Abnormal Signal Transduction in a Patient with Severe Combined Immunodeficiency Disease

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ABSTRACT. We have studied an 8-yr-old male patient with adenosine deaminase-positive severe combined immunodeficiency disease with a normal number of peripheral CD3⁺, T cell receptor- $\alpha\beta$ ⁺ T cells. The majority of these T cells expressed the CD8 molecule and were oligoclonal in nature as proven by Southern blot analysis of the T cell receptor genes. T cells failed to proliferate *in vitro* either upon stimulation with T cell mitogens or when stimulated with a combination of the phorbol ester phorbol myristate acetate and the Ca-ionophore ionomycin. High doses of recombinant IL-2, when added to *in vitro* cultures, were able to restore proliferation induced by phorbol myristate acetate and ionomycin but the response to concanavalin A remained severely defective. However, activation of the patient's T cells with phytohemagglutinin or concanavalin A induced an increase of free cytoplasmic Ca²⁺, which was 2- to 5-fold higher than in normal CD8⁺ T cells. Furthermore, phorbol myristate acetate or phytohemagglutinin induced the translocation of protein kinase C from cytosol to plasma membrane. Analysis of membrane phospholipid composition of the patient's T cells disclosed that the ratio of phosphatidylcholine to phosphatidylserine was 5-fold higher than in normal T cells. The abnormal Ca²⁺ response after activation with T cell mitogens as well as the high phosphatidylcholine/phosphatidylserine ratio may be causally linked to the defective *in vitro* T cell proliferation. Because the capacity of T lymphocytes to produce or respond to IL-2 may vary, the oligoclonality of the T cells of the patient should be considered as well in the explanation of defective cell proliferation. (*Pediatr Res* 29: 306-309, 1991)

Abbreviations

ADA, adenosine deaminase
ConA, concanavalin A
DAG, diacylglycerol
MNC, mononuclear cells
PC, phosphatidylcholine
PHA, phytohemagglutinin
PI, phosphatidylinositol
PKC, protein kinase C
PMA, phorbol myristate acetate
PS, phosphatidylserine
SCID, severe combined immunodeficiency disease
MEM, minimal essential medium
[Ca²⁺]_i, concentration of free cytoplasmic Ca²⁺

SCID is characterized by a functional deficiency of both cellular and humoral immunity. A number of different forms of SCID have been defined by the World Health Organization. This classification is based largely on clinical presentation of the disease because in most cases the underlying molecular defect is unknown. Two notable exceptions are firstly, SCID associated with a deficiency of an enzyme of purine metabolism, *i.e.* ADA (1, 2), and secondly, class I/II MHC deficiency syndrome (3, 4). The latter patients have nearly normal numbers of lymphocytes that are functionally deficient because of the absence of MHC products on the cell membrane (5). Recently, a new form of SCID was defined in a patient with normal numbers of T cells with a defect in signal transduction (6). In this patient's T cells, it was demonstrated that, in contrast to normal T cells, the T cell receptor/CD3 complex was poorly coupled to the phosphatidylinositol second messenger pathway.

We have studied T cell activation pathways in a patient with SCID with normal numbers of peripheral T cells. The majority of T cells were CD8⁺ and oligoclonal in nature (7). Functional studies revealed a complete absence of *in vitro* mitogen-induced proliferation that could only partially be corrected by addition of IL-2. Analysis of mitogen-induced second messengers showed quantitative and qualitative abnormalities in the intracellular calcium response.

MATERIALS AND METHODS

Case report. The patient, a boy, was born in 1979 as the first child of healthy, unrelated parents. The diagnosis ADA⁺ SCID was based on a severe hypogammaglobulinemia, the initial absence of peripheral blood T cells, and presence of ADA activity in the patient's red blood cells (7). From the age of 4 y onward, rising numbers of CD3⁺ T cells could be detected, which were almost exclusively CD8⁺ (CD4:CD8 ratio 0.1). A maternal origin of the T cells could be excluded by HLA-typing (7). Between the ages of 4 and 6 y, the patient gradually developed hepatosplenomegaly and an interstitial pulmonary infiltrate of unknown origin. Biopsies of liver and lung revealed lymphoid infiltrates of CD8⁺ T cells. Southern blot analysis of T cell receptor- β genes showed a restricted banding pattern, which indicated that at least three clonal T cell populations were present in peripheral blood, one of which had invaded the lung (7). The patient died at the age of 10 y of respiratory insufficiency.

Lymphocyte isolation and activation. Peripheral blood MNC were isolated by density gradient centrifugation of heparinized venous blood on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). Cells were cultured for 3-6 d at 37°C, 5% CO₂, 100% relative humidity in RPMI-1640 medium supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), and 10% heat-inactivated human AB serum. MNC were cultured in 96-well tissue culture clusters (150 µL/well) at a density of 0.25 × 10⁶/mL and were activated with PHA (Burroughs Well-

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come, Research Triangle Park, NC; 5 µg/mL final concentration), ConA (Calbiochem, La Jolla, CA: 20 µg/mL), or a combination of PMA (Sigma Chemical Co., St. Louis, MO) and ionomycin (Calbiochem). Cultures were pulsed with 0.08 µCi ³H-thymidine (sp act 44.5 Ci/mol; Amersham International, Amersham, UK) during the last 18 h of a 6-d culture.

Phospholipid analysis. Purified T cells were suspended at a concentration of 10⁷ cells/mL in medium (17 mM Tris in 0.9% NaCl, pH 7.2) containing 100 µCi of carrier-free ³²P_i (Amersham International). Cells were incubated for 2 h at 37°C, washed twice, and resuspended at 10⁷/mL. Samples of cell suspension (250 µL) were transferred to prewarmed test tubes containing 25 µL (20 µg) PHA or 25 µL medium and incubated at 37°C for indicated times. Incubation was stopped by addition of 1 mL chloroform/methanol/concentrated HCl (200:100:2, vol/vol). After phase separation, the upper phase was discarded and the lower phase was washed two times with a mixture of chloroform/methanol/0.6 M HCl (3:48:47, vol/vol). Subsequently, the lower phase, which contains PI bisphosphate, PI phosphate, PI, phosphatidyl acid, and other phospholipids, was subjected to high-performance thin-layer chromatography according to Jolles *et al.* (8). Labeled lipids were visualized by radioautography on Kodak Royal X-Omat film (Eastman Kodak, Rochester, NY). The spots were scraped from the plates and counted for radioactivity.

PKC activity assay. Purified T cells (15 × 10⁶ per time point) were activated with PHA (see above) and lysed with Triton X-100. Cytosol and membrane fractions were obtained by differential centrifugation followed by diethylaminoethyl column chromatography. PKC activity was defined as Ca⁺⁺- and phospholipid-dependent kinase activity and is expressed as pmol ³²P incorporated into substrate (histone III S) per min per mg protein (9).

Intracellular calcium. MNC or purified T cells were washed and resuspended in RPMI-1640 medium at a concentration of 10 × 10⁶/mL. Cells were loaded with 4 µM of the acetoxymethyl ester of indo-1 (indo-1 AM; Molecular Probes, Eugene, OR) by incubation for 20 min at 37°C followed by a 40 min incubation at 37°C at a cell concentration of 2 × 10⁶/mL (10). Samples of 2 × 10⁶ cells were spun down and resuspended in prewarmed assay buffer (145 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄ · 2H₂O, 0.5 mM MgSO₄ · 7H₂O, 5 mM glucose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 7.4). Indo-1 fluorescence was monitored in an Aminco-Bowman (Silver Spring, MD) spectrofluorometer using 349 nm for excitation and 410 nm emission. Cells were activated with PHA (200 µg/sample), ConA (150 µg/sample), or ionomycin (1 µM). Changes in intracellular calcium after activation were recorded continuously over a period of up to 5 min.

Flow cytometry. Patient's and control MNC were cultured for 3 d with PHA or medium (see above). Cultured MNC were washed twice in MEM and resuspended in MEM, 1% BSA, 0.05% Na-azide (MEM/BSA/azide). MNC (0.5–1 × 10⁶) were incubated under gentle shaking for 30 min at 4°C in 10 µL appropriately diluted anti-IL-2 receptor antibody (CD25; Becton

Dickinson, San Jose, CA) and washed three times with ice-cold MEM/BSA/azide. Subsequently, the cells were incubated with fluoresceinated goat anti-mouse Ig (Becton Dickinson) for another 30 min at 4°C. Stained cells were diluted in sheath fluid and run on a FACS Analyzer (Becton Dickinson). From each sample, 10 000 events were stored in list-mode and analyzed using Consort 30 software (Becton Dickinson).

RESULTS AND DISCUSSION

Peripheral blood MNC of the patient consisted predominantly (up to 80%) of CD8⁺ T cells. These cells expressed the α/β-T cell receptor and, as assessed by Southern blot analysis of the T cell receptor-β genes, were oligoclonal in nature (7). The patient's peripheral blood T cells did not proliferate, as assessed by tritiated thymidine incorporation, when activated with mitogens like PHA or ConA. Activation with ConA did not induce expression of the IL-2 receptor (Table 1). Neither could proliferation be induced when cells were activated with the combination of the phorbol ester PMA and the calcium ionophore ionomycin (Table 1). This defective proliferative response can partly be explained by a defective IL-2 production because addition of IL-2 to the *in vitro* cultures normalized the PMA/ionomycin response (Fig. 1). The ConA response, however, was only partially corrected by addition of IL-2 (Fig. 1). Because cell activation with PMA and ionomycin bypasses the need for receptor-mediated transmembrane signalling, the defective proliferative response after ConA activation could be due to a signalling defect in the patient's T cells.

We therefore studied whether activation of the patient's lymphocytes with mitogens would lead to activation of phospholipase C and thus hydrolysis of membrane phosphatidylinositides. Figure 2 shows that the increase in PI and phosphatidyl acid in the patient's cells was comparable to that in control T cells. We next studied whether both products of phospholipase C, *i.e.* inositol trisphosphate and DAG, can mediate their biologic functions. Translocation of PKC from the cytosol to plasma membrane is one of the functions mediated by DAG. Figure 3 shows that this process is intact in the patient's T cells. The increase in cytosolic free Ca⁺⁺ after activation of the patient's MNC with ConA or PHA, which is mediated by inositol trisphosphate, was repeatedly found to be 2- to 5-fold higher than that observed in normal T cells (Fig. 4). Because of the predominance of CD8⁺ T cells in the patient's MNC, we also compared the mitogen-induced Ca⁺⁺ response with that of purified CD8⁺ T cells from normal donors. It appeared, however, that the Ca⁺⁺ response of normal CD8⁺ T cells was lower compared with normal CD4⁺ T cells (data not shown). Therefore, the enhanced Ca⁺⁺ response, as observed in the patient's MNC, cannot be explained on the basis of an imbalance of T cell subsets.

Because of the abnormally high [Ca²⁺]_i after activation with ConA or PHA, we analyzed whether this phenomenon would lead to cell death. However, cell viability (assessed by trypan blue exclusion) was not significantly decreased up to 24 h after acti-

Table 1. *In vitro* induced T cell proliferation and IL-2 receptor (IL-2R) expression

Treatment	Patient			Control		
	IL-2R ⁺ cells (%)	³ H-TdR incorporation*		IL-2R ⁺ cells (%)	³ H-TdR incorporation*	
		Day 3	Day 6		Day 3	Day 6
PHA (40 µg/mL)	2.8	134	53	42.3	10 105	5 015
ConA (5 µg/mL)		138	69		1580	2677
PMA (1 ng/mL) + ionomycin (100 nM)			150			4345
PMA (0.3 ng/mL) + ionomycin (250 nM)	0.1		181	0.3		6250
Medium		87	66		76	71

* Tritiated thymidine incorporation (cpm per 40 × 10³ cells).

Fig. 1. Proliferative response of patient's (circles) and control (triangles) lymphocytes to *in vitro* activation with ConA (left panel) or PMA + ionomycin (right panel). Cells were cultured for 6 d in the presence of variable concentrations of recombinant IL-2. Data shown are mean values of quadruplicate cultures; SD < 10% of mean values.

Fig. 2. Changes in membrane PI (open symbols, broken lines) and phosphatidylcholine (closed symbols, solid lines) in patient's (circles) and control (triangles) lymphocytes after activation with PHA. Data shown are radioactivities in the respective phospholipid fractions of cells prelabelled with inorganic ^{32}P .

vation with ConA. Furthermore, the process of ConA capping was found to be normal on the patient's T cells (data not shown).

Activation of PKC is, next to DAG, also dependent on the phospholipid composition of the plasma membrane. The ratio of PC to PS has been shown to be a critical variable in this respect (11). We therefore performed a thorough analysis of the membrane phospholipid composition of the patient's T cells. All phospholipids (including PS) were within the normal range except for PC, which was approximately 5-fold higher than in normal T cells (Fig. 5). As a consequence, the PC/PS ratio in the patient's T cells was significantly higher than that in normal T cells (7.1 and 1.6, respectively).

Defective signal transduction as the underlying cause in lymphocyte dysfunction has been described in a single patient with SCID (6). The defect in that patient resembles the defect in a somatic mutant, derived from the human leukemic T cell line Jurkat, with altered signal transduction through the T cell receptor/CD3 complex (12). T cell receptor-mediated signaling has also been studied in immunodeficient patients with decreased

Fig. 3. Translocation of PKC. Patient's (circles) or control T cells (triangles) were activated with PHA. At various times after activation, cells were lysed and separated in cytosolic (solid lines) and membrane fractions (broken lines) and assayed for PKC activity, which is expressed as cpm of inorganic ^{32}P incorporated into substrate (histone III S) per min.

Fig. 4. Mitogen-induced changes in intracellular free Ca^{2+} . Purified T cells from the patient and a control were prelabelled with the calcium indicator dye indo-1 and then stimulated with PHA, ConA, or ionomycin. Changes in fluorescence reflect changes in intracellular Ca^{2+} levels (relative $[\text{Ca}^{2+}]_i$). Data are from a single experiment that was repeated at three separate occasions with similar results.

lymphocyte proliferative responses. No significant differences between the PHA- or CD2-antibody-induced rise of $[\text{Ca}^{2+}]_i$ in normal and AIDS lymphocytes were observed (13, 14). Human immunodeficiency virus-1 infected T cells were, however, unable to mobilize Ca^{2+} after stimulation with anti-CD3 (14). We do not know whether the enhanced Ca^{2+} response observed in the T cells of our patient provides the biochemical mechanism to explain, in part, the decreased proliferative response. We also did find abnormalities in the T cell membrane phospholipid composition. PS is the natural phospholipid cofactor in the Ca^{2+} -dependent activation of PKC, whereas PC inhibits PKC activation (15–17). PKC activation after mitogenic stimulation (Fig. 3) was measured in isolated membrane preparations and not in intact cells. We therefore cannot exclude that the high PC/PS ratio in the T cell membrane impairs PKC activity in intact cells. Finally, it has to be kept in mind that the T cells in our patient are oligoclonal in nature and that their characteristics were compared with normal polyclonal T cells. Apart from $[\text{Ca}^{2+}]_i$, the biochemical changes associated with lymphocyte activation cannot be studied at the single-cell level. As far as changes in

Fig. 5. PC (open columns) and PS (hatched columns) in T cell membrane preparations from the patient and a control, measured as radioactivity in the respective fractions after prelabeling the cells with inorganic ^{32}P .

$[\text{Ca}^{2+}]_i$ after activation with mitogens are concerned, the response observed in the patient's T cells is well above the range found in normal polyclonal T cells (10). The normal ranges in single individual lymphocytes of the other biochemical parameters studied are unknown. Therefore, the oligoclonality of the T cells of the patient should be considered as well in the explanation of the defective mitogen-induced T cell proliferation.

Although the causal relation between the observed signaling defect and the T cell anergy is not proven as yet, this patient may represent another case of an immunodeficiency with abnormal signal transduction.

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