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Specific immunoglobulin E responses in ZAP-70-deficient patients are mediated by Syk-dependent T-cell receptor signalling

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SUMMARY

ZAP-70 deficiency is a rare primary immunodeficiency characterized by the absence of peripheral $CD8^+$ T cells and defects in T-cell receptor (TCR) signalling. T cells in ZAP-70-deficient patients are assumed to have no helper functions for B-cell immunoglobulin synthesis, whereas the patients rarely have antigen-specific antibodies. We experienced a ZAP-70-deficient patient, who had immunoglobulin E (IgE) antibodies specific to food allergens, and we investigated the mechanisms of switching to IgE in the patient. Peripheral blood mononuclear cells from the patient did not proliferate upon stimulation with the antigens but produced distinct levels of interleukin-4 (IL-4). Cell sorting analysis indicated that the cells that produced IL-4 in response to the antigens were enriched in $CD4^+$ T cells. Purified $CD4^+$ T cells from the patient produced IL-4 and expressed CD40L upon stimulation with anti-CD3. Moreover, $CD4^+$ T cells pretreated with anti-CD3 induced mature ε transcript on naive B cells. Since the results indicated that there remained sufficient T-cell receptor (TCR)-signalling in the patient's T cells to exert antigen-specific IgE switching on B cells, we next investigated the expression of the ZAP-70-homologous kinase Syk. Syk was present in high levels in patient's $CD4^+$ T cells and was tyrosine-phosphorylated after TCR stimulation. Inhibition of Syk by piceatannol resulted in decreased production of IL-4 and expression of CD40L on patient's $CD4^+$ T cells. Moreover, Syk was expressed on all human T-cell leukaemia virus (HTLV-1)-transformed T-cell lines derived from peripheral blood of the patient, whereas it was low or undetectable in control lines. It was therefore concluded that specific IgE responses in the patient were most likely to be mediated by Syk-dependent TCR-signalling.

INTRODUCTION

ZAP-70 deficiency is a rare type of severe combined immunodeficiency (SCID) characterized by the absence of $CD8^+$ T cells and by the presence of non-functional $CD4^+$ T cells in peripheral blood.[1,2] In patients with ZAP-70 deficiency, T cells cannot respond to T-cell receptor (TCR)-mediated stimuli because of mutations in a TCR-associated protein tyrosine kinase, ZAP-70.[1–3] Absent proliferation responses *in vitro* to allogeneic antigens and to mitogenic stimuli mediated through surface TCRs, such as anti-CD3, have been reported in patients with ZAP-70 deficiency. Because T-cell help for immunoglobulin synthesis in B cells is impaired, patients with ZAP-70 deficiency often show hypogammablobulinaemia and usually lack specific antibody production.[4,5] However, some patients have normal or increased levels of serum immunoglobulins and rarely have the ability to produce antigen-specific antibodies.[6,7]

Differentiation of naive B cells into antibody-secreting cells is largely controlled by the direct interaction of B cells with activated T cells expressing the CD40 ligand (CD40L)[8–10] and by cytokines secreted from activated T cells. Switching to each isotype of immunoglobulin is strongly influenced by the cytokine milieu where B cells respond with T cells. For example, interleukin-4 (IL-4) is a major cytokine that induces immunoglobulin E (IgE) switching on B cells.[11–13]

We report here a case of a ZAP-70-deficient patient who had IgE antibodies specific to food allergens. Although peripheral T cells in the patient did not proliferate upon TCR-mediated stimuli *in vitro*, the cells retained the capacity to produce IL-4 and to express CD40L in response to the stimuli. These two signals were sufficient to induce IgE switching on naive B cells. These findings suggested that there are alternative TCR-signalling pathways independent of ZAP-70. Our speculation is that ZAP-70-homologous kinase Syk is most likely to participate in the alternative TCR-signalling pathway.

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MATERIALS AND METHODS

Patient

An 8-month-old girl with a history of recurrent respiratory infection was admitted to our hospital. Flow cytometric analysis of her peripheral blood mononuclear cells (PBMNC) revealed a lack of $CD8^+$ T cells ($<1\%$) but a sufficient number of $CD4^+$ T cells (Fig. 1). The patient's PBMNC did not proliferate upon stimulation with phytohaemagglutinin (PHA), concanavalin A (con A), or anti-CD3 monoclonal antibody (mAb; anti-CD3) but responded to stimulation with phorbol myristate acetate (PMA) plus ionomycin (Table 1). These results suggested that the patient had ZAP-70-deficient SCID, and this was confirmed by the results of Western blots, which showed an absence of ZAP-70 protein in $CD4^+$ T cells (Fig. 2). DNA sequencing revealed that the genomic DNA of the patient contained a G to A homozygous transition at nucleotide position 1603, resulting in an arginine to histidine transition at position 465 of the kinase domain. Serum immunoglobulin levels were normal: 788 mg/dl IgG, 96 mg/dl IgA, 154 mg/dl IgM, and 183 IU/l IgE. At 6 months of age, no IgE antibodies specific to food allergens such as milk, soybean, and egg white were detected ($<0{\cdot}35$ IU/ml); however, IgE responses to egg white (2·46 IU/ml), milk (4·21 IU/ml), soybean (15·0 IU/ml), and wheat (2·43 IU/ml) were detected at 8 months of age. In spite of the presence of IgE antibodies specific to food allergens, she showed no obvious allergic reactions to the foods. Peripheral blood stem cell transplantation from her haploidentical father was performed twice at 9 and 10 months of age but engraftment was not successful.

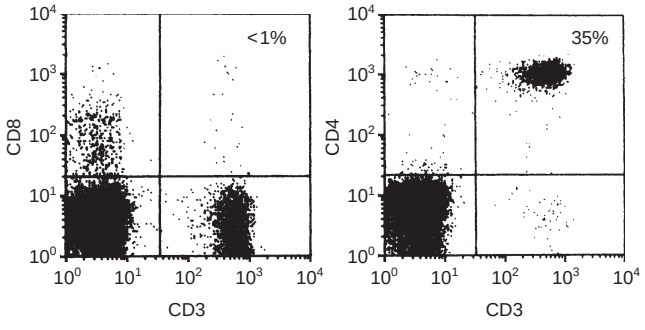

Figure 1. Results of flow cytometry of the patient's PBMNC. PBMNC of the patient were stained with FITC-or PE-conjugated anti-CD3, anti-CD4, and anti-CD8 mAb and then analysed using FACScan.

Table 1. Proliferation responses of the patient's PBMNC

Stimulation	Normal control (c.p.m.)	Patient (c.p.m.)
(–)	1080 ± 15	1250 ± 12
PHA	39780 ± 785	1578 ± 25
PMA + ionomycin	62570 ± 1428	47850 ± 875
Anti-CD3	33150 ± 658	1625 ± 22
Ovalbumin	NT	1440 ± 87
Ovomucoid	NT	1329 ± 54

The patient's PBMNC were stimulated with PHA (5 μg/ml), PMA (10 ng/ml) and ionomycin (1 μg/ml), biotinylated anti-CD3 (100 ng/ml) plus streptavidin, ovalbumin (25 μg/ml), or ovomucoid (25 μg/ml) for 3 days. Cell proliferation was assessed by incorporation of 3H-thymidine. Each value represents the mean value for three experiments ± SEM. NT, not tested.

Cell preparation and phenotype analysis

PBMNC were isolated by Ficoll–Hypaque gradient centrifugation. Cells were stained with fluoroscein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD3, anti-CD4, or anti-CD8 (Pharmingen, San Diego, CA) and analysed by two-colour flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). The cells positive for each antibody were separated using a preparative magnetic cell sorter (MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of each cell population was $>95\%$.

Analysis of ZAP-70 and Syk expression

Cell lysates of purified 1×10^6 $CD4^+$ T cells were analysed by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham-Pharmacia, Uppsala, Sweden). The blots were incubated with anti-ZAP-70 antibody (Santa-Cruz Biotechnology, Santa-Cruz, CA) and then visualized using ECL (Amersham-Pharmacia) according to the manufacturer's instructions.

Cell proliferation assay

PBMNC (2×10^5/well) were incubated at 37° in 5% CO_2 with RPMI-1640 in 96-well round-bottomed tissue culture plates with or without 5 μg/ml PHA, 5 μg/ml con A, 10 ng/ml phorbol 12-myristate 13-acetate (PMA) plus 1 μg/ml ionomycin, 100 ng/ml biotinylated anti-CD3 (Pharmingen) plus streptavidin, 25 μg/ml ovalbumin, or 25 μg/ml ovomucoid. Cell proliferation was assessed by the incorporation of ^{3}H-thymidine after 3 days.

IL-4 production

PBMNC or purified $CD4^+$ T cells (2×10^5/well) were stimulated with various mitogens and antigens as indicated in the cell proliferation assay for 2 days. In some cases, cells were cultured with 10 μM piceatannol[14] (Sigma, St. Louis, MO). The levels of IL-4 in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA; MBL, Nagoya, Japan).

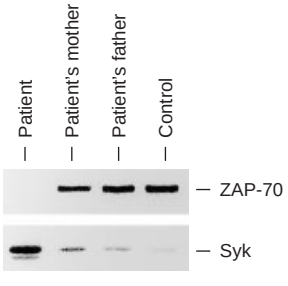

Figure 2. ZAP-70 and Syk expressions on $CD4^+$ T cells. $CD4^+$ T cells from the patient, her parents, and a normal control were purified from PBMNC using MACS. Cell lysates of the $CD4^+$ T cells (2×10^6 each) were analysed by SDS–PAGE and were subjected to anti-ZAP-70 and anti-Syk blots.

IL-4 mRNA expression

Cells positive for CD3, CD4, CD20, CD56, and FcεRI+ (CRA1; Kyokuto Pharmaceutical, Ibaraki, Japan) were purified from patient's PBMNC using MACS. Cells (10^6) were cultured with or without 25 μg/ml ovalbumin for 48 hr. After cultivation, total RNA was extracted from sorted cells using ISOGEN (Nippon gene, Tokyo, Japan) and reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase (BRL Live Technologies, Gaithersburg, MD) using oligo (dT) primers (BRL). IL-4 and β-actin cDNA were amplified by polymerase chain reaction (PCR) with specific primers (IL-4 sense primer, 5′-GATCGT-TAGCTTCTCCTGATAAACT-3′; IL-4 antisense primer, 5′-AGATTCTATATATACTTTATTTTAT-3′;[15] β-actin sense primer, 5′-CGTGACATCAAAGAGAAGCTGTG-3′;[16] and β-actin antisense primer, GCTCAGGAGGAGCAATGA-TCTTGA-3′). Thirty-five cycles of denaturation (94° for 45 s), annealing (60° for 45 s), and elongation (75° for 1·5 min) were performed in a thermocycler (Perkin-Elmer, Norwalk, CT). PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

CD40 ligand expression

Purified $CD4^+$ T cells (2×10^5) were stimulated with biotinylated anti-CD3 plus streptavidin in the presence or absence of 10 μM piceatannol for 3 hr. The cells were then stained with PE-conjugated anti-CD40L mAb (Pharmingen) and analysed using FACScan. The stained cells were sorted into $CD40L^-$ and $CD40L^+$ populations using FACS Vantage (Becton Dickinson). Cell lysates of the sorted cells (2×10^6 each) were analysed by SDS–PAGE and were subjected to anti-Syk blots as indicated above.

Induction and detection of germline and mature ε transcript on B cells

Surface IgD^+ B cells were purified using MACS as described above. One 10^6/ml $sIgD^+$ naive B cells from the patient were incubated with or without 15 ng/ml IL-4 (Genzyme, Cambridge, MA), 100 ng/ml anti-CD40 antibody (Serotec, Oxford, UK), or 1×10^6/ml $CD4^+$ T cells pretreated with biotinylated anti-CD3 plus streptavidin for 24 hr. After 10 days of cultivation, total RNA was extracted from the cells and subjected to reverse transcription (RT)–PCR analysis using specific primers for germline ε transcripts (sense primer, 5′-AGGCTCCACTGCCCGGCACAGAAAT-3′; antisense primer, 5′-ACGGAGGTGGCATTGGAGGGAATGT-3′), mature ε transcripts (sense primer, 5′-GACACGGCTGTG-TATTACTG-3′; antisense primer, 5′-ACGGAGGTGGCAT-TGGAGGGAATGT-3′)[17] and β-actin. PCR products were analysed by electrophoresis on 2% agarose gels followed by ethidium bromide staining.

Expression and tyrosine-phosphorylation of Syk and ZAP-70

Purified $CD4^+$ T cells were stimulated with biotinylated anti-CD3 and streptavidin for 2 min. Cells (10^6) were lysed in NP-40 lysis buffer (10 mM Tris–HCl, ph 7·4, 5 mM ethylenediamine tetra-acetic acid (EDTA), 150 mM NaCl, 0·1% SDS, 0·1% sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 μg/ml aprotinin, and 30 mM $Na_4P_2O_7$). Cell lysates were incubated with anti-ZAP-70, anti-Syk, or anti-TCR ζ antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and then with protein A-Sepharose (Amersham-Pharmacia). The precipitates were subjected to 10% SDS–PAGE and separated proteins were transferred to PVDF membranes (Amersham-Pharmacia). The blots were incubated with anti-ZAP-70, anti-Syk, anti-TCR ζ, or anti-phosphotyrosine antibodies (Santa-Cruz Biotechnology Inc.) and then visualized using ECL (Amersham-Pharmacia) according to the manufacturer's instructions.

Syk expression on HTLV-1-transformed T-cell lines

HTLV-1 transformation of PBMNC from the patient and normal individuals was performed as described elsewhere.[18] Cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and IL-2 (20 U/ml). Each HTLV-1 line expressed αβ-TCR and CD4 similarly. Cells (10^6) were lysed in NP-40 lysis buffer as described above. Cell lysates were analysed by 10% SDS–PAGE and Western blots using anti-Syk antibody.

RESULTS

IL-4 production by PBMNC in response to food allergens

Because IL-4 is one of the main cytokines that induces IgE switching on B cells,[11–13] we investigated the levels of IL-4 production by PBMNC taken from the patient. When the PBMNC were stimulated with PMA plus ionomycin over a period of 2 days, high levels of IL-4, comparable to those of controls, were detected in culture supernatants (Table 2). Stimulation of the PBMNC with ovalbumin and ovomucoid, which are major allergens of egg white,[19,20] resulted in the production of small but distinct amounts of IL-4, although no proliferation responses of PBMNC were observed by stimulation with these allergens (Table 1).

IL-4 production by $CD4^+$ T cells in response to food allergens

To clarify the lineage of the cells producing IL-4 in response to food allergens in the patient's PBMNC, cell sorting followed by RT–PCR analysis of IL-4 mRNA were performed. When PBMNC were fractionated by their surface marker, transcripts for IL-4 were detected in $CD3^+$ and $CD4^+$ T cells but not in $FcεRI^+$ cells after stimulation with OVA (Fig. 3). Moreover, purified T cells, especially $CD4^+$ T cells, produced IL-4 in response to anti-CD3 (Table 2). These results indicated that $CD4^+$ T cells, but not $FcεRI^+$ cells, produced IL-4 in response to the allergens.

CD40L expression on TCR-stimulated $CD4^+$ T cells

Besides the action of cytokines, switching to IgE requires direct contact between T cells and B cells through interaction with CD40 and CD40L.[8–10] We therefore investigated the expression of CD40L on the patient's $CD4^+$ T cells, which were stimulated with anti-CD3 for 3 hr. CD40L expression was recognized after 1 hr of TCR stimulation and continued to increase up to 6 hr. Figure 4 shows the results at 3 hr of cultivation. On the other hand, there were no proliferation responses of T cells as well as $CD4^+$ T cells to TCR stimulation (Table 1). These results indicated that the patient's $CD4^+$

Table 2. IL-4 production by PBMNC and $CD4^+$ T cells

	PBMNC (pg/ml)		$CD4^+$ T cells (pg/ml)	
Stimulation	Control	Patient	Control	Patient
(–)	<2·0	<2·0	<2·0	<2·0
PMA + ionomycin	2542±123	2854±151	3745±873	2538±1025
Anti-CD3	1423±151	583±54	2523±622	1785±211
Ovalbumin	NT	85±12	NT	NT
Ovomucoid	NT	73±18	NT	NT
Anti-CD3 + Piceatannol	NT	NT	2055±855	173±98

The patient's PBMNC and purified $CD4^+$ T cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml), biotinylated anti-CD3 (100 ng/ml) plus streptavidin, ovalbumin (25 μg/ml), or ovomucoid (25 μg/ml) in the presence or absence of 10 μM piceatannol for 2 days. The amount of IL-4 in culture supernatants was measured by ELISA. Each value represents the mean value for three experiments ± SEM. NT, Not tested.

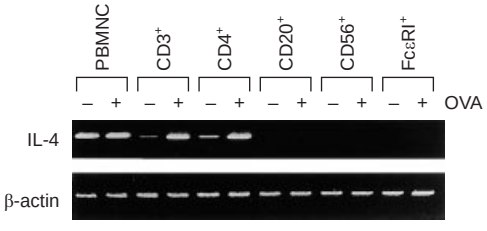

Figure 3. IL-4 mRNA expressions on various cell populations of the patient's PBMNC. Cells positive for CD3, CD4, CD20, CD56, or FcεRI were purified using MACS. Each cell population was stimulated with (+) or without (–) OVA (25 μg/ml) for 48 hr. Total RNA was extracted from 1×10^6 cells and subjected to RT–PCR analysis for IL-4 and β-actin mRNA expression.

T cells had the capacity to express CD40L and produce IL-4 in response to TCR-mediated stimuli without cell proliferation.

Induction of germline and mature ε transcripts on naive B cells by $CD4^+$ T cells

Because these two signals, namely IL-4 production and CD40L expression, are thought to be sufficient to evoke IgE class switching on B cells, we investigated whether TCR-stimulated T cells really induce IgE transcripts in naive B cells. When purified naive $sIgD^+$ B cells were cultured in the presence of IL-4, germline ε transcripts were induced (Fig. 5). A combination of IL-4 and cross-linking of CD40 on B cells by anti-CD40 mAb induced the expression of mature ε transcripts as well as germline ε transcripts. On the other hand, TCR-stimulated $CD4^+$ T cells from the patient could substitute for these two stimuli and could induce germline and mature ε transcripts on naive B cells.

Expression of Syk in $CD4^+$ T cells and its tyrosine-phosphorylation after TCR stimulation

Since it was thought that there remained signalling pathways via TCR in the patient's T cells that bypassed ZAP-70, we investigated the expression of the ZAP-70-homologous kinase Syk. High levels of Syk were detected in the patient's $CD4^+$ T cells by Western blot analysis, whereas faint or undetectable in normal controls (Fig. 2). In $CD4^+$ T cells of the patient, Syk was expressed predominantly in the cells that expressed high levels of CD40L in response to TCR stimulation (Fig. 6). We next investigated the tyrosine-phosphorylation and association with TCR ζ chain of Syk in $CD4^+$ T cells of the patient

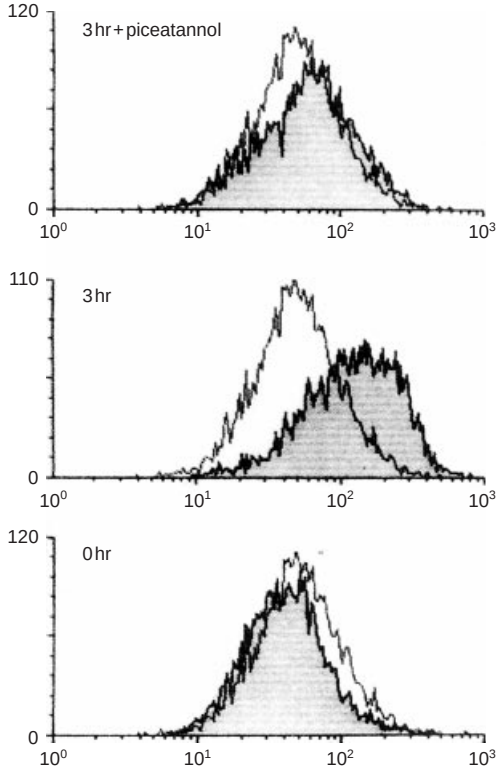

Figure 4. CD40L expression on TCR-stimulated $CD4^+$ T cells of the patient. Purified $CD4^+$ T cells were stimulated with biotinylated anti-CD3 plus streptavidin for 3 hr with or without 10 μM piceatannol. Cells were stained with PE-conjugated anti-CD40L antibody and were analysed using FACScan. The clear histogram indicated CD40L expression before stimulation, whereas the shaded histogram corresponded to that after stimulation.

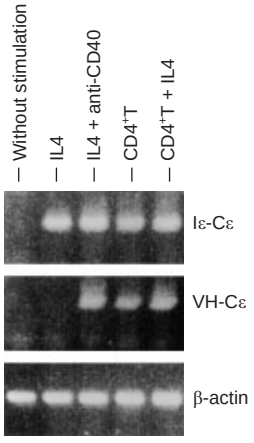

Figure 5. Induction of germline and mature ε transcripts on naive B cells by the patient's T cells. $CD4^+$ T cells were prestimulated with biotinylated anti-CD3 plus streptavidin for 24 hr. Purified IgD^+ naive B cells were cultured with IL-4, anti-CD40, or the preactivated $CD4^+$ T cells for 10 days. Total RNA was extracted from the cells and subjected to RT–PCR for the expression of germline (Iε-Cε) and mature (VH-Cε) ε transcripts.

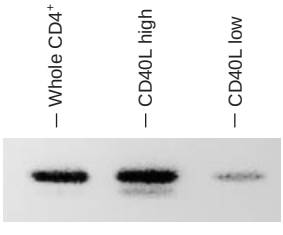

Figure 6. Syk expression on patient's $CD4^+$ T cells that express high levels of CD40L. Purified $CD4^+$ T cells of the patient were stimulated with biotinylated anti-CD3 plus streptavidin for 3 hr. The cells were stained with PE-conjugated anti-CD40L antibody and were sorted into $CD40L^+$ (CD40L high) and $CD40L^-$ (CD40L low) populations using FACS Vantage. Cell lysates of the sorted cells (2×10^6 each) were analysed by SDS–PAGE and were subjected to anti-Syk blots.

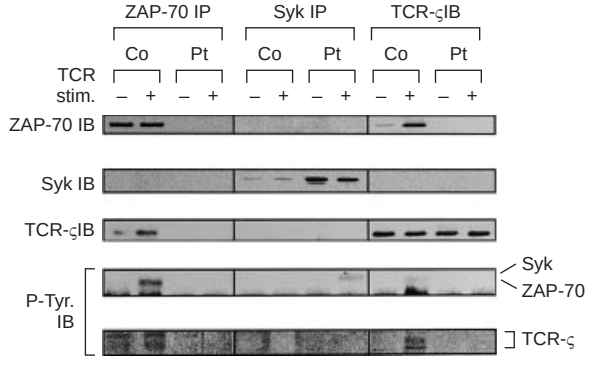

Figure 7. Expression and tyrosine phosphorylation of Syk, ZAP-70, and TCR ζ chain in the patient's $CD4^+$ T cells. $CD4^+$ T cells obtained from the patient and normal control were cultured with (+) or without (–) biotinylated anti-CD3 plus streptavidin for 2 min. The cells (2×10^6 each) were lysed in 1% NP-40 lysis buffer, and immunoprecipitated with anti-Syk, anti-ZAP-70, and anti-TCR ζ antibodies. Immunoprecipitates were resolved on 10% SDS–PAGE and immunoblotted with anti-Syk, anti-ZAP-70, and anti-TCR ζ, and anti-phosphotyrosine (P-Tyr.) antibodies.

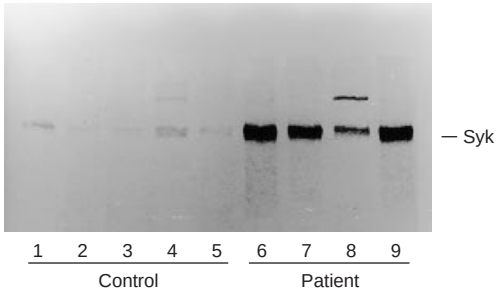

Figure 8. Syk expression on T-cell lines. HTLV-1-transformed T-cell lines were established from the patient and normal control. Cell lysates of T-cell lines (2×10^6 each) obtained from the patient (lanes 6–9) and control (lanes 1–5) were analysed by SDS–PAGE and were subjected to anti-Syk blot.

after TCR stimulation by immunoprecipitation followed by immunoblotting. Syk immunoprecipitates were tyrosine-phosphorylated after TCR stimulation of the patient's $CD4^+$ T cells (Fig. 7). The ζ chain was not tyrosine-phosphorylated after TCR stimulation and there was no association between Syk and the ζ chain.

Syk expression in T-cell lines derived from the patient

We hypothesized that high levels of Syk expression in the whole lysate of the patient's $CD4^+$ T cells reflect increased numbers of T cells, which expressed high levels of Syk. To test this hypothesis, we established HTLV-1-transformed T-cell lines from the patient and controls, and we studied the Syk expression in each cell line. As shown in Fig. 8, Syk expression was low or undetectable in five control cell lines, whereas every cell line obtained from the patient expressed high levels of Syk. These results suggested that the number of $CD4^+$ T cells that expressed high levels of Syk was increased in the peripheral blood of the patient.

DISCUSSION

In the present study, we found that T cells in a ZAP-70-deficient patient have the capacity to induce antigen-specific IgE production from B cells in response to TCR stimulation. $CD4^+$ T cells from our patient produced IL-4 in culture supernatants and expressed CD40L on their surfaces in response to TCR stimulation. These two signals are necessary, and sufficient, for induction of IgE class switching on naive B cells. In fact, co-cultivation of prestimulated $CD4^+$ T cells with naive B cells resulted in the induction of germline and mature ε transcripts. These results suggested that $CD4^+$ T cells in our patient were able to induce switching to IgE on naïve B cells and subsequent IgE production from B cells *in vitro*. It is unlikely that cells other than $CD4^+$ T cells were engaged in IgE switching, because only $CD4^+$ T cells, among various cells in peripheral blood, produced IL-4 in response to food antigens. As well as T cells, it is known that $Fc\varepsilon RI^+$ cells, namely,

basophils, eosinophils and monocytes, have the capacity to induce IgE switching on naïve B cells. These cells can produce IL-4[21–23] and express CD40L[24,25] after cross-linking of FcεRI. Therefore, antigen-specific activation of FcεRI$^+$ cells requires antigen-specific IgE antibodies that are bound to multivalent antigens. In the absence of antigen-specific IgE antibodies, FcεRI$^+$ cells cannot produce IL-4 in response to antigens. The fact that food-specific IgE antibodies in the serum of the patient were not detected at 6 months of age but were detected at 8 months of age suggests that *de novo* synthesis of antigen-specific IgE occurred after exposure to food allergens. Moreover, IL-4 production by peripheral blood FcεRI$^+$ cells of the patient was not observed by cell sorting followed by RT–PCR. Thus, FcεRI$^+$ cells were not to be candidate helper cells for IgE production in our patient.

Previous studies have demonstrated that peripheral blood CD4$^+$ T cells of ZAP-70-deficient patients are not functional cells.[2,4] However, we found that the peripheral T cells in our patient were partially functional, although there were no proliferation responses of these cells to TCR-mediated stimuli. The existence of the functional T cells cannot be attributed to engraftment of maternal lymphocytes,[26–28] because maternal cells were not detected in the patient's PBMNC by sensitive PCR analysis of polymorphic short tandem repeat markers[29] (data not shown). This kind of T-cell activation, namely partial activation without cell proliferation, is observed when T cells respond to ligands as partial agonists.[30] Partial agonists have subtle alterations in their peptide sequences and evoke incomplete activation of T cells in the absence of cell proliferation, for example, cytokine production,[31,32] expression of surface molecules,[33] or induction of anergy.[34] Interestingly, tyrosine-phosphorylation of ZAP-70 and the ζ chain are impaired in partial agonism in spite of induction of the mobilization of a small amount of intracellular Ca^{2+}.[35] Thus, it is thought that there are other signalling pathways that bypass the ζ chain and ZAP-70 also in normal T cells.

Our results suggest that Syk can compensate for the loss of ZAP-70 not only in thymocytes[5] but also in peripheral blood T cells. Syk was present in high levels in peripheral blood T cells as well as T-cell lines that were obtained from the patient. After TCR stimulation, the extent of CD40L induction in patient's T cells was correlated with the levels of Syk expression. TCR stimulation of the patient's T cells resulted in tyrosine phosphorylation of Syk. Moreover, inhibition of Syk by piceatannol[14] aborted IL-4 production and CD40L induction on stimulated T cells of the patient. Previous studies have shown that Syk is predominantly expressed on B cells, myeloid cells, and thymocytes but is expressed at only very low levels on peripheral T cells.[36] However, the overall low expression of Syk in peripheral blood T cells does not imply the absence of T cells that express high levels of Syk. Actually, it represents the average of the majority of T cells that express low or undetectable levels and a small subpopulation of T cells that express high levels of Syk.[37] It is therefore thought that the high levels of Syk expression in the patient's T-cell population reflect an increased number of T cells in the peripheral blood that expressed high levels of Syk. This speculation was supported by the fact that every T-cell line from the patient expressed high levels of Syk, whereas control lines expressed low or undetectable levels of Syk. The roles of Syk in normal T cells are less clear, because the levels of ZAP-70 in the T cells with high Syk expression are comparable to those in T cells with low Syk expression, and therefore the roles of Syk are masked by ZAP-70.[37] However, functional similarities between Syk and ZAP-70 are obvious in experiments using mice deficient in Syk and ZAP-70. Syk can restore the development of thymocytes in ZAP-70-deficient mice,[38] and ZAP-70 can reconstitute the BCR function in Syk-deficient B cells.[39] Accordingly, it was likely that Syk compensated for the loss of ZAP-70 not only in thymocytes but also in peripheral blood T cells in our patient.[40]

There was no association between Syk and the ζ chain in activated T cells in our patient. Because no association has been found between Syk and the ζ chain in thymocyte lines of ZAP-70-deficient patients,[5] this phenomenon does not seem to be unique to peripheral blood T cells in our patient. It is not known how Syk was tyrosine-phosphorylated after TCR stimulation in the absence of phosphorylation of the ζ chain in our patient. One possibility for this phenomenon is that interaction between Syk and the ζ chain is so transient and weak that it cannot be detected by the method we used.[5] Another explanation is that there are other TCR-signalling pathways that bypass the ζ chain, such as is observed in partial agonism as described above.[40]

It is not clear whether the compensation of T-cell functions by Syk is a common phenomenon in ZAP-70-deficient patients. Most ZAP-70-deficient patients lack specific antibody production and some patients show severe hypogammaglobulinaemia, which requires γ-globulin replacement therapy.[1,2] Occasionally, ZAP-70-deficient patients show normal or increased levels of serum immunoglobulin and rarely have the ability to produce antigen-specific antibodies.[6,7] These studies suggested that the contribution by T cells to antibody production is not a common phenomenon in ZAP-70-deficient patients. On the other hand, Katamura *et al.* reported a patient with ZAP-70 deficiency who had CD4$^+$ T cells with memory phenotypes in the peripheral blood and in the perivascular area of erythematous skin lesions.[41] The T cells in this patient could proliferate in response to a high concentration of PHA and could produce IL-4 and interferon-γ (IFN-γ) in response to TCR stimulation. These data indicate that an alternative signalling pathway, which is independent of ZAP-70, also exists in this patient. Moreover, Noraz *et al.* recently reported two ZAP-70-deficient patients who had a subset of peripheral T cells that expressed high levels of Syk and could proliferate *in vitro*.[40] The Syk^{high}/ZAP-70$^-$ T cells had a signalling pathway distinct from conventional T cells. A discrepancy between their data and ours is that Syk^{high}/ZAP-70$^-$ T cells of our patient could not proliferate upon stimulation of TCR. Although the true reason for this discrepancy is unclear, there is a difference in the characteristics of employed T cells between theirs and ours. We used T cells that were freshly isolated from the patient, whereas they used the cells that were maintained by IL-2 and feeder cells for a long time. Moreover, proliferation responses of Syk^{high}/ZAP-70$^-$ T cells were markedly decreased compared with control T cells in their report, which was agreed with our observation.

Taken together, it is thought that at least some ZAP-70-deficient patients retain a TCR-signalling pathway via Syk that is insufficient to evoke complete activation of peripheral T cells but is sufficient to induce some T-cell functions such as assistance in specific IgE production.

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