chimeric cDNA, and stable transfectants expressing Ly-6E.1/hIgC$_{\gamma 1}$ fusion protein were isolated.

For electroporation, SP2/0-Ag14 cells were harvested and washed twice with ice-cold PBS and resuspended at a concentration 2×10^7 cells per 0.8 ml in PBS. After mixing with 10 μg of linearized plasmid DNA in a cuvette, the cell-DNA mixture was incubated on ice for 10 min, then electric shock (250 V, 960 μFD) was delivered (Bio-rad). The cuvette was immediately incubated on ice for 10 min further. Cell were then distributed in 96 well plate at a concentration of $2\text{-}5 \times 10^4$ cells per well. After 48 h, 1 g/L G418 was added to each well. Two or three weeks later, drug-resistant colonies were visible. The supernatant from each clone was screened for the secretion of Ly-6E.1/hIgC$_{\gamma 1}$ fusion protein by Western dot blot.

Western blot and Western dot blot

The supernatant from each clone was subjected to SDS-PAGE using Laemmli method (Laemmli, 1970). The proteins were then transferred onto Immobilon-P membrane (Millipore) by electrophoretic transfer at 40 V for 2 h. The membrane was then blocked with 4% BSA in TBS solution (10 mM Tris, pH 7.0, 150 mM NaCl) at room temperature for 1 h. After washing with TTBS (0.5% Tween-20, 10 mM Tris, pH 7.0, 150 mM NaCl) three times, the membrane was incubated with SK70.94, and followed by AP-conjugated goat anti-mouse IgG antibody to detect Ly-6E.1 epitope. To detect epitope(s) on hIgC$_{\gamma 1}$, the duplicated membrane was incubated with AP-conjugated goat anti-human IgG antibody. Substrate solution (BCIP/NBT) was added and the reaction was stopped by adding 10 mM EDTA/PBS.

For dot blotting, Immobilon-P membrane was pre-wetted with methanol and washed in KP buffer (25 mM KH_2PO_4, 25 mM K_2HPO_4, pH 4.0). Culture supernatant was then bound using HYBRI-SLOT manifold (Gibco-BRL) onto the membrane. After air-drying briefly, the paper was immersed in 4% BSA/TBS for 1 h at room temperature. Following procedures were same as in Western blot.

Purification of fusion protein

Purification of secreted fusion protein was performed using a modification of the protocol of Linsley (1991a). Spent culture media from one of best clone (IIE7) were used as source for the purification of Ly-6E.1/hIgC$_{\gamma 1}$ fusion protein. Gammabind G-sepharose column (Pharmacia) was stripped with a buffer (1 M glycine-HCl, pH 2.7, 1 M NaCl) and equilibrated with a running buffer (20 mM sodium phosphate, pH 7.0). After removal of cellular debris by low speed centrifugation and filtration through 0.45 μm filter paper, the medium was applied to the column. After washing with the running buffer extensively, bound protein was eluted with 0.1 M glycine- HCl, pH 2.7. Fractions were collected and neutralized immediately with 1 M Tris, pH 9.0. For quantitation of purified fusion protein, absorbance at 280 nm was measured and double checked by Lowry method using BSA as standard (Pierce). The fractions containing the fusion protein were pooled and dialyzed against PBS before use.

FACS analysis

Cells ($1X10^6$ cells) were harvested and washed twice with ice-cold staining buffer (1% fetal bovine serum, 0.02% NaN_3 in PBS). After incubation with the purified fusion protein for 30 min, cells were washed twice with the staining buffer. After incubation with FITC-conjugated F(ab')$_2$ fragment of goat anti-human IgG antibody, cells were washed twice with the staining buffer and once with PBS, then fixed in 1% paraformaldehyde/PBS. Quantitation of cell surface staining was performed using a Becton Dickson FACStar. In most cases, list mode files of 10,000

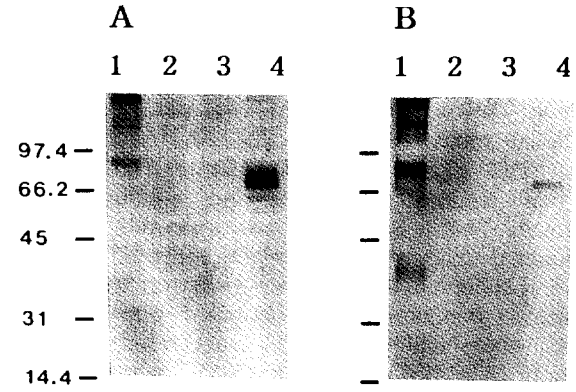

Fig. 2. Analysis of secreted Ly-6E.1/hIgC$_{\gamma 1}$ fusion protein by Western blot. Detection of fusion protein was performed with anti-Ly-6E.1 antibody (SK70.94) and AP-conjugated goat anti-mouse IgG antibody (A), or AP-conjugated goat anti-human IgG antibody directly (B). Lane 1 is normal human serum, lane 2 is culture supernatant of SP2/0-Ag14, lane 3 is culture supernatant of clone transfected with pNeoSR αII vector only, lane 4 is culture supernatant from IIE7 clone.

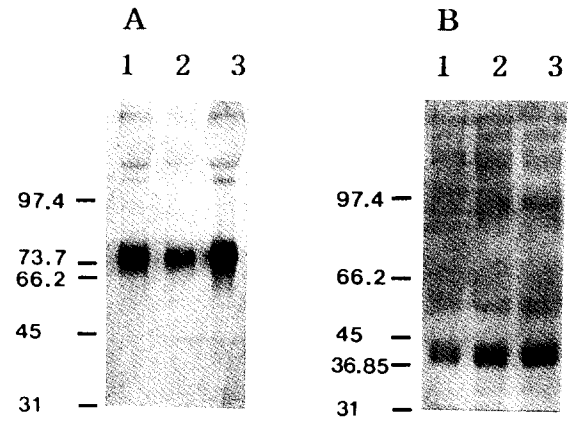

Fig. 3. Analysis of purified fusion protein through Gammabind G column by Western blot. The purified fusion protein of three different preparations was subjected to SDS-PAGE under non-reduced condition (A) and reduced condition (B), and transferred onto Immobilon-P membrane. The fusion protein was detected by incubation with SK70.94 and AP-conjugated goat anti-mouse IgG antibody.

viable cells were collected for analysis.

Results

Expression of soluble Ly-6E.1/hIgCγ1 fusion protein

To make a soluble fusion protein of Ly-6E.1 and hIgC$_{\gamma 1}$, a chimeric cDNA expression vector encoding Ly-6E.1, thrombin target sequence, and hIgC$_{\gamma 1}$ hinge /CH2/CH3 domain sequence was constructed (Fig. 1). The original Ly-6E.1 cDNA encodes 26 leader and 108 structural amino acids. Because the carboxy-terminal 29 amino acids are cleaved off during glycosyl phosphatidylinositol-linkage formation after translation (Shevach and Korty, 1989), the chimeric cDNA was designed to encode the 105 amino acids of Ly-6E.1 including leader. To isolate the Ly-6E.1 protein for structural study, primers were designed for PCR to contain 20 extra nucleotides coding thrombin target sequence (LVPRGS) between Ly-6E.1 and hIgC$_{\gamma 1}$ hinge region. The hIgC$_{\gamma 1}$ hinge region contains three cysteine residues, one is for interdisulfide bond formation with light chain, the other two are for interdisulfide bonds with other heavy chain (Ellison et al., 1982). The chimeric cDNA was subcloned into the pNeoSRαII eukaryotic expression vector, and transfected into SP2/O-Ag14 myeloma cell line. Transfected cells were selected in the G418-containing medium, and the drug-resistant clones were screened for secretion of proper fusion protein using Western dot blot analysis with culture supernatant from each clone. The Ly-6E.1/hIgC$_{\gamma 1}$ fusion protein from a clone (IIE7) migrated predominantly as a 73 kDa protein in SDS-PAGE under non-reducing condition, and 36 kDa under reducing condition, indicating that it is expressed as a dimer (Fig. 2).

Yields of fusion protein purified through affinity chromatography on Gammabind G-sepharose was estimated to approximately 100 µg/liter of spent culture medium. The purified protein maintained the epitopes for SK70.94 antibody, and for goat anti-human IgG antibody (Fig. 3). The Ly-6E.1 specific antibodies, D7 and Sca-1, also recognized the fusion protein in Western blot (data not shown). Because there are 10 cysteine residues in Ly-6E.1 involved in intradisulfide bond formation, deterioration of Ly-6E.1 structure in fusion protein was possible (Shevach and Korty, 1989). However, the recognition of purified fusion protein by various anti-Ly-6E.1 antibodies and goat anti-human IgG antibody indicates that the cysteine residues in Ly-6E.1 and IgG hinge region take part in proper inter- or intra-disulfide bond formation, and the affinity purification procedures does not disrupt general structure of each domain in the fusion protein.

Analysis of Ly-6E.1 ligand expression on cell lines

To investigate the expression of Ly-6E.1 ligand, various cell lines were stained with the purified fusion protein (Fig. 4, summarized in Table 1). Among the various cell lines, A20 (B cell, Balb/c), J774A.1 (monocyte, Balb/c), RAW264.7 (monocyte, Balb/c), P815 (mastocytoma, DBA/2) and EL-4 (T cell, C57BL/6) cell lines were stained with the fusion protein, but BW5147 (T cell, C57BL/6), S49 (T cell, Balb/c), Yac-1 (T cell, As/Sn) and P388D1 (monocyte, DBA/2) were not. The positive staining of EL-4 and P815, which are strains originating from the Ly-6b allotype mice, suggests that Ly-6E.1 interacts with Ly-6A.2 ligand as well as the Ly-6E.1 ligand regardless of the allotype. Furthermore, Ly-6E.1 igand was detected on B cell, T cells and monocytes. When normal ouse antibody was pretreated as blocker to remove the possibility of the interaction between fusion protein and Fc receptors on cells,

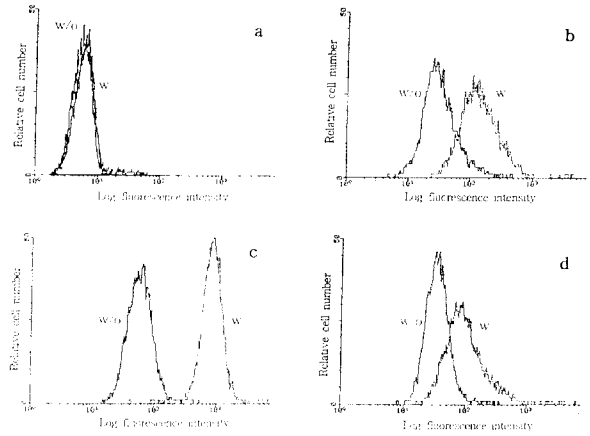

Fig. 4. Expression of Ly-6E.1 ligand on cell lines. BW5147 (a), A20 (b), J774A.1 (c), and EL-4 (d) cells were incubated with medium only (w/o marked) or purified fusion protein (w marked) followed by FITC-conjugated $F(ab')_2$ fragment of goat anti-human IgG antibody as secondary antibody.

the staining pattern did not change (data not shown). Since FITC-conjugated $F(ab')_2$ fragment of goat anti-human IgG antibody was used as secondary antibody, these positive staining were not caused by Fc receptor-goat antibody interaction, but by interaction specific to the Ly-6E.1 ligand.

Analysis of Ly-6E.1 ligand in lymphoid tissues

We next investigated the expression of Ly-6E.1 ligand on the cells from various lymphoid tissues from Balb/c (Ly-6^a allotype) and C57BL/6 (Ly-6^b allotype) mice. Cell suspensions from thymus, bone marrow, lymph node, and spleen were analyzed with fusion protein (Fig. 5). For FACS analysis, both lymphoid cells and non-lymphoid cells were scored. Cells from bone marrow were stained best, but those from thymus were stained least. These results envisage that the cells from bone marrow, in which most stem cells (Ly-6E.1 positive) are populated, express Ly-6E.1 ligand best. This phenomenon was more obvious in C57BL/6. The least expression in thymic cells suggests that the interaction of Ly-6E.1 ligand and Ly-6E.1 may not be prominent during thymocyte maturation, or that the number of cells expressing Ly-6E.1 ligand is few. The positive staining of cells from spleen and lymph node may reflect the functional involvement of Ly-6E.1 ligand in lymphoid cell activation, because activated cells usually express Ly-6E.1.

The expression of Ly-6E.1 ligand on various cell lines as well as on the cells from primary and secondary lymphoid organs suggests that the interaction of Ly-6E.1 ligand with Ly-6E.1 may function both in the stem cell environment and in the activation environment of mature lymphoid cells.

Discussion

A lot of studies were made on the immunological functions of Ly-6 antigens (reviewed in Rock et al., 1989; Shevach and Korty, 1989; Gumley et al., 1995). Originally, the TAP molecule, which was named by its function, is apparently involved in T cell activation (Ortega et al., 1980; Rock et al., 1986; Malek et al., 1986). Antibody-cross linking experiments, which also sometimes generates negative signals to T cells, suggested a corresponding ligand on antigen presenting cells (Flood et al., 1985; Codias et al, 1990). Also the blockage of CTL function by treatment of target cells with monoclonal antibody specific for the Ly-6C strongly argues the presence of a recognition apparatus on CTL (Johnson et al., 1993). Furthermore, T cells or B cells expressing forcibly Ly-6A.2 in transgenic mice showed

Table 1. Expression of Ly-6E.1 ligand on various cell lines

Name	Type	Origin	Ly-6 allotype	Reactivity*
A20	B	Balb/c	a	++
J774A.1	Macrophage	Balb/c	a	++
RAW 264.7	Monocyte	Balb/c	a	++
2C	CTL	Balb.B(H-2^b)	a	+
S49	T	Balb/c	a	-
3T3	Fibroblast	Balb/c	a	-
WEHI-3	Mast cell	Balb/c	a	-
LM	Connective	C3H/An	a	-
YAC-1	T	A/Sn	a	-
EL-4	T	C57BL/6	b	++
P815	Mastocytoma	DBA/2	b	++
BW5147	T	C57BL/6	b	-
D10	Th2	AKR/J	b	-
PD31	B	C57BL/6	b	-
P388D1	Mo	DBA/2	b	-
CH1	B	B10.H-2^aH-4^bp/Wts	b	-
CH27	B	B10.H-2^aH-4^bp/Wts	b	-
CTLL-2	CTL	C57BL/6	b	-

*FACS analyses were performed as described in Materials and Methods with the purified fusion protein. The staining profile with only the secondary antibody (FITC-conjugated goat anti-human IgG antibody) was compared with fusion protein followed by the secondary antibody. ++ indicates more than 5 scale shift in mean log fluorescence intensity; +, 2-5 scale shift; -, no significant shift.

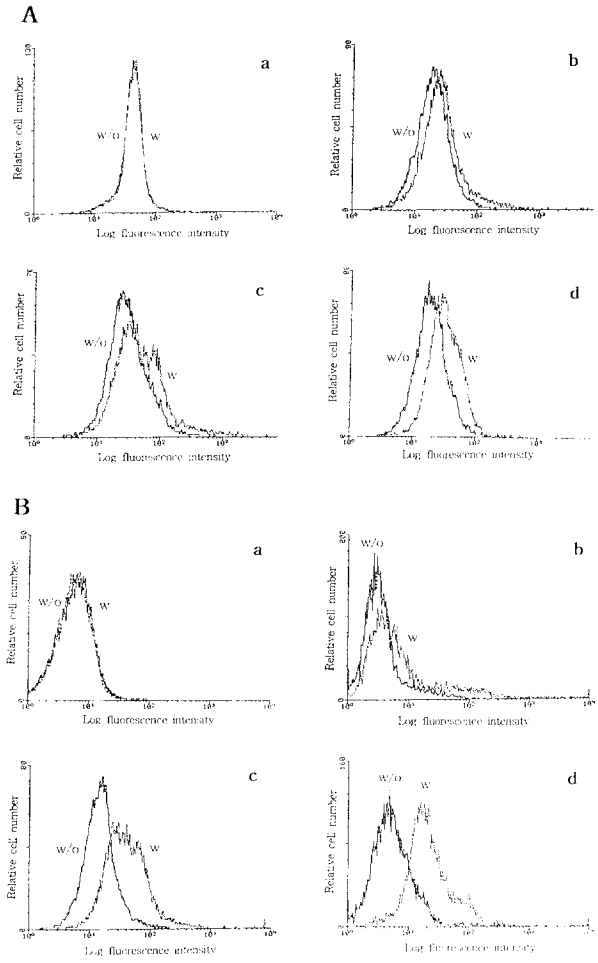

Fig. 5. Expression of Ly-6E.1 ligand on cells of thymus, lymph node, bone marrow and spleen cells. Cell suspensions from each tissue of female Balb/c (A) or C57BL/6 (B) mouse were prepared; thymus (a), lymph node (b), spleen (c), and bone marrow (d). Cells were incubated with purified fusion protein followed by FITC-conjugated $F(ab')_2$ fragment of goat anti-human IgG antibody.

homotypic aggregation *in vitro* (Bamezai and Rock, 1995). The constitutive overexpression of Ly-6A.2 *in vivo* leads to a marked impairment in the generation of thymocytes (Bamezai et al., 1995). Thymocyte development was arrested at the time in thymic development when Ly-6A.2 expression is normally extinguished. The negative signaling event by Sca-2 (TSA-1) on thymocyte apoptosis induced by anti-TcR antibody may be the functional opposite of Ly-6A.2 in thymus (Noda et al., 1996). These results indicate the presence of a putative ligand on T cells, B cells and target cells.

In this study, the fusion protein of Ly-6E.1 and human IgG Fc portion has been generated to use as a probe to Ly-6E.1 ligand and to screen Ly-6E.1 ligand expression. The fusion protein interacted with several cell lines of hematopoietic origin, and cells from several lymphoid organs. We made several new observations on the Ly-6E.1 ligand as follows.

First, Ly-6E.1/hIgC$_{\gamma 1}$ interacts with T cells, B cells, or monocytes, suggesting the presence of the Ly-6E.1 ligand on these cells. This result corroborates previous results that a putative ligand may be present on T cells and B cells (Bamezai et al., 1995), or antigen presenting cells (Johnson et al., 1993; Izon et al., 1996), though expression analysis results with established cell lines can not be directly extrapolated to natural cells *in vivo*.

Second, Ly-6E.1/hIgC$_{\gamma 1}$ recognizes a putative ligand on the cells regardless of Ly-6 allotype. It is natural that allele has same function but contains different epitopes on the molecule. Though there were difference in expression extent between Ly-6E.1 and Ly-6A.2 in a and b allotypic mice, respectively, they were always co-segregated in functional studies (Malek et al., 1986; Ortega et al., 1986; Rock et al., 1986). The Ly-6E.1 seems to interact with the ligand to Ly-6A.2, though we can not rule out the possibility that alleles of the ligand are also present for each allotype. Furthermore, the expression of Ly-6E.1 ligand is much higher in C57BL/6 (Ly-6^b) than in Balb/c (Ly-6^a). This fact may reflect that Ly-6A.2 expression in Ly-6^b allotype mice is much higher than that of Ly-6E.1 in Ly-6^a allotype mice (Kimura et al., 1984; Ortega et al., 1986).

Third, staining of the cells from various lymphoid organs with fusion protein indicates that the bone marrow cells express the most Ly-6E.1 ligand. The fact that Sca-1$^+$ cells are most prominent in bone marrow cells (Spangrude et al., 1988b) strengthens the interpretation that the environmental cells around the Ly-6E.1 positive cells may express more Ly-6E.1 ligands.

Our results seem not to reconcile with those of Bamezai et al. (1995) that suggested the presence of the ligand on thymocytes. Our FACS staining of thymic cells with the fusion protein failed to detect ligand expressing cells. Since the Ly-6E.1 is expressed only before double negative stage, which constitutes 0.5% of total thymocyte, it is possible that the cells expressing the Ly-6E.1 ligand will be too few to detect by FACS analysis. Immunohistochemistry is planned to visualize the location of cells expressing Ly-6E.1 ligand in each lymphoid organ.

Shevach and Korty (1994) reported that Ly-6A.2/IgG fusion protein binds to IgM for T cell-B cell interaction, and to an unknown Ly-6 ligand. However, the staining of IgM-positive CH1 and CH27 cell lines with Ly-6E.1/hIgC$_{\gamma 1}$ was negative in our experiment. More extensive analysis of lymphoid organ cells with known marker antigens will give a clear picture of the expression of Ly-6E.1 ligand. The cDNA cloning by library screening and the identification of Ly-6E.1 ligand protein by immunoprecipitation or Western blotting with Ly-6E.1 ligand-positive cell lines is now in progress.

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