age, frequently involving the skin, bone, or lymph nodes. Owing to the rareness of B-LBL and its morphological and immunophenotypic similarities to mature B-cell lymphomas in some cases, distinguishing between these diseases is of great importance, especially in the field of pediatric oncology, because the treatment strategies for these two diseases are quite different. In addition, other tumors, including precursor T-cell lymphoblastic lymphoma (T-LBL), extramedullary myeloid tumors, and Ewing sarcoma, must also be included in a differential diagnosis of B-LBL.

In an attempt to characterize B-LBL using the expression of Ig-related molecules and to examine the utility of such a method for diagnosis, we examined CD179a/b expression in B-LBL tissues using immunohistochemistry. CD179a/b was found to be specifically expressed in B-LBL, but not in mature B-cell lymphomas and other tumors in childhood. The usefulness of CD179a/b as diagnostic markers for B-LBL is discussed.

Materials and methods

Materials

The human pre-B-cell line HPB-NULL[10] and the Burkitt cell line Ramos (Japanese Cancer Research Resources Bank, Tokyo, Japan) were used in this study. Cells were maintained in RPMI1640 supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere.

Biopsy specimens from pediatric patients, including 11 patients with B-LBL, seven patients with Burkitt lymphoma, three patients with diffuse large B-cell lymphoma, seven patients with T-LBL, three patients with extramedullary myeloid tumors, and three patients with Ewing sarcoma, were selected from medical files collected between 1985 and 2003 at the Department of Developmental Biology, National Research Institute for Child Health and Development. In each case, the initial diagnosis was based on morphological observations of hematoxylin and eosin (H&E)-stained, formalin-fixed, paraffin-embedded tissues, the immunophenotypic characteristics revealed by immunohistochemistry using acetone-fixed, fresh frozen sections, and the patient's clinical features. In some cases, immunophenotyping was also performed using flow cytometric analysis of a single-cell suspension prepared from the tissue. To examine CD179a/b expression, snap-frozen tissues in OCT compounds stored at −85°C after the initial diagnosis were used.

The following mouse monoclonal antibodies (mAbs) were used in this study: anti-CD179a (HSL96), anti-CD179b (HSL11), anti-conformational pre-BCR (HSL2),[10] anti-CD20 (L26),[13] anti-HLA-DR,[14] and anti-CD10 (IF6).[15] HSL2 is a unique mAb that does not bind to each component of the pre-BCR, but recognizes a conformational epitope formed only when the μ HC and CD179a/b surrogate LC associate with each other to make the pre-BCR complex.[10] In addition, several commercially available mAbs were also used: anti-μ (G20-127), anti-CD179a (VpreB8 and VpreB9), and anti-CD19 (Leu12) from BD Pharmingen (San Diego, CA, USA); anti-κ (HP6053) and anti-λ (HP6054) from Zymed Laboratories Inc. (San Francisco, CA, USA); anti-CD79a (HM-57), anti-CD22 (4KB128), and anti-TdT (HT-1/3/4) from DAKO (Glostrup, Denmark); anti-CD179a (4G7) from Coulter/Immunotech Inc. (Westbrook, MA, USA); anti-TdT (SEN28) from Nichirei Co. (Tokyo, Japan); and anti-CD179a (B-MAD-688) from Biocarta (San Diego, CA, USA). The anti-CD77 (1A4) used in this study was a generous gift from Dr S Hakomori of the University of Washington, Seattle, WA, USA and Otsuka Assay Laboratories, Kawauchi-cho, Tokushima, Japan. Secondary Abs, including fluorescence- and enzyme-conjugated Abs, were purchased from Jackson Laboratory, Inc., West Grove, PA, USA.

Flow Cytometry

The cells were stained with mAbs and analyzed by flow cytometry (EPICS-XL, Coulter) as described previously.[15] Cytoplasmic antigens were stained using CytoStain™ Kits (BD Pharmingen), according to the manufacturer's protocol.

Immunohistochemistry

Immunohistochemical staining of acetone-fixed fresh frozen sections was performed as described elsewhere.[16] Briefly, fresh frozen sections from each tissue were prepared using a cryostat apparatus and fixed in acetone for 15 min at 4°C. After washing in phosphate-buffered saline (PBS) and blocking with normal rabbit serum, the sections were incubated with mAbs at appropriate dilutions for 30 min at room temperature. Sections were then washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibodies for 30 min at room temperature. After washing with PBS, color development was performed in diaminobenzidine solution (10 mM in 0.05 M Tris-HCl, pH 7.5) with 0.003% H_2O_2.

For the cell line samples, the cells were cytocentrifuged on slide glasses using Cytospin III (Shandon Scientific Ltd., Pittsburgh, PA, USA). After fixation with acetone, immunohistochemical staining was performed as described above. In addition, other fixatives, including paraformaldehyde and Zamboni's fixative, were also tested.

The formalin-fixed, paraffin-embedded tissue specimens were initially deparaffinized and then treated using the heat-induced epitope retrieval method in 10 mM of citrate buffer, pH 6.0; immunohistochemical staining was performed using the CSA system (DAKO) according to the manufacturer's protocol.

Results

Immunohistochemical Staining of CD179a/b in Acetone-fixed Cytocentrifuged Cell Lines

As reported previously and presented in Figure 1, the mAbs HSL96, HSL11, and HSL2 recognized CD179a/VpreB, CD179b/λ5, and conformational pre-BCR, respectively, in membrane-permeabilized cells when analyzed using flow cytometry.[10] We first examined whether these mAbs could also be used for immunohistochemical staining in acetone-fixed cells. When acetone-fixed, cytocentrifuged pre-B-ALL HPB-NULL cells expressing conformational pre-BCR were tested, the HSL11 mAb was able to detect CD179b at a concentration of 5 μg/ml; neither the HSL96 nor the HSL2 mAbs detected this molecule (Figure 1). Typically, a cytoplasmic staining pattern was observed in HPB-NULL cells using HSL11. In contrast, HSL11 did not react with similarly treated Ramos Burkitt cells, which express the complete form of Ig (μλ), but lack the surrogate LCs, suggesting that CD179b binds specifically to SL11.

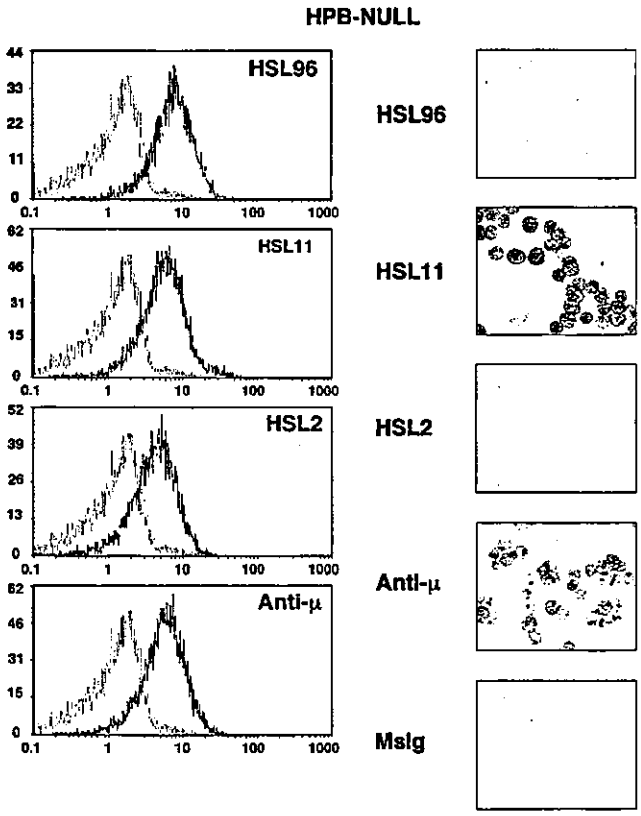

Figure 1 Immunohistochemical detection of CD179b by HSL11 in acetone-fixed, cytocentrifuged precursor B-ALL cell lines. Pre-BCR-expressing HPB-NULL cells were permeabilized and stained with specific mAbs, as indicated, and analyzed using flow cytometry (left panels). The resulting histograms (solid lines) were superimposed on those of the negative control (cells stained with isotype-matched control mouse Ig, broken light lines) and displayed. *X*-axis, fluorescence intensity; *Y*-axis, relative cell number. In parallel, HPB-NULL cells were cytocentrifuged, acetone-fixed, and stained with each mAb, as indicated, using immunohistostaining (right panels). HSL11 is strongly positive and anti-μ is moderately positive, but others are negative. MsIg, iso-type matched control mouse immunoglobulin.

We also examined the staining patterns produced by commercially available anti-CD179a mAbs: VpreB8, VpreB9, 4G7, and B-MAD-688. When these four anti-CD179a mAbs were examined, only the VpreB8 mAb reacted with CD179a in acetone-fixed HPB-NULL cells (data not shown). However, VpreB8 mAb exhibited a weak nonspecific binding with the nuclei of acetone-fixed Ramos cells at high mAb concentrations. A concentration of 1.25 μg/ml was optimized as a sufficient and specific condition for CD179a detection in precursor B-ALL cells, which does not produce a nonspecific reaction in Burkitt cells (data not shown).

We further examined whether SL11 and VpreB8 could be used for immunohistochemical staining in cells treated with other fixatives and observed that both mAbs react with Zamboni's fixative-treated cells, but not with paraformaldehyde-treated cells (data not shown).

Immunohistochemical Staining of CD179a/b in Acetone-fixed Fresh Frozen Tissues

Next, we used immunohistochemistry to examine whether VpreB8 and HSL11 could detect CD179a/b in clinical childhood B-LBL specimens. When acetone-fixed fresh frozen sections prepared from biopsy specimens obtained from B-LBL patients were examined using immunohistochemical staining, both VpreB8 and HSL11 were found to react with the tissues (Figure 2 and Table 1). Typically, a diffuse cytoplasmic staining pattern was observed in B-LBL tissues using both mAbs (Figure 2). Cases were considered as positive if most of the blasts present in the tissue were clearly stained. As summarized in Table 1, nine out of 10 (90%) B-LBL patients and eight out of 11 (73%) B-LBL patients were positive for VpreB8 and HSL11, respectively. In contrast, no positive staining for VpreB8 or HSL11 was seen in either the Burkitt lymphoma tissues (seven cases) or the diffuse large B-cell lymphoma tissues (three cases), suggesting that both VpreB8 and HSL11 react specifically with B-LBL cells, but not with mature B-cell lymphomas in childhood.

We also examined the other pediatric tumors that must also be included in a differential diagnosis of B-LBL. As presented in Table 2, when acetone-fixed fresh frozen sections prepared from biopsy specimens obtained from seven T-LBL cases, three extramedullary myeloid tumors, and two Ewing sarcoma cases were examined similarly, all of these cases were negative for both VpreB8 and HSL11, indicating the specificity of these mAbs to B-LBL cells.

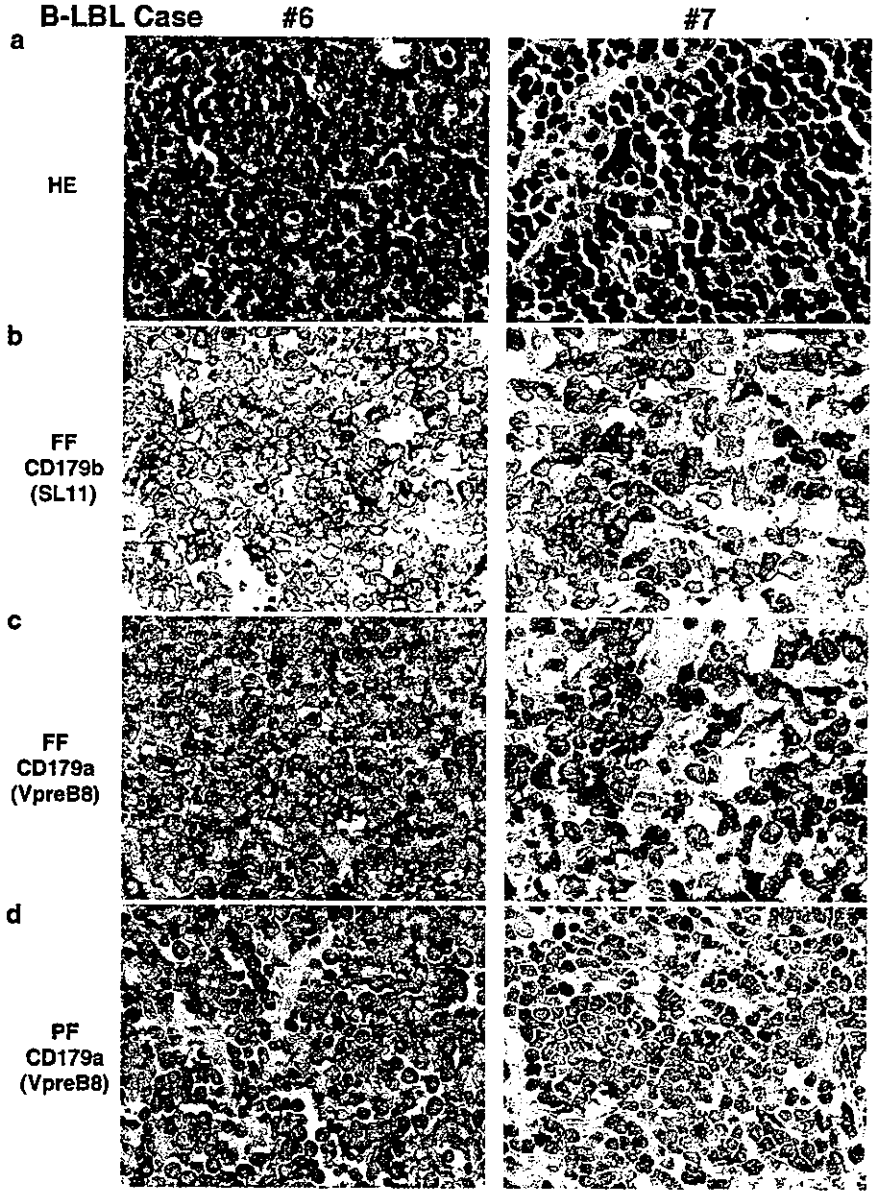

Figure 2 Immunohistochemical detection of CD179a and CD179b. CD179a and CD179b were detected in B-lymphoblastic lymphoma tissues using immunohistochemical staining on acetone-fixed fresh frozen sections ((**b**), (**c**), FF) and formalin-fixed, paraffin-embedded tissue sections ((**d**), PF) from biopsy tissues. The H&E-staining of formalin-fixed and paraffin-embdded tissues is also shown ((**a**), HE).

Immunohistochemical Staining of CD179a in Formalin-fixed, Paraffin-embedded Tissues

Next, we examined whether mAbs against CD179a and CD179b could be used in formalin-fixed, paraffin-embedded tissues. When paraffin-embedded tissues prepared from clinical specimens obtained from B-LBL patients were examined using immunohistochemical staining with the heat-induced epitope retrieval treatment, only VpreB8 reacted with the tissue. The staining results were consistent with those obtained from the immunostaining of acetone-fixed frozen sections. None of the other mAbs reacted with the B-LBL samples. Since higher concentrations of VpreB8 resulted in nonspecific nuclear staining in paraffin sections of Burkitt lymphomas, care must be taken when deciding the appropriate conditions for the use of this mAb.

Discussion

In the current study, we clearly presented that both VpreB8 and HSL11 are useful for the immunohistochemical detection of CD179a and CD179b, respectively, in acetone-fixed B-LBL tissues. Furthermore, VpreB8 can also be used in paraffin-embedded sections. The reactivities of these Abs were highly specific for B-LBL. Reactivity was not seen in tissues of Burkitt lymphoma, diffuse large B-cell

Table 1 Detection of CD179a and CD179b in B-lineage lymphoma tissues using immunohistochemical staining in acetone-fixed fresh frozen sections

Case no.	*Age (years)*	*Sex*	*Origin*	*CD179a*	*CD179b*	*TdT*	*CD34*	*CD19*	*CD79a*	*DR*	*CD20*	μ	*LC*	*CD10*	*CD77*
B-LBL															
1	4	M	Bil-CL	+	+	+	−	+	+	+	+P	−	−	+	−
2[a]	9	M	R-testis	+	+	+	−	+	+	+	+M	NT	NT	+	−
3	7	M	L-CL	+	+	+	−	+	+	+	−	+	−	+	−
4	5	F	L-CL	+	+	+	−	+	+	+	−	−	−	+	−
5	7	F	L-CL	+	+	+	+	+	+	+	−	+	−	+	−
6	1	F	R-CL	+	+	+	−	+	+	+	−	−	−	+	−
7	12	M	AT	+	+	−	−	+	+	+	−	−	−	+	−
8	5	F	L-upper arm	+	−	−	−	+	+	+	+M	−	−	+	NT
9	7	M	L-CL	−	−	+	−	+	+	+	+P	−	−	+	NT
10	4	F	R-radius	+	−	−	+	+	+	+	−	−	−	+	−
11	9	M	CNS	NT	+	+	NT	+	+	NT	NT	+	NT	NT	NT
Burkitt															
1	6	F	AT	−	−	−	−	+	+	+	+	+	−	−	+
2	7	M	AT	−	−	−	−	+	+	+	+	+	Lamda	+	+
3	15	M	AT	−	−	−	−	+	+	+	+	+	Lamda	+	+
4	4	M	AT	−	−	−	−	+	+	+	+	+	Kappa	+	+
5	6	M	AT	−	−	−	−	+	+	+	+	+	Kappa	+	−
6	5	M	AT	−	−	−	−	+	+	+	+	+	Kappa	+	+
7	4	M	AT	−	−	−	−	+	+	+	+	+	Lamda	+	+
B-DL															
1	7	F	R-CL	−	−	−	−	+	+	+	+	+	Lamda	−	−
2	6	M	AT	−	−	−	−	+	+	+	+	+	Lamda	−	−
3	8	M	R-CL	−	−	−	−	+	+	+	+	+	Lamda	+	−

B-LBL, precursor B-cell lymphoblastic lymphoma; DL, diffuse large cell lymphoma; Bil, bilateral; L, left; R, right; CL, cervical lymph nodes; AT, abdominal tumor; LC, light chains; NT, not tested; P, patchy staining pattern; M, membranous staining pattern.
[a]Testicular relapse of precursor B acute lymphoblastic leukemia.

Table 2 Immunohistochemical staining of CD179a and CD179b on acetone-fixed fresh frozen sections of non-B-cell lineage neoplasm tissues

	Positivity	
	CD179a	*CD179b*
T-LBL	0/7	0/7
Extramedullary myeloid tumors		
Granulocytic sarcoma	0/2	0/2
AMoL, skin infiltration	0/1	0/1
Ewing sarcoma	0/2	0/2

T-LBL, precursor T-cell lymphoblastic lymphoma; AMoL, acute monocytic leukemia.

lymphoma, T-LBL, extramedullary myeloid tumors, and Ewing sarcoma.

In pediatrics, the three major types of B-cell lymphoma are B-LBL, Burkitt lymphoma, and diffuse large B-cell lymphoma; the latter two types must be distinguished from B-LBL since the therapeutic protocols for these diseases are quite different from that for B-LBL. In the Berlin Frankfurt Munster (BFM) study group, for example, B-LBL cases were treated using ALL-type protocol with a total therapy duration of at least 24 months.[17] In contrast, mature B-cell lymphoma cases, including Burkitt lymphoma and diffuse large B-cell lymphoma, are treated using a short course of treatment that is usually completed within a year.[18] Each type of B-lineage lymphoma is morphologically unique and distinctive upon histological examination. In the practical pathological diagnosis of lymphomas, however, pathologists may experience difficulties in differentiating B-LBL from other B-lineage lymphomas, especially when only poor-quality biopsy specimens are available.[12] Unfortunately, pathologists are not always familiar with B-LBL because of its rarity among childhood lymphomas; as a result, patients with B-LBL may be misdiagnosed as having mature B-cell lymphoma, such as Burkitt lymphoma. The similarity in marker expression patterns for B-LBL and Burkitt lymphoma is also partly responsible for the risk of misdiagnosis.[11,12]

TdT is considered to be a reliable marker for the diagnosis of cases of precursor lymphocyte origin,[11,12] but TdT is not always positive in B-LBL cases as reported by several different groups.[19–22] For example, Mertelsmann *et al*[20] reported that TdT was absent in approximately 5% of ALL and LBL cases. Orazi *et al*[21] also reported that 6% (two out of 35) of LBL cases was TdT-negative assessed by immunohistochemical staining. On the other hand, CD34 is expressed on human bone marrow progenitor cells and leukemic blasts, and is considered to be an immature marker. Although the expression of CD34 on B-lineage lymphomas suggests their precursor B-cell origin, the positivity of CD34 among the B-LBL cases is approximately 50%. In addition, both TdT

and CD34 are not restricted to the precursor of B cells. In contrast, CD20 is a B-cell-specific marker and its expression increases with B-cell maturation. Therefore, the absence of CD20 expression in B-lineage lymphomas suggests their precursor B-cell origin. However, CD20 expression is variable among cases of B-LBL and approximately 50% of B-LBL cases are CD20-positive, exhibiting sometimes a strong membranous staining pattern.[11] Therefore, it is difficult to specify a B-precursor origin using CD20 expression alone. Based on the above evidences, the development of other markers capable of revealing a precursor B-cell origin is urgently required; in this regard, the results described here are expected to assist in the proper diagnosis of B-LBL among B-cell lymphomas in childhood.

CD179a and CD179b are essential for the development of precursor B cells. Although their biological significance is not fully understood, they are believed to serve as surrogate LCs expressed with μ HCs in pre-BCR to determine whether the clone should survive or die. After subsequent rearrangements in κ or λ LC genes, the expression of the surrogate LCs is suppressed.[6–9] The utilization of such functional molecules in the diagnosis of precursor B-cell lymphomas is appropriate if the expression is conserved even in tumor cells. In precursor B-ALL cells, we previously reported that CD179a, CD179b, and the complete form of pre-BCR were detected by HSL96, HSL11, and HSL2, respectively, and were expressed in most of the CD10-positive precursor B-ALL cases,[10] suggesting that these markers may be useful for the further classification of this disease. Consistent with this observation, CD179a and CD179b, detected by VpreB8 and HSL11, respectively, were frequently expressed in B-LBL cases, whose origin is comparable to that of precursor B-ALL. Thus, the successful employment of these functional molecules in the diagnosis of B-cell lymphomas is another important aspect emphasized in this study.

As shown here, CD179a and CD179b immunohistochemistry can identify more than 90% of B-LBL cases. In our series, the positivity of TdT among the B-LBL cases examined was lower (73%) than that of previous reports.[19–22] The reason for this discrepancy is not known, but it is noteworthy that three TdT-negative cases were positive for either CD179a or CD179b or both. Thus, by combining the TdT and CD179 markers, we believe that virtually all B-LBL cases can be properly judged as having a precursor B-cell origin. The absence of CD179a/b reactivity in Burkitt and diffuse large B-cell-type lymphomas further supports the reliability of this marker.

Occasionally, B-LBL may be misdiagnosed as Ewing sarcoma, since these two diseases have similar morphologies and immunostaining patterns.[23] CD99 (MIC2) was previously considered to be a specific marker for Ewing sarcoma, but this molecule has now been shown to be frequently expressed in B-LBL. Bone tumors with a blastic morphology and a CD45−, CD20−, MIC2+ phenotype can be diagnosed as Ewing sarcoma. In such cases, immunostaining for CD179a/b along with TdT and CD79a will lead to a proper diagnosis. In addition, immunostaining for CD179a/b is also useful for distinguishing B-LBL from either T-LBL or extramedullary myeloid tumors, both of which are included in frequent differential for B-LBL.

Diagnostic markers must be usable in paraffin sections for practical diagnostic procedures. In this regard, the utilization of mAb VpreB8 in paraffin sections, as demonstrated in this report, should facilitate its use in daily diagnostics. Caution must be exercised, however, when using VpreB8 because this antibody may produce nonspecific binding. After careful examination, we selected a concentration of 1.25 μg/ml for our system; however, this value should be evaluated for each laboratory in which the mAb is used, since differences in detection systems may affect the results. Other than VpreB8, unfortunately, none of the mAbs against CD179a/b tested in this study was useful for immunohistochemical detection in paraffin-embedded sections. Since the expression of CD179b was always accompanied by that of CD179a in our cases assessed using fresh frozen section staining (Table 1), paraffin section staining with VpreB8 may be sufficient for the diagnosis of B-LBL. However, the generation of novel mAbs against CD179a/b and preBCR that can react in paraffin sections would be useful and may provide more convincing results.

In conclusion, we have demonstrated that mAbs against CD179a/b specifically detect B-LBL tissues. Although an examination of a larger number of lymphoma tissues is required to confirm their reliability, the application of these mAbs in the immunohistochemical examination of lymphoma tissues should contribute to a precise diagnosis of B-lineage lymphomas.

Acknowledgements

This work was supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan and MEXT, KAKENHI 15019129, JSPS, KAKENHI 15390133 and 15590361. This work was also supported by a grant from the Japan Health Sciences Foundation for Research on Health Sciences Focusing on Drug Innovation. Additional support was provided by a grant from Sankyo Foundation of Life Science.

We thank M Sone and S Yamauchi for their excellent secretarial works. We also thank Dr S Hakomori (Washington University) and Otsuka Assay Laboratories for gifting CD77 mAb 1A4.

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Immunology 2004 **112** 575–582 doi:10.1111/j.1365-2567.2004.01918.x

Deficiency of BLNK hampers PLC-γ2 phosphorylation and Ca^{2+} influx induced by the pre-B-cell receptor in human pre-B cells

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SUMMARY

B-cell linker protein (BLNK) is a component of the B-cell receptor (BCR) as well as of the pre-BCR signalling pathway, and BLNK$^{-/-}$ mice have a block in B lymphopoiesis at the pro-B/pre-B cell stage. A recent report described the complete loss or drastic reduction of BLNK expression in approximately 50% of human childhood pre-B acute lymphoblastic leukaemias (ALL), therefore we investigated BLNK expression in human pre-B ALL cell lines. One of the four cell lines tested, HPB-NULL cells, was found to lack BLNK expression, and we used these human pre-B ALL cell lines that express and do not express BLNK to investigate the intracellular signalling events following pre-BCR cross-linking. When pre-BCR was cross-linked with anti-μ heavy-chain antibodies, significant phosphorylation of intracellular molecules, including Syk, Shc, ERK MAP kinase, and AKT, and an activation of Ras were observed without regard to deficiency of BLNK expression, suggesting that BLNK is not required for pre-BCR-mediated activation of MAP kinase and phosphatidyl-inositol 3 (PI3) kinase signalling. By contrast, phospholipase C-γ2 (PLC-γ2) phosphorylation and an increase in intracellular Ca^{2+} level mediated by pre-BCR cross-linking were observed only in the BLNK-expressing cells, indicating that BLNK is essential for PLC-γ2-induced Ca^{2+} influx. Human pre-B cell lines expressing and not expressing BLNK should provide an *in vitro* model for investigation of the role of BLNK in the pre-BCR-mediated signalling mechanism.

Keywords B-cell receptor; B cells; signalling/signal transduction

INTRODUCTION

Signals transduced through antigen receptors play essential roles in B-cell development and fate determination. The B-cell antigen receptor (BCR), which consists of a μ heavy chain (HC), conventional light chain (LC), immunoglobulin α (Igα; CD79a), and Igβ (CD79b), mediates different biological responses in B cells, i.e. proliferation, differentiation, growth arrest, or induction of apoptosis, depending on the differentiation and activation stage of the B cell.[1–3]

In contrast to mature B cells, B-cell progenitors do not possess the complete forms of the BCR, but do express BCR-related components. For example, pro-B cells express the Igα/Igβ heterodimer in association with calnexin,[4] an integral membrane protein, and the surrogate light (SL) chain encoded by the VpreB (CD179a) and λ5 (CD179b) genes.[5,6] These molecules have been found to be competent for transducing differentiation signals for pro-B cells.[4]

In addition, pre-B cells that have successfully accomplished rearrangement of the HC genes start to express a premature form of the antigen receptor, i.e. a pre-B-cell

Received 15 December 2004; revised 21 April 2004; accepted 10 May 2004.

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receptor (pre-BCR) consisting of μ HC, SL chains and the Igα/Igβ heterodimers.[7–9] Several studies have shown the vital importance of pre-BCR as a mediator of pre-B-cell differentiation signals.[10–12] Expression of pre-BCR on the cell surface suppresses further recombination of μ HC genes and induces rearrangement of the conventional LC genes, indicating that signals through pre-BCR facilitate the proliferation of successfully developed pre-B cells.

Although the μ HC does not have any enzymatic activity in its cytoplasmic domain to transduce intracellular signals, the regulatory cascade of molecules is involved in BCR-mediated signalling.[2,3] The stimuli conveyed by antigens through BCR activate a number of BCR-associated cytoplasmic protein tyrosine kinases (PTKs), including the Src-family PTKs, Syk and Brutons tyrosine kinase (BTK).[13,14] These PTKs then phosphorylate numerous intracellular proteins and couple BCR stimulation to intracellular signallings, such as phosphoinositide hydrolysis, protein kinase C activation, and activation of Ras-mitogen-activated protein (MAP) kinase pathways.[2,3] A similar molecular cascade for signal transduction has been postulated for pre-BCR signalling.[4,15]

B-cell linker protein (BLNK), also known as SLP-65, BASH and BCA, is a B-cell adaptor molecule that links the cytoplasmic PTKs with phosphorylation of downstream effector molecules[16–18] and plays a crucial role in the BCR signalling system. Since BLNK does not encode any intrinsic enzymatic activity, its function is to serve as a scaffold for assembling molecular complexes that include enzymes and additional linker proteins. Upon BCR stimulation, BLNK couples activated Syk to phospholipase C-γ (PLC-γ), Vav, Grb2 and NCK.[19] In addition, it binds Btk[20,21] and is required for activation of the transcription factor NF-κB.[22] It has been reported consistently that B cells lacking BLNK fail to elicit Ca^{2+} influx following BCR cross-linking and exhibit attenuated activation of all three families of MAP kinases.[19]

BLNK has also been shown to play important roles in pre-BCR signalling, and BLNK-deficient mice show a partial block at the pre-B cell stage characterized by impaired developmental progression from large cycling $CD43^+$ pro-B cells into small resting $CD43^-$ pre-B cells,[23–26] suggesting an essential role of BLNK in pre-BCR signalling that mediates the growth and differentiation of B-cell precursors.

More importantly, it has been reported that some BLNK-deficient mice spontaneously develop pre-B-cell lymphomas that express large amounts of pre-BCR on their surface.[27,28] Consistent with this, approximately 50% of human childhood pre-B acute lymphoblastic leukaemias (ALL) show complete loss or drastic reduction of BLNK expression.[29] These findings indicate that BLNK functions as a tumour suppressor and that loss of BLNK and the accompanying block in pre-B-cell differentiation is one of the primary causes of pre-B ALL, although the precise mechanism is unknown.

We employed human pre-B cell lines that express and do not express BLNK and examined the intracellular signalling events following pre-BCR cross-linking in an attempt to investigate the role of BLNK in pre-BCR-mediated signalling. In this paper, we report the absence of Ca^{2+} influx following pre-BCR ligation in BLNK-negative human pre-B-cell lines, but not interference with pre-BCR-mediated phosphorylation of intracellular molecules. This suggests that BLNK is essential to Ca^{2+} signalling in human pre-B cells but not to other signalling cascades and it should provide an *in vitro* model for studying the role of BLNK in pre-BCR-mediated signalling.

MATERIALS AND METHODS

Cells and reagents

The human pre-B cell lines, NALM-17, HPB-NULL, P30/OHK[30] and NALM-6[31] were used in this study. The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum at 37° in a humidified 5% CO_2 atmosphere. The mouse monoclonal antibodies (mAbs) used were; anti-μ (G20-127), anti-κ (G20-193), and anti-λ (JDC-12) from Pharmingen (San Diego, CA); anti-BLNK (2B11), anti-Syk (4D10), and anti PLC-γ2 (B-10), from Santa Cruz Biotechnology (Santa Cruz, CA); anti-extracellular signal-regulated kinase (ERK)-1 (MK12) from Transduction Laboratories (Lexington, KY); anti-phosphotyrosine (PY) (4G10) from Upstate Biotechnology Inc. (Lake Placid, NY); anti-μ (AF6) from Beckman/Coulter Inc. (Westbrook, MA); anti-β actin (ZSA1) from Seikagaku Co. (Tokyo, Japan); and anti-μ (DA4.4) from the American Type Culture Collection (Rockville, MD). Anti-λ5 (HSL11), anti-Vpre-B (HSL96) and anti-conformational pre-BCR (HSL2) were also used.[30] As the negative control for flow cytometric analysis, isotype-matched mouse immunoglobulins, IgG1 (KOPC-31C) and IgG2a (G155-178), from Pharmingen were used. The rabbit polyclonal antibodies used were; $F(ab')_2$ fragment of anti-μ HC from Jackson Laboratory, Inc. (West Grove, PA); anti-PLC-γ1, anti-phospho-ERK, anti-phospho-MAP kinase/ERK kinase (MEK), anti-phospho-PLC-γ1, anti-phospho-PLC-γ2 and anti-phospho-AKT from New England Biolabs, Inc. (Beverly, MA); anti-PLC-γ2 from Pharmingen; and anti-Shc from Transduction Laboratories. The goat polyclonal anti-BTK antibody from Santa Cruz Biotechnology was also used. Secondary antibodies, including fluorescein-conjugated and enzyme-conjugated antibodies, were purchased from Jackson.

Immunofluorescence study

The cells were stained with mAbs and analysed by flow cytometry (EPICS-XL, Coulter) as described previously.[32] Staining of cytoplasmic antigens was performed with CytoStain™ Kits (Pharmingen) according to the manufacturer's protocol.

Immunoblotting and immunoprecipitation

Immunoblotting was performed as described previously.[33] Briefly, cell lysates were prepared by solubilizing the cells in lysis buffer (containing 20 mM Na_2PO_4, pH 7·4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 5 mM phenylmethylsulphonyl fluoride, 100 mM NaF and 2 mM Na_3VO_4). After centrifugation, supernatants were obtained and the protein concentration of each cell lysate was

determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of each cell lysate were electrophoretically separated on sodium dodecyl sulphate–polyacrylamide gel and transferred onto a nitrocellulose membrane using a semi-dry transblot system (Bio-Rad). After blocking, the membranes were incubated with the appropriate combination of primary and secondary antibodies as indicated, washed intensively, then examined using the enhanced chemiluminescence reagent system (ECL, Amersham Life Science, Buckinghamshire, UK). The results obtained from a 1-min exposure of the ECL-treated membrane to film are presented.

For the immunoprecipitation, 500 μg of the cell lysates was incubated with 1 μg of antibody and 50 μl of 50% protein-G agarose (Boehringer Mannheim Biochemica, Mannheim, Germany) for 1 hr. After intensive washing, the immunoprecipitates were separated by electrophoresis and analysed as described above.

To measure Ras activation, EZ-Detect™ Ras Activation Kits from PIERCE Biotechnology (Rockford, IL) were used according to the manufacturer's protocol.

Ca^{2+} mobilization assay

Intracellular levels of Ca^{2+} were measured by flow cytometry using Fluo 3-AM (Dojin, Kumamoto, Japan) after pre-BCR cross-linking with anti-μ antibodies. Ten million cells were washed and resuspended in 1 ml of OPTI-MEM containing 0·5% bovine serum albumin, and incubated with 1 mM of Fluo 3-AM for 30 min at 37°. After washing, the cells were resuspended in 10 ml of medium, stimulated by adding different concentrations of rabbit anti-human μ HC antibody as described in Figure 4 and the intracellular calcium concentration was measured by flow cytometry as described previously.[34] Calcium ionophore (ionomycin, Sigma-Aldrich Fine Chemicals, St Louis, MO) was used as the positive control. The data obtained were analysed using WINMDI software (distributed by Dr Joe Trotter) and presented as a kinetics line.

RESULTS

Expression of cell surface pre-BCR and BLNK in human pre-B-cell lines

It has been reported that some pre-B-cell lines express pre-BCR on their cell surface, even if not abundantly.[35] To identify cell lines that express pre-BCR on their cell surface, we tested a series of human pre-B-cell lines for surface expression of μ HC. Flow cytometry showed that all four cell lines tested, i.e. lines NALM-6, HPB-NULL, NALM-17, and P30/OHK, expressed μ HC on their cell surface, and that two of them, NALM-17 and HPB-NULL cells, expressed μ HC on their cell surface more abundantly than the others (Fig. 1a). To investigate the cell surface expression of pre-BCR, we examined reactivity to mAb against SL chains. The mAbs HSL11, HSL96 and HSL2 specifically recognize λ5, VpreB and the conformational epitope of pre-BCR, respectively.[30] When evaluated by flow cytometry, the cell lines stained positive with all three mAbs (Fig. 1a), but not with anti-κ and -λ mAb (data not shown), indicating that the pre-BCR is indeed expressed on the cell surface of some pre-B cell lines. It is noteworthy that although NALM-6 cells clearly reacted with both HSL11 (λ5) and HSL96 (VpreB), they revealed much weaker reactivity with HSL2 (conformational epitope of pre-BCR), for which the precise reason is unknown (Fig. 1a).

Since complete loss or drastic reduction of BLNK expression has also been reported in approximately 50% of childhood precursor-B ALL cases[29] we tested pre-B cell lines for expression of BLNK. Immunoblotting revealed abundant expression of BLNK by NALM-17 cells, whereas no BLNK expression was detectable in HPB-NULL cells (Fig. 1b). The NALM-6 and P30/OHK cells showed an intermediate amount of BLNK expression (Fig. 1b). We also investigated HPB-NULL and NALM-17 cells for intracellular BLNK expression by flow cytometry. When membrane-permeabilized cells were stained with fluorescein-labelled anti-BLNK mAb, clear expression of BLNK was observed only in NALM-17 cells (Fig. 1c), consistent with the results of immunoblotting. In contrast, when tested for expression of other B-cell-related signalling molecules, i.e. BTK, Syk, PLC-γ1 and PLC-γ2, by immunoblotting, all four molecules were comparably expressed in all four pre-B cell lines (Fig. 1b). Based on these findings, we decided to use NALM-17 and HPB-NULL cells in the following experiments as *in vitro* models of BLNK-positive and negative pre-B cells, respectively.

Phosphorylation of intracellular proteins induced by pre-BCR cross-linkage in both BLNK-positive and -negative pre-B cells

Next, we investigated whether cross-linking of pre-BCR with anti-μ antibodies would induce tyrosine phosphorylation of intracellular proteins in pre-B cell lines. Evaluation by immunoblotting with the anti-PY mAb, DA4.4 directed against human μ HC was found to cross-link pre-BCR strongly enough to induce tyrosine phosphorylation of intracellular proteins in HPB-NULL cells (Fig. 2a), but isotype-matched control mouse immunoglobulin did not (data not shown). Phosphorylation of the tyrosine residues peaked at 1–5 min after pre-BCR cross-linking and then decreased to a resting level by 30 min (Fig. 2a). Testing of other antibodies specifically reacting with μ HC, including the other clone of anti-μ HC mAb (AF6 and G20-127) and $F(ab')_2$ fragment of rabbit polyclonal anti-μ HC, yielded identical results (data not shown). Based on the results obtained under various conditions, incubation with 10 μg/ml of anti-μ mAb DA4.4 for 5 min was considered the optimal condition for pre-BCR cross-linking.

Next, immunoprecipitation and immunoblotting were used to identify signal transduction molecules located downstream in the pre-BCR signalling cascade in HPB-NULL cells. When Syk PTK (Fig. 2b) and Shc adapter molecules (Fig. 2c) were immunoprecipitated with specific antibodies, a pre-BCR-mediated increase in tyrosine phosphorylation was observed on anti-PY immunoblotting, and use of phospho-specific antibodies revealed that ERK MAP

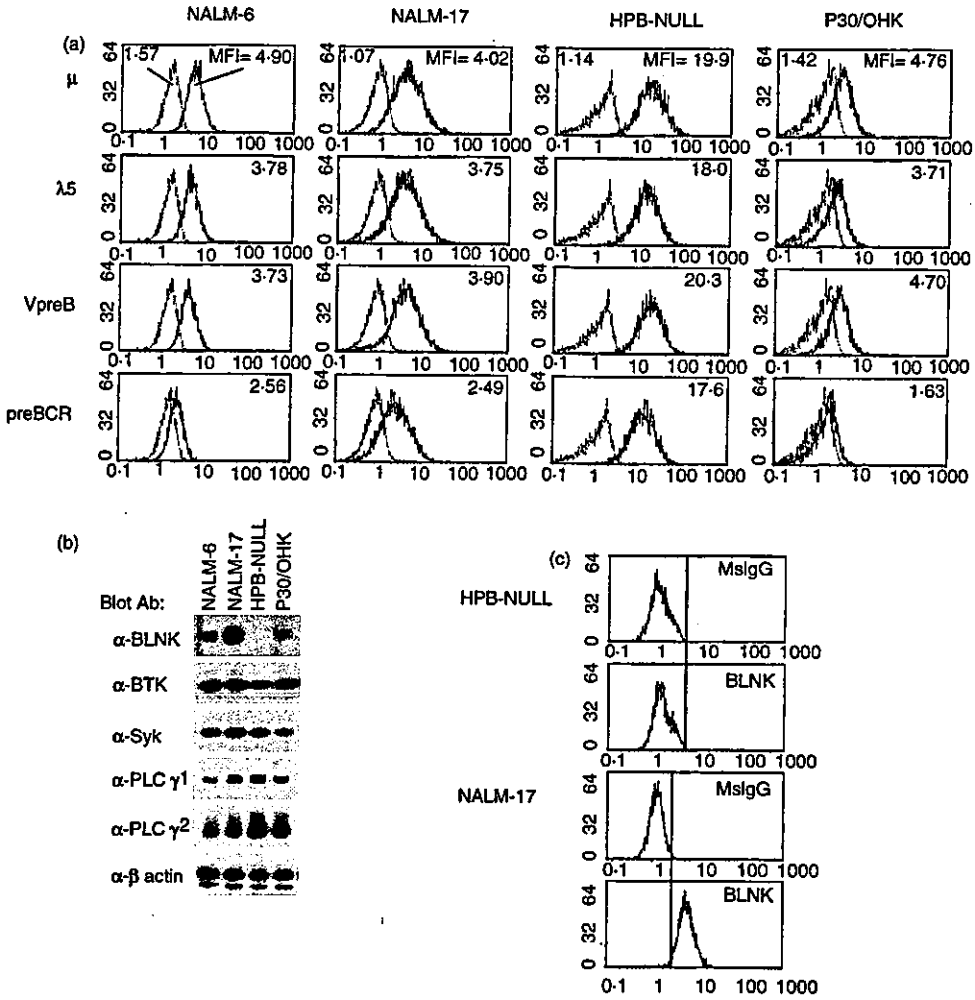

Figure 1. Expression of pre-BCR and BLNK in human pre-B-cell lines. (a) The pre-BCR-related molecules, i.e. μ heavy chain, $\lambda 5$, VpreB and conformational pre-BCR (preBCR), expressed on the cell surface of human pre-B cell lines, NALM-6, NALM-17, HPB-NULL, and P30/OHK, were stained with the specific monoclonal antibodies indicated and analysed by flow cytometry as described in the Materials and methods. The histograms obtained (solid lines) have been superimposed on those of the negative control (cells stained with isotype-matched control mouse immunoglobulin, IgG1 KOPC-31C, light broken lines). The mean fluorescence intensity (MFI) for each staining is presented in the top right hand corner of each panel. The MFI for each negative control staining is presented in the top left hand corner of the top panel of each cell line. The *x*-axis represents fluorescence intensity; the *y*-axis represents relative cell number. (b) Human pre-B-cell lines were tested for expression of BLNK and signal-transduction-related molecules by immunoblotting analysis with the specific antibodies indicated. For PLC-γ2 blotting, anti-PLC-γ2 monoclonal antibody (B-10) was used. (c) Human pre-B cell lines were tested for expression of BLNK by flow cytometry. Cells were permeabilized, stained with a combination of anti-BLNK monoclonal antibody and fluorescein isothiocyanate-conjugated secondary goat anti-mouse IgG, and analysed as described in the Materials and methods. As a negative control, cells were also stained with isotype-matched control mouse immunoglobulin (IgG2a G155-178, MsIgG).

kinase and MEK kinase were phosphorylated immediately after exposure to the anti-μ HC mAb DA4.4 (Fig. 2d). The time course of the phosphorylation state of MEK and MAP kinase showed kinetics similar to those of intracellular proteins detected with anti-PY mAb (Fig. 2a,d), and clear phosphorylation of PLC-γ1 and AKT was also detected in HPB-NULL cells after pre-BCR cross-linking (Fig. 3a). We also examined the activation of Ras, an upstream signalling molecule of ERK MAP kinase. As shown in Fig. 3(b), we observed an activation of Ras in HPB-NULL cells after pre-BCR cross-linking.

Similar testing of BLNK expressing NALM-17 cells revealed that pre-BCR cross-linking induced phosphorylation of these intracellular molecules as well as Ras activation that was as immediate and clear as we observed in HPB-NULL cells (Figs 3a,b). However, it is noteworthy that examination of phosphorylation of PLC-γ2 in the same manner showed that pre-BCR cross-linking induced phosphorylation of PLC-γ2 only in NALM-17 cells, and not in HPB-NULL cells (Fig. 3a).

A major mechanism by which BLNK has been proposed to regulate PLC-γ2 is through the juxtaposition of BTK and

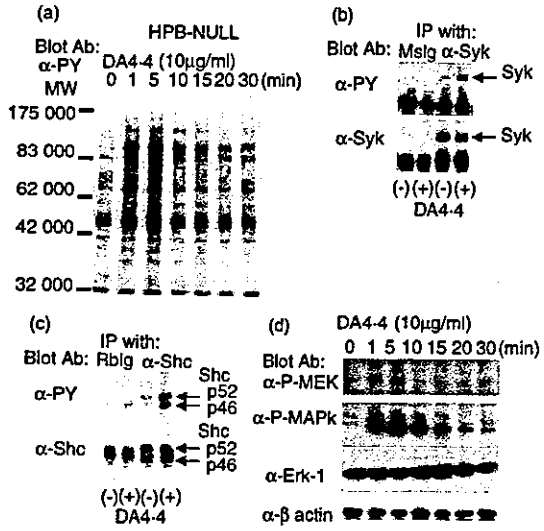

Figure 2. Increase in phosphorylation of intracellular proteins in HPB-NULL cells after exposure to anti-μ antibodies. (a) Immunoblot analysis with anti-phosphotyrosine (α-PY) monoclonal antibody was performed on cell lysates prepared from HPB-NULL pre-B cells exposed to 10 μg/ml of anti-μ mAb DA4.4 for the periods indicated. A molecular weight standard is indicated on the left. (b) Proteins immunoprecipitated with either isotype-matched control mouse immunoglobulin (MsIg, lanes 1 and 2) or mouse monoclonal antibody against Syk (α-Syk, lanes 3 and 4) from HPB-NULL lysates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis in duplicate. After transfer onto a nitrocellulose membrane, the samples were analysed by immunoblotting with either anti-phosphotyrosine (α-PY) monoclonal antibody (upper panel) or anti-Syk monoclonal antibody (lower panel). Corresponding bands for Syk are indicated by arrows. (c) An experiment similar to that in (b) was performed using a combination of HPB-NULL lysates and rabbit polyclonal anti-Shc antibody (α-Shc). (d) The cell lysates prepared from HPB-NULL as in (a) were also tested by immunoblotting with antibodies indicated.

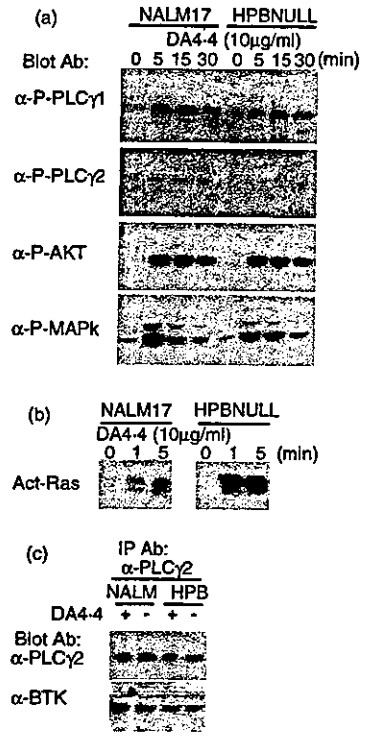

Figure 3. Pre-BCR-mediated signalling in pre-B cell lines. (a) Immunoblot analysis using phospho-specific antibodies on NALM-17 and HPB-NULL cells after exposure to anti-μ monoclonal antibody DA4.4. Cell lysates prepared from NALM-17 and HPB-NULL cells as in Fig. 2(a) were also tested by immunoblotting with the anti-phospho-specific antibodies indicated. (b) NALM-17 and HPB-NULL cells were exposed to DA4.4 and cell lysates were prepared as in (a). Active form of Ras (Ras-GTP) proteins were captured with Raf1-immobilized resin and detected by immunoblotting using anti-Ras antibody. (c) Cell lysates were prepared from NALM-17 and HPB-NULL cells treated with (+) or without (–) DA4.4 (10 μg/ml) for 5 min as in (a). Proteins were immunoprecipitated with rabbit anti-PLC-γ2 antibody from the cell lysates and were separated by SDS–PAGE in duplicate. Immunoblotting was performed with either mouse anti-PLC-γ2 monoclonal antibody (B-10, α-PLC-γ2, upper panel) or goat anti-BTK antibody (lower panel).

PLC-γ2.[3,20,21,28] Therefore we examined the complex formation between BTK and PLC-γ2 in both cell lines. As shown in Fig. 3(c), immunoprecipitation revealed that a portion of BTK was detected in anti-PLC-γ2 immunoprecipitates from the lysates prepared from both NALM-17 and HPB-NULL cells without pre-BCR cross-linking. Interestingly, the total amount of BTK protein precipitated with anti PLC-γ2 antibody was increased after pre-BCR cross-linking in NALM-17 cells, while pre-BCR cross-linking did not affect the amount of BTK protein precipitated with anti PLC-γ2 antibody in HPB-NULL cells (Fig. 3c).

Ca^{2+} influx does not occur in BLNK-negative cell lines after cross-linking of pre-BCR

Next, we investigated whether the cross-linkage of pre-BCR leads to an increase in intracellular Ca^{2+} level. Measurement of the intracellular Ca^{2+} level of HPB-NULL cells by flow cytometry with Fluo 3-AM showed no significant increase in intracellular Ca^{2+} level after exposure to anti-μ antibodies, while treatment with calcium ionophore led to a clear increase in the intracellular Ca^{2+} level (Fig. 4). By contrast, however, when NALM-17 cells, which express BLNK were examined, an increase in intracellular Ca^{2+} level after pre-BCR cross-linkage was observed under identical experimental conditions (Fig. 4). We therefore concluded that the cross-linking of pre-BCR failed to increase intracellular Ca^{2+} in pre-B cells that lack BLNK expression.

DISCUSSION

Several different groups have reported that 5–10% of BLNK-deficient mice spontaneously develop pre-B cell leukaemia/lymphomas expressing large amounts of pre-BCR on their surface.[27,28] Injection of immunodeficient mice with a $BLNK^{-/-}$ pre-B-cell line has been found to result in the development of pre-B-cell leukaemia that was

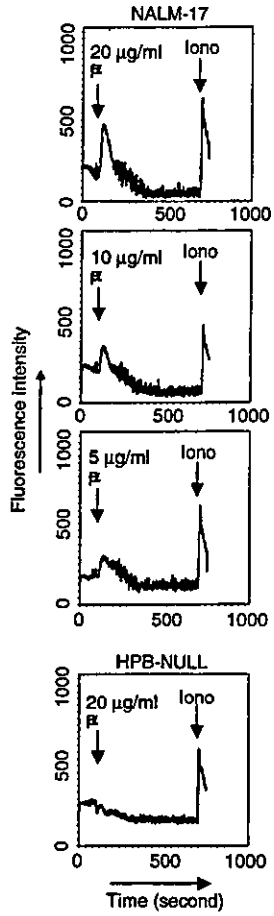

Figure 4. Flow cytometric analysis of Ca^{2+} mobilization in human B-cell lines. NALM-17 or HPB-NULL cells were loaded with Fluo 3-AM as described in the Materials and methods, and the intracellular Ca^{2+} levels were measured by flow cytometry. Rabbit anti-μ monoclonal antibody (α-μ, 5, 10, and 20 μg/ml) or the calcium ionophore ionomycin (Iono, 0·1 μg/ml) was added to the cells at the times indicated by the arrows. Other anti-μ antibodies were also used, and identical results were obtained (data not shown).

prevented by reconstitution of BLNK expression in $BLNK^{-/-}$ pre-B-cell line.[29] More important, it is also reported that 16 of 34 human childhood precursor-B ALL cases showed complete loss or drastic reduction of BLNK expression.[29] Thus, it was hypothesized that BLNK acts as a tumour suppressor and that somatic loss of BLNK and the accompanying block of pre-B-cell differentiation is one of the primary causes of childhood ALL.[27,28] Consistent with the above observations, the results of the present study demonstrated the existence of a human pre-B ALL cell line that lacks BLNK expression, and our findings indicate that the BLNK-deficiency phenotype in human precursor-B ALL cells is maintained in the cell lines established from these ALL cells.

The function of BLNK in pre-B cells is still a matter of controversy. Since, as stated above, reconstitution of BLNK expression in the $BLNK^{-/-}$ pre-B-cell line prevented the development of the leukaemia in immunodeficient mice, Jumaa *et al.* suggested that BLNK is essential to limiting pre-B-cell proliferation.[27,29] By contrast, Hayashi *et al.* observed that the pre-B cells that accumulate in BLNK-deficient mice are mostly non-cycling large pre-B cells, and they therefore concluded that BLNK is critical to pre-BCR signalling that induces proliferation of large pre-B cells.[28] Although the function of BLNK in pre-B ALL cell lines has not yet been clarified, the variable expression levels of BLNK in the pre-B ALL cell lines that we described in this study may mean that the level of expression of BLNK no longer critically affects growth and survival in established human pre-B ALL cell lines. In fact, we did not observe any significant difference in growth rate between the human pre-B ALL cell lines regardless of their BLNK expression level (data not shown).

In the present study we examined the downstream events mediated by cross-linking of pre-BCR in both BLNK-positive and -negative human pre-B-cell lines. The analysis with HPB-NULL cells that lack BLNK clearly indicated that the cross-linking of pre-BCR induces activation of Syk, Shc, Ras, ERK MAP kinase, and AKT, suggesting that the pre-BCR-mediated signalling in the MAP kinase pathway and the phosphatidyl-inositol 3 (PI3) kinase-AKT pathway do not require BLNK. In contrast, cross-linking of pre-BCR induced phosphorylation of PLC-γ2 and an increase in the intracellular Ca^{2+} level in NALM-17 cells alone, and not in HPB-NULL cells, suggesting that BLNK is essential to the pre-BCR-mediated Ca^{2+} influx via PLC-γ2 activation.

Kuwahara *et al.* analysed the downstream events of pre-BCR signalling in the human pre-B cell lines NALM-6 and 796 cells and reported a significant difference in comparison with the events mediated by the conventional BCR expressed on mature B cells, namely, less tyrosine phosphorylation of the cytoplasmic proteins, including Syk, and failure of Ca^{2+} mobilization.[35] However, the fact that the pre-B lines that they studied express only a small amount of pre-BCR on their cell surface may have been a limitation. Thus, their failure to detect signals following pre-BCR cross-linkage may have been because the signals were below the threshold of the detection system.[36] In fact, Nakamura *et al.* analysed the downstream signalling events after pre-BCR stimulation in $\mu\kappa$-transfected NALM-6 cells, which express larger amounts of the reconstituted BCR, and observed that cross-linkage of BCR on pre-B cells caused an elevation in intracellular Ca^{2+} that was qualitatively indistinguishable from the elevation following cross-linkage of BCR on mature B cells.[36]

In addition, we observed that NALM-6 cells clearly reacted with both anti-λ5 and anti-VpreB antibodies, while they revealed much weaker reactivity with antibody against conformational epitope of pre-BCR (Fig. 1a). Although the precise reason for the discrepancy is unknown, our finding may be related to the failure of Ca^{2+} mobilization by pre-BCR cross-linking in NALM-6 cells.

By contrast, our findings also indicated that cross-linkage of pre-BCR expressing on pre-B NALM-17 cells induces hyperphosphorylation of the tyrosine residues in numerous intracellular proteins as well as elevation of the intracellular Ca^{2+} level. Therefore, NALM-17 cells should

provide an *in vitro* model for investigation of the pre-BCR-mediated intracellular signalling mechanism. In addition, since HPB-NULL cells that lack BLNK also revealed similar signalling events induced by pre-BCR cross-linking, with some exceptions such as Ca^{2+} mobilization, comparison between these two cell lines could provide a means of analysing the function of BLNK in human pre-B cells.

BLNK was demonstrated to couple activated Syk to PLC-γ, Vav and Grb2, and participate in the BCR-mediated signalling in mature B cells.[19] Although BLNK has been suggested to also play important roles in pre-BCR signalling, the details remain poorly understood. Based on the molecular similarity of pre-BCR to BCR, however, BLNK is thought to play a role in the pre-BCR signalling system similar to that of BCR, and consistent with this, our results clearly indicated that BLNK is essential for pre-BCR-mediated PLC-γ2 activation and the following Ca^{2+} mobilization. By contrast, they also demonstrated that BLNK is unnecessary for the activation of MAP kinase and the PI3 kinase signalling pathway.

Based on models of transmembrane receptor tyrosine kinases,[37,38] recruitment of the Grb2 adaptor protein, which is associated with Sos GEF, to the plasma membrane was thought to be a likely mechanism for Ras and ERK activation by antigen receptor signalling. However, recent studies have shown that PLC-γ-Ras GRP plays a critical role in Ras activation in the TCR signalling context, in addition to the Grb2-Sos pathway.[39,40] It was also suggested that the latter pathway is dominant in DT40 chicken B cells, because a PLC-γ2-deficient DT40 mutant manifested a large decrease in BCR-mediated ERK activation, whereas it has less of an effect on ERK activation in Grb2-deficient cells, and ablating Sos2, a dominantly expressed Sos isoform in DT40 cells, barely affected ERK activation.[41] As we showed in this study, pre-BCR cross-linking effectively activated Ras and ERK in BLNK-deficient HPB-NULL cells in spite of the impairment of PLC-γ2 phosphorylation and the failure of an increase in complex formation between BTK and PLC-γ2. Thus, our findings may mean that PLC-γ2 activation is unnecessary for the activation of ERK MAP kinase in human pre-B cells. Alternatively, it is also possible that HPB-NULL cells possess a mechanism that partially compensates for the function of BLNK in pre-BCR-mediated signalling, thereby enabling pre-BCR cross-linking to induce ERK activation without PLC-γ2 activation. Interestingly, we observed that PLC-γ1 was expressed and phosphorylated by pre-BCR cross-linking both in BLNK-expressing and BLNK-deficient pre-B-cell lines. The data may indicate that PLC-γ1 and PLC-γ2 have distinct functions in pre-B-cell lines.

As stated above, BLNK-deficient mice have a block in B-cell development at the pre-B-cell stage, but the block is incomplete, and a small number of mature B cells are still present in the periphery.[23–26] This suggests that other signalling molecule(s) may partially neutralize the BLNK deficiency in B cells, and indeed, combined expression of adaptor proteins SLP-76 and LAT has been reported to reconstitute BCR function in $BLNK^{-/-}$ DT40 chicken B cells.[42,43] In addition, most recent reports have suggested that LAT and SLP-76 are involved in pre-BCR signalling and rescue arrested murine $BLNK^{-/-}$ pre-B cells.[44] Thus, alternative signalling molecule(s) that partially compensate for the function of BLNK may be present in HPB-NULL cells.

In conclusion, human pre-B-cell lines NALM-17 and HPB-NULL should provide an ideal *in vitro* model for analysis of pre-BCR-mediated signalling and the role of BLNK in pre-B cells. Further characterization including an analysis on the effect of transfection of the BLNK gene into HPB-NULL cells are clearly necessary. However, experiments using these cell lines will be of assistance in understanding the signalling mechanism in early B-cell development.

ACKNOWLEDGMENTS

We thank M. Sone and S. Yamauchi for their excellent secretarial work. This work was supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan and MEXT. KAKENHI 15019129, JSPS. KAKENHI 15390133 and 15590361. This work was also supported by a grant from the Japan Health Sciences Foundation for Research on Health Sciences Focusing on Drug Innovation. Additional support was provided by a grant from the Sankyo Foundation of Life Science. T. Taguchi is in receipt of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research (Japan) for the 2nd Term Comprehensive 10-Years-Strategy for Cancer Control.

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HYBRIDOMA AND HYBRIDOMICS
Volume 23, Number 3, 2004

Development of Novel Monoclonal Antibody 4G8 against Swine Leukocyte Antigen Class I α Chain

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ABSTRACT

A mouse monoclonal antibody (MAb) was generated against swine leukocyte antigen (SLA) class I α chain. A newly developed series of MAb clones that react with pan leukocytes were selected and tested by immunohistochemistry using SLA class I α chain expressing Cos-7 cells. Among them, MAb 4G8 was characterized by the following features: (1) 4G8 reacted with Cos-7 cells transfected with SLA class I α chain from the *d* haplotype, (2) 4G8 recognized epitopes that were different from those of commercially available anti-SLA class I MAbs 74-11-10 and PT85A, and (3) 4G8 could be used to immunostain frozen sections of thymus, spleen, lymph node, kidney, and liver tissues with good results.

INTRODUCTION

THE PORCINE SYSTEM has received much attention as a suitable model for transplantation medicine. Therefore, an accurate understanding of human immune responses to porcine tissues has become increasingly important. However, the details of the porcine immune system, especially those features that are novel to the pig, remain unclear. We thus attempted to develop new MAbs that could be used to analyze the porcine immune system.

The immune response to foreign antigens is determined by the expression of specific major histocompatibility complex (MHC) molecules that can bind and present peptide fragments of that protein to T cells. There are two different types of MHC gene products, termed Class I and Class II MHC molecules, and any given T cell recognizes foreign antigens bound to only one Class I or Class II MHC molecule. Antigens associated with Class I molecules are recognized by $CD8^+$ cytolytic T cells, whereas class II–associated antigens are recognized by $CD4^+$ helper T cells. Class I molecules are located on every nucleated cell surface, except those of neurons and trophoblasts. In contrast, the expression of Class II molecules is limited to certain cell types. In pigs, MHC molecules are known as swine leukocyte antigens (SLA). All SLA class I molecules contain two separate polypeptide chains: an MHC-encoded α chain of 45 kD and a non–MHC-encoded β chain of 12 kD.

Recently, the profound involvement of SLA Class I molecules in human anti-porcine cell reactions has been described. Several studies have shown that human T cells can directly recognize porcine MHC molecules and that such recognition can lead to the killing of the porcine cells. Porcine cells have recently been shown, moreover, to be targets for human NK cells. Since human MHC class I molecules deliver a negative signal to human NK cells, protecting syngeneic cells from lysis, we surmised that differences in the gene sequences of porcine MHC class I molecules may be responsible for the lack of recognition by human NK cell receptors and subsequent cytolysis of the porcine cells. In addition, it was reported that a single treatment with a monoclonal antibody (MAb) directed against the SLA class I provides an attractive approach to the induction of T cell tolerance, possibly enabling long-term graft survival in porcine-to-human cell transplantations.[1] These studies indicate that SLA class I molecules play critical roles in transplantation medicine.

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Here, we report a novel MAb 4G8 against the SLA class I α chain that was proven to be different from four commercially available anti-SLA class I MAbs. The utilization of 4G8 in tissue sections was also examined.

MATERIALS AND METHODS

Animals and tissues

Landrace or (Landrace × Large White) F1 pigs were used in this study. Peripheral blood (PB) and tissues were obtained from anesthetized animals and were processed. PB was collected in acid citric buffer to avoid coagulation. Tissues were immediately snap frozen and kept in the deep freezer until use.

Monoclonal antibodies

PB leukocytes were treated using RBC lysis with NH_4Cl lysis buffer followed by centrifugation at 1,500 rpm for 10 min. After washing twice in phosphate-buffered saline (PBS), approximately 1×10^8 cells were injected into the abdominal cavity of 8-week-old female Balb/c mice. Boost injections were performed twice at 2-week intervals. At 4 days after the last boost, splenocytes were fused with P3U1 mouse myeloma cells and incubated in hypoxanthine and thymidine (HAT) medium. Supernatants of growing hybridomas were screened on porcine PB leukocytes by flow cytometry and clones secreting antibodies reactive with porcine PB leukocytes were subcloned twice by limiting dilution. Clones were grown in the abdominal cavity of Pristane-treated Balb/c mice, and ascites were obtained. Purification of MAbs was performed by Protein-A or Protein-G column (Bio-Rad Laboratories, Hercules, CA). After purification, MAb was fluorescence isothiocyanate (FITC) conjugated as described previously.[2] Commercially available MAbs against SLA class I 74-11-10, PT85A, H17A*, H58A* (* indicates known as cross-reactive with pig and other species) were obtained from Veterinary Medical Research and Development (Pullman, WA).

Flowcytometry and immunohistochemistry

Flowcytometrical analysis of MAbs was carried out as follows. Briefly, aliquot of porcine PB was incubated with appropriate amount of MAb for 30 min at 4°C. After washing with PBS, cells were incubated with FITC-conjugated (Jackson Laboratory, West Grove, PA) for 30 min at 4°C. Cells were washed with PBS and analyzed by EPICS XL analyzer (Beckman/Coulter, Westbrook, MA).

Reactivity of MAbs on tissues were analyzed by immunohistochemistry on frozen sections. Briefly, porcine tissues were snap-frozen in optimal cutting temperature (OCT) compounds and frozen sections were made by cryostat apparatus. Sections were fixed by acetone for 15 min at 4°C. After washing in PBS and blocked with normal rabbit serum, sections were incubated with MAbs at appropriate dilutions for 30 min at room temperature. Sections were then washed with PBS and incubated with horseradish peroxidase (HRP)–conjugated rabbit anti-mouse antibodies (Jackson Laboratory) for 30 min at room temperature. After washing with PBS, color development was done in diaminobenzidine solution (10 mM in 0.05 M Tris-HCl, PH 7.5) with 0.003% H_2O_2.

Binding competition assay

Binding competition assay was carried out as follows. Briefly, after aliquot of porcine PB leukocytes were incubated with 2ug saturated amount of commercially available MAbs for 30 min at 4°C. The cells saturated with these commercially available MAbs were stained with FITC-4G8 for 30 min at 4°C. Then, PB leukocytes were treated using RBC lysis with NH_4Cl lysis buffer followed by centrifugation at 1,500 rpm for 10 min. FITC-mouse immunoglobulin (MsIg) was used as control antibody. Cells were washed with PBS and analyzed by EPICS XL analyzer (Beckman/Coulter).

Cloning and expression of porcine cDNA library

As another purpose for analysis of $\gamma\delta$TCR against MAb (7G3) and CD8 against MAb (6F10), cDNA libraries of 7G3-positive as well as 6F10-positive PB leukocytes were first constructed. A brief description is shown below. Porcine PB labeled with FITC-7G3 antibody was incubated with magnetic-activated cell sorting (MACS) beads conjugated with anti-FITC antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) and was loaded onto AutoMACS cell separator (Miltenyi Biotec). 7G3-positive cells were positively selected and a cDNA library was constructed using the oligo-capping method[3] and plasmid vector pME18S-FL3, which contains the SR-α promoter for expression in mammalian cells. To 7G3-negative pass-through fractions, FITC-6F10 was added and labeled. These cells were also positively selected by AutoMACS and used for the cDNA library construction. Out of several thousand clones sequenced from both cDNA libraries, one clone was selected which exhibited homologies to known porcine MHC class I sequences from d haplotype and contained full-length open reading frames.

Complementary DNA coding for porcine MHC class I under SRα promoter was introduced into COS7 cells by lipofec-

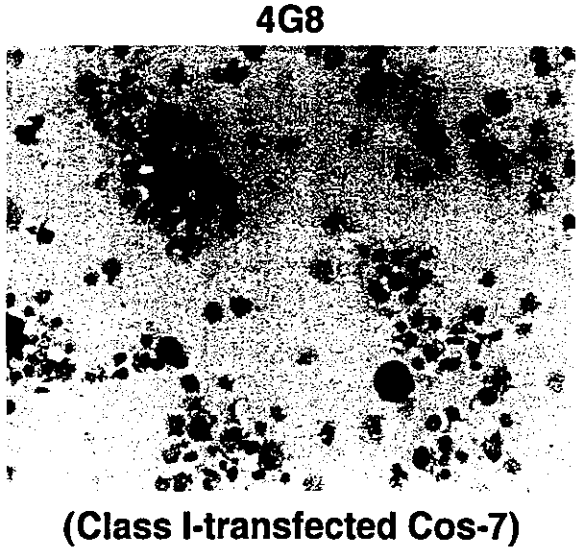

FIG. 1. Reactivity of 4G8 on Cos-7 cells transfected with SLA class I PD1. Mammalian expression vectors containing SLA class I PD1 were introduced into Cos-7 cells, and the cells were stained with 4G8 using immunohistochemistry.

tion (LIPOFECTAMIN, Invitrogen, Groningen, Netherlands) and cells were stained with 4G8 MAb after 3 days.

RESULTS AND DISCUSSION

Anti-SLA Class I MAb 4G8 recognizes a distinct epitope from those of commercially available antibodies

From one hybridization experiment, 45 hybridoma clones were established. The MAbs produced by these clones reacted differently to the porcine PB leukocytes, as revealed by flow cytometry (data not shown). To determine whether a MAb against SLA class I α chain was included among these clones, MAb clones that reacted with pan leukocytes were selected and tested by immunohistochemistry using SLA class I α chain expressing Cos-7 cells. As shown in Figure 1, when a mammalian expression vector of SLA class I PD1 from d haplotype was transfected into Cos-7 cells, clone 4G8 was found to stain the cells, whereas control MsIg failed to stain the cells (data not shown). Therefore, 4G8 was considered to recognize the SLA class I α chain, including the d haplotype.

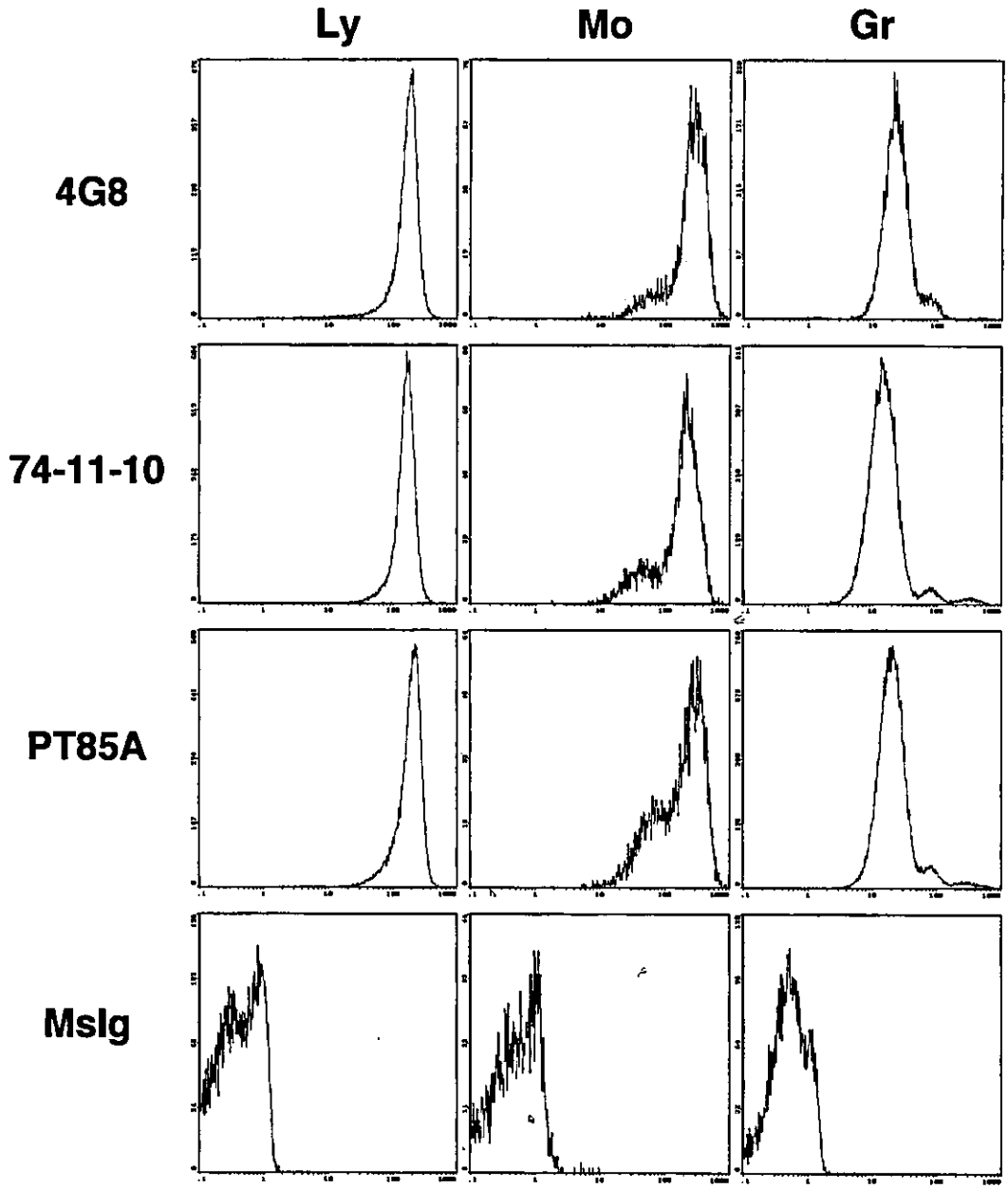

FIG. 2. Comparison of reactivity profiles of 4G8 and commercially available anti-SLA class I monoclonal antibodies. Porcine PB leukocytes were stained with 4G8 and commercially available anti-SLA class I MAbs, 74-11-10, and PT85A, using flow cytometry. MsIg was used as a control antibody.

Next, we compared the reactivity of 4G8 with commercially available anti-SLA class I Abs 74-11-10, PT85A, H17A, H58A to PB from outbred domestic pigs. As shown in Figure 2, flow-cytometrical analysis demonstrated that the reactivity of 74-11-10 and PT85A to the porcine PB was similar to that of 4G8. Although multiple samples from individual animals were tested, all of these MAbs always revealed a pan leukocyte positive staining pattern. In contrast, H17A did not react with the domestic porcine PB samples (data not shown), indicating that H17A is polymorphic in pigs. H58A, exhibited variable reactivity from one animal to another (data not shown). This data indicates that the reactivity of H17A and H58A is different from that of 4G8.

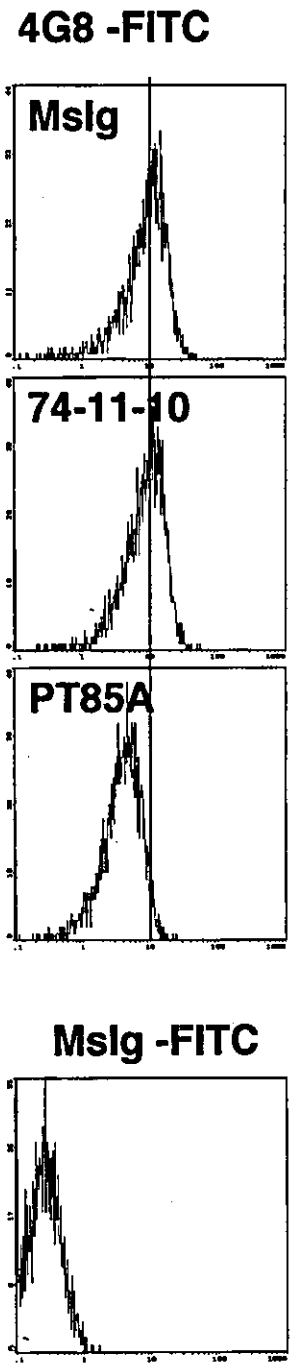

FIG. 3. Competition binding assay using flow cytometry. 4G8 continued to react with porcine PB leukocytes that had been saturated with commercially available MAbs (74-11-10 and PT85A). MsIg-FITC was used as a negative control.

To determine whether the epitope recognized by 4G8 was the same as that of 74-11-10 and PT85A, we examined whether 4G8 could still bind to the cells after the cells had been saturated with 74-11-10 or PT85A. As shown in Figure 3, 4G8 continued to react with PB leukocytes that had been saturated with 74-11-10 or PT85A. These results indicate that 4G8 recognizes a novel epitope distinct from those recognized by 74-11-10, PT85A, H17A and H58A. MHC class I molecules are extremely polymorphic, and polymorphism often occurs in the α1 domain, or the α2 domain, but the α3 domain is nonpolymorphic.[4] 74-11-10 and PT85A have been reported to require the PD1 α1/α2 domains, but not the α3 domain, to exhibit reactivity.[1] A precise analysis to clarify which domain is recognized by 4G8 is now underway.

Analysis of 4G8 using immunohistochemistry

We examined whether 4G8 could be used to immunostain frozen sections. As shown in Figure 4, 4G8 produced high-quality immunostaining results when used on frozen sections. In thymus tissues, 4G8 produced a dense and diffuse staining pattern in the medullar region and a lighter, scattered staining pattern in the cortex, suggesting that SLA class I molecules are mainly expressed on mature thymocytes in the medulla, but not on immature thymocytes in the cortex. In spleen, lymph node, kidney and liver tissues, 4G8 produced an ubiquitous staining pattern, as shown in Figure 4. These results demonstrate that 4G8 can be effectively used to immunostain frozen sections. Therefore, 4G8 may be a useful reagent for immunopathology studies and improving our general understanding of the porcine immune system.

In conclusion, a novel MAb, 4G8, that recognizes the SLA class I α chain has been identified and used to produce high-quality immunostaining results on tissues sections. 4G8 is expected to become a useful tool for investigating the immune system of domestic pigs.

ACKNOWLEDGMENTS

We thank M. Sone and S. Yamauchi for their excellent secretarial work. This research was supported in part by Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan and MEXT (KAKENHI 15019129, JSPS. KAKENHI 15390133 and 15590361). Support was also provided by a grant from the Japan Health Sciences Foundation for Research on Health Sciences Focusing on Drug Innovation and a grant from the Sankyo Foundation of Life Science.

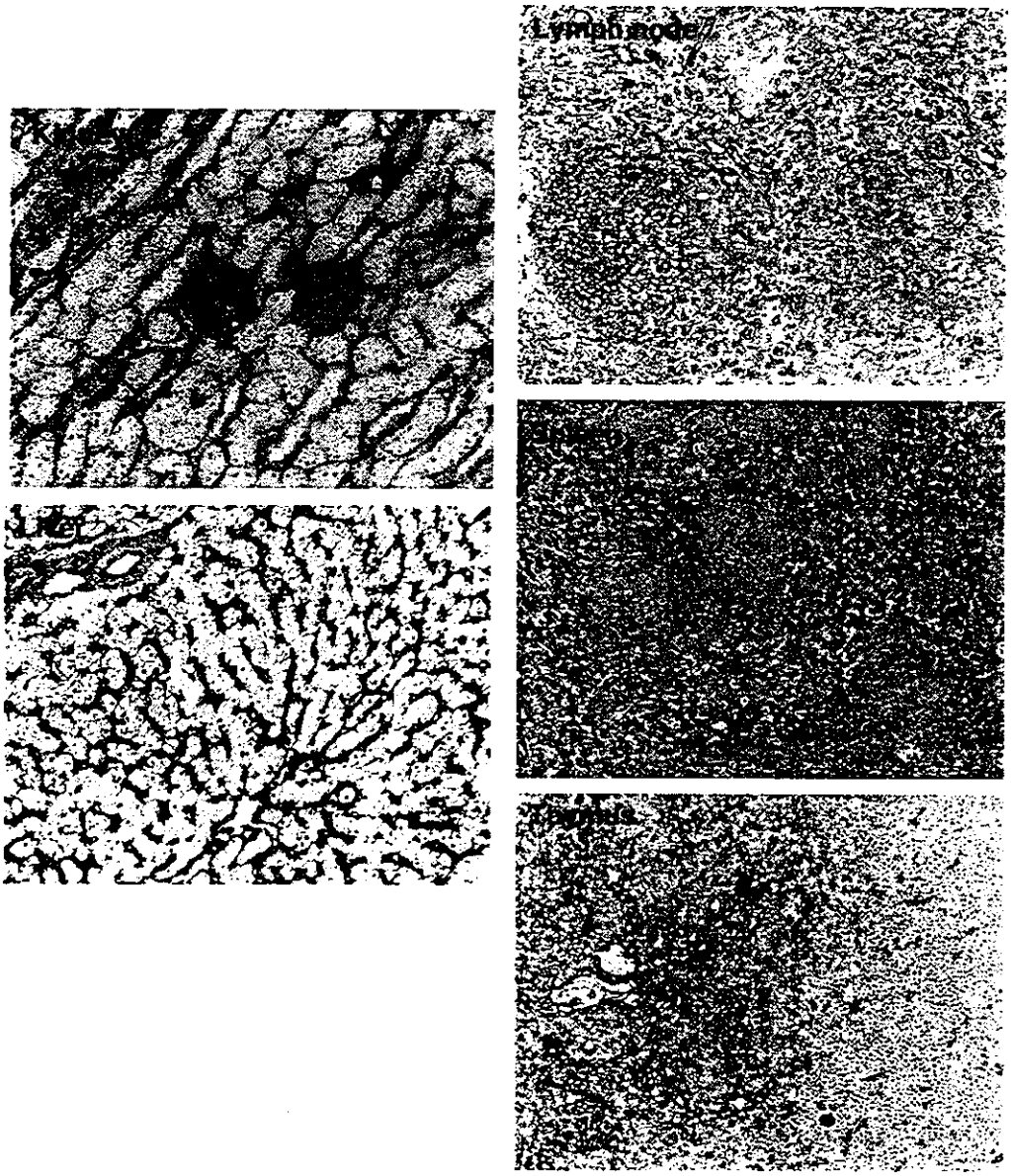

FIG. 4. Reactivity of 4G8 on frozen sections of porcine tissues. Frozen sections of porcine thymus, spleen, lymph node, kidney, and liver tissues were stained with 4G8 using immunohistochemistry.

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Received for publication February 27, 2004. Accepted for publication March 24, 2004.

Shiga toxin binding to globotriaosyl ceramide induces intracellular signals that mediate cytoskeleton remodeling in human renal carcinoma-derived cells

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Accepted 6 April 2004
Journal of Cell Science 117, 3911-3922 Published by The Company of Biologists 2004
doi:10.1242/jcs.01246

Summary

Shiga toxin is a bacterial toxin consisting of A and B subunits. Generally, the essential cytotoxicity of the toxin is thought to be mediated by the A subunit, which possesses RNA cleavage activity and thus induces protein synthesis inhibition. We previously reported, however, that the binding of the Shiga toxin 1-B subunit to globotriaosyl ceramide, a functional receptor for Shiga toxin, induces intracellular signals in a manner that is dependent on glycolipid-enriched membrane domains, or lipid rafts. Although the precise role of this signaling mechanism is not known, here we report that Shiga-toxin-mediated intracellular signals induce cytoskeleton remodeling in ACHN cells derived from renal tubular epithelial carcinoma. Using confocal laser scanning microscopy, we observed that Shiga toxin 1-B treatment induces morphological changes in ACHN cells in a time-dependent manner. In addition, the morphological changes were accompanied by the redistribution of a number of proteins, including actin, ezrin, CD44, vimentin, cytokeratin, paxillin, FAK, and α- and γ-tubulins, all of which are involved in cytoskeletal organization. The transient phosphorylation of ezrin and paxillin was also observed during the course of protein redistribution. Experiments using inhibitors for a variety of kinases suggested the involvement of lipid rafts, Src family protein kinase, PI 3-kinase, and RHO-associated kinase in Shiga toxin 1-B-induced ezrin phosphorylation. Shiga toxin 1-B-induced cytoskeletal remodeling should provide an in vitro model that can be used to increase our understanding of the pathogenesis of Shiga-toxin-mediated cell injury and the role of lipid-raft-mediated cell signaling in cytoskeletal remodeling.

Key words: Shiga toxin, Globotriaosylceramide, Cytoskeleton

Introduction

Shiga toxin (Stx) is a protein toxin produced by Stx-producing strains of *Escherichia coli* (STEC) and has been postulated to be the substance responsible for the development of serious complications associated with STEC infection, such as hemolytic uremic syndrome (HUS) (Kaplan et al., 1990; Richardson et al., 1988). Stx consists of two major types, Stx1 and Stx2, that both contain an A-subunit monomer and a B-subunit pentamer (Lingwood, 1996). The A-subunit is a 30 kDa cytotoxic chain that exhibits RNA *N*-glycohydrolase activity and cleaves a specific adenine residue on the 28S ribosomal RNA in the cytosol, thereby inhibiting protein synthesis (Lingwood, 1996). In contrast, the 7 kDa B-subunit can bind to globotriaosyl ceramide (Gb3), the functional receptor for Stx found on the surface of target cells (Lingwood, 1996). Although Stx cytotoxicity is thought to be caused by the A-subunit-mediated inhibition of protein synthesis, a number of recent studies have clarified that the B-subunit also has a biological effect on the target cells.

For example, Stx-B binding to Gb3 has been shown to trigger intracellular signaling events in Burkitt's lymphoma cells (Taga et al., 1997). We further observed that Gb3 was only distributed in glycolipid-enriched membrane (GEM) domains (Rodgers and Rose, 1996), also known as lipid rafts (Simons and Ikonen, 1997), indicating that lipid rafts are deeply involved in Stx-mediated signal transduction in both Burkitt's lymphoma cells (Mori et al., 2000) and renal carcinoma ACHN cells (Katagiri et al., 1999). However, the physiological role of Gb3-mediated cell signaling remains unknown. Although Stx1-B binding is sufficient to induce apoptosis in Burkitt's lymphoma cells (Kiyokawa et al., 2001; Mangeney et al., 1993; Taga et al., 1997), this effect of Stx1-B appears to be limited to this species of cells, and the A-subunit of Stx is reportedly required to induce apoptosis in other cell types, including Vero cells and monocytic THP-1 cells (Kojio et al., 2000; Williams et al., 1997).

Ezrin, radixin and moesin are members of the ERM protein family and are mainly distributed just beneath the plasma membranes of cellular protrusions, such as microvilli. ERM proteins are thought to function as general cross-linkers between plasma membranes and actin filaments (Arpin et al., 1994; Bretscher et al., 1997; Tsukita and Yonemura, 1997; Tsukita et al., 1997; Tsukita and Yonemura, 1999). The highly conserved NH2-terminal halves of ERM proteins possess the