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APPEARANCE OF ANTIBODIES AGAINST PHOSPHOTYROSINE AND TYROSINE PHOSPHORYLATION OF LYMPHOCYTE PROTEINS IN HUMAN T CELL LEUKEMIA VIRUS TYPE-I INFECTION

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Antibodies reactive with O-phosphotyrosine (Ptyr) were detected in all of 56 patients with adult T cell leukemia (ATL) with high titers ranging from 1:1,600 to 1:25,000 in 91% of the patients, whereas 97% of 70 healthy subjects showed titers of 1:400 or less. Ptyr-containing proteins (molecular weights of 70k, 45k and 30k) were detected in peripheral lymphocytes from patients with ATL. These proteins might be involved as antigens in the induction of such antibodies.

Key words: Anti-phosphotyrosine antibody — Adult T cell leukemia

A number of oncogene-encoded proteins and receptors for growth factors share the ability to phosphorylate cellular protein substrates on tyrosine (reviewed, e.g., in 1, 2). Using antibody raised against O-phosphotyrosine (Ptyr), we have shown that proteins phosphorylated on tyrosine (Ptyr-proteins) increase in various types of malignant cells from patients[3,4] and in established cell lines of leukemic cells.[5,6] We have also found that anti-Ptyr antibodies appear frequently in patients with some types of malignant diseases, including leukemia.[7] Although the mechanism for the induction of such antibodies is unknown, it is reasonable to implicate the increased tyrosine phosphorylation of proteins in malignant tumor cells.

We report here the appearance of high titers of anti-Ptyr antibodies in the majority of patients with adult T cell leukemia (ATL) and a significant rise of such antibodies also in asymptomatic carriers of human T cell leukemia virus type-I (HTLV-I). In addition, we attempted to find putative antigens in peripheral lymphocytes from patients, antigens which might be responsible for the induction of Ptyr-antibodies, in view of the fact that Ptyr-proteins are accumulated in lymphoid cell lines transformed with HTLV-I.[6]

Human sera were obtained from 56 patients with ATL and 36 asymptomatic carriers of HTLV-I. The diagnosis of ATL was based on the following criteria: (i) the presence of typical pleomorphic cells with markedly deformed nuclei and T cell markers such as CD2 and CD4, and (ii) the presence of anti-HTLV-I antibodies in peripheral blood of patients. Asymptomatic carriers of HTLV-I were those having anti-HTLV-I antibodies. Control sera were obtained from healthy normal subjects. Lymphocyte preparations were obtained by centrifugation of heparinized blood from patients with ATL on a layer of Ficoll-paque solution (Pharmacia Fine Chemicals, Uppsala, Sweden).

As shown in Table I, the anti-Ptyr antibody titers of the sera from 70 healthy normal subjects were less than 1:800. Of these, 97% were $\leq$1:400 and 70% were $\leq$1:200. In contrast, sera from patients with ATL showed significantly higher values. In 91% of them, high titers in the range from 1:1,600 to 1:25,600 were found. A significant rise of the antibodies was also observed in a group of asymptomatic carriers of HTLV-I ($P < 0.001$), but the rise was only of moderate degree and the titers were significantly lower than those of the patient group ($P < 0.001$).

A blocking test with 2m*M* Ptyr was negative for all control sera from healthy persons with titers $\geq$1:400. Therefore, the control group possibly include a considerable number

Table I. Frequency Distribution of Anti-Ptyr Antibody Titers in Three Groups

Antibody titer	Number of patients		
	Normal control[a]	HTLV-I carrier[b]	ATL patient[c]
≦1:100	28	0	0
1:200	21	3	0
1:400	19	9	2
1:800	2	14	3
1:1,600	0	5	15
1:3,200	0	1	11
1:6,400	0	3	18
1:12,800	0	1	4
1:25,600	0	0	3

Enzyme-linked immunosorbent assay for anti-Ptyr antibody was performed as described previously.[7,8] Peroxidase-conjugated anti-human IgG (H and L chains) (Miles-Yeda Ltd., Rehovot, Israel) was used for color development. Antidody titers were expressed as the final dilution of sera to give absorbance ≧0.2 at 410 nm. For blocking tests, serum samples were preincubated with 2 m*M* Ptyr for 1 hr at room temperature. The difference between *a* and *b*, and that between *b* and *c* are both significant ($P < 0.001$) by the two-tailed *t*-test.

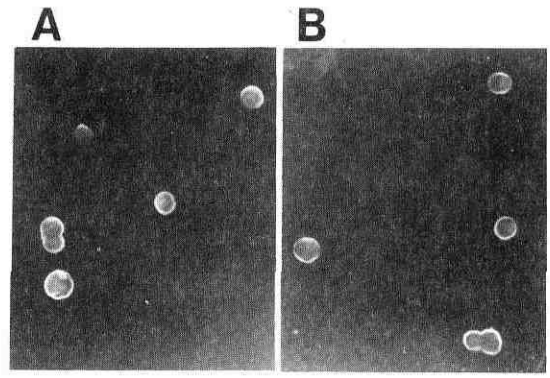

Fig. 1. Immunofluorescent staining of peripheral lymphocytes from a patient with ATL. Acetone-fixed or intact cells were stained as described,[6,8] using anti-rabbit Ptyr antibody and fluorescein-conjugated anti-rabbit IgG (Medical and Biological Laboratories Co. Ltd., Nagoya). (A) cells fixed with acetone (×400): (B) intact cells (×400). For blocking tests, anti-rabbit Ptyr antibody was preincubated with 2m*M* Ptyr for 1 hr at room temperature (not shown).

of subjects with false positive response, this being consistent with the previous observation.[7] In contrast, all the sera from HTLV-I carriers as well as patients were blocking-positive with a blocking rate of 50% and over. This fact emphasizes the significance of difference in titers between the healthy control group and the groups infected with HTLV-I.

Since we are unable to specify in any of these cases the stage of disease at the time when serum was taken from the patient, it is not possible to correlate antibody titers with the clinical condition of the patients.

We reported previously that Ptyr-proteins with molecular weights of 70k, 65k, and 45k were commonly detectable in established cell lines either derived from ATL patients or transformed *in vitro* with HTLV-I. Of these, the 65k and 45k components were found, at least partly, on the external surface of cells, and the former had the characteristics of the interleukin-2 (IL-2) receptor.[6] In the present study, to examine whether peripheral lymphocytes directly obtained from ATL patients share such properties, we isolated lymphocytes from five ATL patients and analyzed them for Ptyr-proteins first by immunofluorescence staining using rabbit anti-Ptyr antibody. When fixed with acetone, over 95% of the fixed cells showed strong fluorescence in the cytoplasm and plasma membrane area (Fig. 1A). Staining of intact cells showed bright membrane fluorescence in 10–20% of cells (Fig. 1B). Since the peripheral lymphocytes from patients had narrow cytoplasmic areas on the periphery of large nuclei, the cytoplasmic fluorescence of fixed cells looks similar in photographs to the membrane fluorescence of intact cells. The fluorescent staining could be blocked completely with 2m*M* Ptyr but not with phosphoserine or phosphothreonine, and lymphocytes from healthy persons were fluorescence-negative (data not shown), in agreement with our previous reports.[3,5,6]

Ptyr-proteins were extracted from peripheral lymphocytes of two patients with ATL and then analyzed by immunoblotting with rabbit anti-Ptyr antibodies (Fig. 2, lanes A and C). Ptyr-proteins with molecular weights of 70k, 45k and 30k were commonly detected as the major components. No bands were detected in blocking tests in which anti-Ptyr antibody pretreated with Ptyr was used for immunostaining (Fig. 2, lanes B and D), supporting the view that the detection of these proteins is

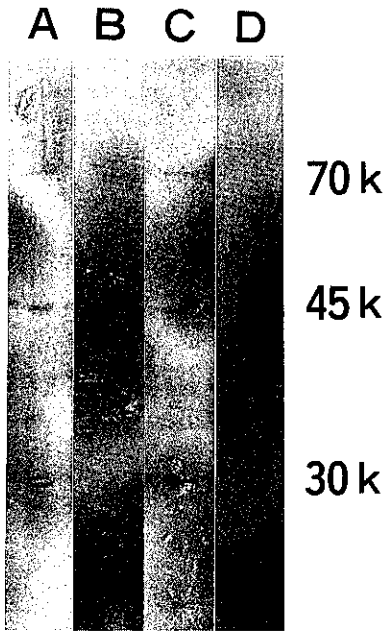

Fig. 2. Immunoblotting analysis of Ptyr-proteins in lymphocytes from two patients with ATL. The procedure was essentially the same as described previously.[6,9] Briefly, 1×10^5 cells were homogenized in 0.2 ml of 1% Triton X-100, 10m*M* Tris-HCl, pH 7.3, and 5m*M* EDTA, and centrifuged at 15,000*g* for 10 min. The supernatant was subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Proteins were electroblotted onto a Durapore filter (Millipore Co., Bedford, MA) and stained with rabbit anti-Ptyr antibody with the aid of biotinylated anti-rabbit IgG antibody and streptavidin bridge reagents (Amersham Int. plc, Buckinghamshire, UK). Lanes A and C; Ptyr-proteins from two different patients. Lanes B and D; Control experiments corresponding to lanes A and C, respectively, in which anti-Ptyr antibody was blocked by pretreatment with 2m*M* Ptyr.

based on specific recognition of Ptyr-proteins by the antibody. These results show that the pattern of protein tyrosine phosphorylation in the peripheral lymphocytes from patients differs from that observed in lymphoid cell lines derived from ATL or transformed *in vitro* with HTLV-I: tyrosine phosphorylation of the 64k component of IL-2 receptors was not detectable in peripheral lymphocytes from two patients examined and, instead, Ptyr-proteins of 30k were detected as one of the three major Ptyr-proteins. This lack of tyrosine phosphorylation may be an important characteristic of lymphocytes in the peripheral blood of patients with ATL, distinguishing them from ATL-derived lymphoid cells cultured *in vitro*. Its biological significance must be investigated.

The mechanism by which anti-Ptyr antibodies are induced in patients with some types of malignant diseases[7] is not clear. The present study shows that such antibodies are highly induced in most patients with ATL, and that Ptyr-proteins are accumulated in a large number of peripheral lymphocytes. It is tempting to implicate these Ptyr-proteins, particularly those on the cell surface, as putative antigens responsible for the induction of anti-Ptyr antibodies. In addition, HTLV-I-mediated changes in the regulatory mechanism of the immune system must be considered, because the anti-Ptyr antibody-positive rate in ATL is must higher than the figure, 38.6%, obtained for other various types of leukemia.[7]

Another important question is whether or not the appearance of high titers of anti-Ptyr antibodies in a certain fraction of HTLV-I carriers is in any way related to the eventual development of ATL. A systematic follow-up study will be needed to answer this question.

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