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Accumulation of p60^{lck} in HTLV-I-transformed T Cell Lines Detected by an Anti-Lck Monoclonal Antibody, MOL 171

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The *lck* gene encodes a protein tyrosine kinase of nonreceptor type, p56^{lck}, whose expression occurs almost exclusively in T lymphocytes. MOL 171, an anti-p56^{lck} monoclonal antibody, was produced by using a 25-amino-acid synthetic polypeptide as the antigen, its structure corresponding to the N-terminal region deduced from the *lck* cDNA sequence. Immunoblot analysis with MOL 171 showed the accumulation of 60 kD form of Lck protein, p60^{lck}, and the decrease of p56^{lck} in human T cell leukemia virus type I (HTLV-I)-transformed T cell lines. Another anti-Lck monoclonal antibody, MOL 294, raised by using a synthetic peptide corresponding to the C-terminal region deduced from the *lck* cDNA sequence, also detected the accumulation of p60^{lck} in those HTLV-I-transformed T cell lines. The appearance of p60^{lck} with the decrease of p56^{lck} in normal T lymphocytes after stimulation suggested the origin of p60^{lck} in HTLV-I-transformed T cells.

Key words: *lck* — HTLV-I — Anti-Lck monoclonal antibody

lck is a member of the *src*-related family of genes that encodes a class of closely related membrane-bound, nonreceptor protein tyrosine kinases (PTK[4]).[1-4] The genes grouped in this family include c-*src*, c-*yes*, c-*fgr*, *fyn*, *hck*, *tkl* and *lyn*.[5] The *lck* gene product p56^{lck} is normally expressed exclusively in T lymphocytes. The functional domains of p56^{lck} can be divided largely into two regions based on the sequence comparison with p60$^{c\text{-}src}$. These regions include the membrane-bound and substrate interactive domain, which is the N-terminal half where the sequences of the *src*-family members diverge most extensively, and the kinase domain, which is the C-terminal half, where the sequences of *src*-family members are most homologous. The homology of the deduced protein sequence of *lck* to that of c-*src* is 51.5% as a whole, whereas it increases to a level of about 80% within the C-terminal half.[3]

The association of p56^{lck} with CD4 and CD8 T cell surface glycoproteins,[6,7] and the phosphorylation of p56^{lck} by the cross-linking of CD4 antigen,[8] suggested a specialized role for p56^{lck} in signal transduction pathways mediating T cell differentiation and activation. Overexpression of p56^{lck} in LSTRA cell line, a murine T cell tumor line, induces an increased level of phosphotyrosine in many protein species and may contribute to its uncontrolled cell growth.[9] The substitution of phenylalanine for tyrosine-505 of p56^{lck} results in an increase in its PTK activity and reveals an oncogenic activity of this protein.[10] These results, thus, raise the possibility that *lck* may be involved in some processes of malignant transformation of human T cells.

We reported previously that the expression level of *lck* mRNA in human T cell leukemia virus type I (HTLV-I)-transformed T cell lines is closely associated with interleukin-2 (IL-2) dependency of their cell growth.[11] That is, IL-2-dependent HTLV-I-transformed cell lines contain *lck* message as abundantly as HTLV-I-negative T cell lines, whereas IL-2-independent HTLV-I-transformed cell lines express little or no *lck* mRNA, although they are derived from T cells. In the HTLV-I-transformed T cell lines used in this study, NOBE, TOM-1, ILT-Su and KAN-Y are IL-2-dependent cell lines, while MT-2 is an IL-2-independent one.

To assess the possible role of p56^{lck} in HTLV-I-induced transformation, the expression of p56^{lck} in such IL-2-dependent HTLV-I-transformed T cell lines was examined in this study by using anti-Lck monoclonal antibodies recognizing the N- or C-terminal region of p56^{lck}. Multiple bands of Lck proteins ranging from 56 kD to 60 kD were detected specifically in the HTLV-I-infected T cell lines by immunoblot analysis.

MATERIALS AND METHODS

Cells Peripheral blood mononuclear cells were obtained from normal healthy volunteers by multiple Ficoll-Hypaque centrifugations, and peripheral blood T lymphocytes (PBTL) were enriched by passage through a

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[4] Abbreviations used are: PTK, protein tyrosine kinase; HTLV-I, human T cell leukemia virus type I; IL-2, interleukin-2; PBTL(s), peripheral blood T lymphocyte(s); BSA, bovine serum albumin; PMA, phorbol myristate acetate.

nylon wool column as previously described.[12] The resulting cell population was >90% T cells as judged by immunofluorescence analysis with a monoclonal antibody, Leu1 (anti-CD5 antibody; Becton Dickinson, San Jose, CA). HTLV-I-negative human cell lines (JURKAT, HUT78, U937 and RPMI1788) were cultured in RPMI 1640 medium containing 10% fetal calf serum in humidified air with 5% CO_2 at 37°C. HTLV-I-transformed T cell lines which require IL-2 for their growth (NOBE, TOM-1, ILT-Su and KAN-Y) were cultured in the same medium supplemented with human recombinant IL-2 (TGP-3; Takeda Pharmaceutical, Osaka) at a concentration of 2 U/ml. Although MT-2 is an HTLV-I-integrated T cell line, it does not express *lck* mRNA, as reported previously.[11] The characters of the cell lines used in this study were mentioned elsewhere.[11, 13, 14] Briefly, NOBE was derived from adult T cell leukemia cells; TOM-1 and ILT-Su were established from an HTLV-I carrier's PBTLs; and KAN-Y was established by coculturing MT-2 cells with normal PBTLs. For activation of cells, phorbol myristate acetate (PMA, Sigma Chemical Co., St. Louis, MO) was added to PBTL or JURKAT cells at the concentration of 50 ng/ml and cells were harvested 15 min or 30 min later for examination.

Preparation of anti-Lck monoclonal antibodies, MOL 171 and MOL 294 Two different synthetic peptides were made corresponding to the N-terminal or C-terminal amino acid sequence deduced from the human *lck* cDNA sequence,[3, 4] i.e., SYEPSHDGDLGFEKGEQLRILE-QSG (N-SP), or KERPEDRPTFDYLRSVLEDFF-TATEGQYQPQP (C-SP), respectively. These peptides, named N-SP and C-SP, were conjugated to bovine serum albumin through the C-terminal cysteine residue in the case of N-SP and the N-terminal cystein residue in the case of C-SP as described[15] and were used as immunogens. For production of anti-Lck monoclonal antibodies recognizing epitopes expressed on N-SP, Balb/c mice were immunized on day 0 by subcutaneous injection with 50 μg of BSA-Cys-N-SP in Freund's complete adjuvant, and three subsequent subcutaneous injections with 50 μg of BSA-Cys-N-SP in Freund's incomplete adjuvant were carried out at 14-day intervals. Three days after the final injection, splenic cells were fused with cells of the P3U1 mouse myeloma cell. The cell fusion, hypoxanthine-aminopterine-thymidine selection, and cloning of the hybridoma lines by ELISA using N-SP as the antigen were performed as described.[16] Finally, one hybridoma clone producing IgG_1 class antibody, MOL171, was obtained. For production of anti-Lck monoclonal antibodies recognizing C-SP, the same procedures were performed using BSA-conjugated C-SP and C-SP, and one hybridoma clone producing IgG_1 class antibody, MOL 294, was selected.

Immunoblot analysis Cells were suspended in lysis buffer (0.5% Triton-X, 300 m*M* NaCl, 50 m*M* Tris-HCl (pH 7.6), 1 m*M* sodium orthovanadate, 0.25 m*M* EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 m*M* phenylmethylsulfonyl fluoride) and lysed by sonication. Cell lysates containing 100 μg of proteins were denatured in an equal volume of SDS sample buffer (0.5 *M* Tris-HCl (pH6.8) containing 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue and 10% glycerol) and electrophoresed on 8% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, FRG) overnight at 30 V in a buffer containing 20 m*M* Tris-HCl, 150 m*M* glycine and 20% methanol. Then the filter was pretreated with Blotto/Tween (5% nonfat dry milk and 0.05% Tween 20 in 0.5 *M* NaCl) for 1 h at room temperature, prior to a 2 h incubation with the anti-Lck monoclonal antibodies (containing 50 μg/ml IgG protein). The filter was then washed in Blotto/Tween for 30 min twice and incubated with 5×10^5 cpm/ml of ^{125}I-labeled anti-mouse IgG antibody (Amersham, Buckinghamshire, England) or peroxidase-conjugated anti-mouse IgG (Tago Inc., Burlingame, CA) diluted 500 times for 2 h at room temperature as described elsewhere.[13]

Immunofluorescence Cells washed twice with PBS were spread on a slide glass, dried and fixed at room temperature. Then the cells were treated for 10 min with acetone at −20°C to permeabilize the cell membranes and desiccated. MOL 171 was added at a concentration of 100 μg/ml, and the sample was incubated for 60 min at 37°C. After being washed with PBS three times, the cell sample was stained with FITC-conjugated anti-mouse IgG (Tago Inc., Burlingame, CA) diluted 40 times for 30 min at room temperature and then washed with PBS for examination under a fluorescence microscope.

RESULTS

Specificity of anti-Lck monoclonal antibody, MOL 171 The specificity of MOL 171, an anti-Lck monoclonal antibody raised against N-SP, was examined by immunoblot analysis (Fig. 1a). MOL 171 at a concentration of 50 μg/ml detected a 56 kD protein band in JURKAT cells, which express *lck* mRNA, but not in U937 cells which do not express *lck* mRNA. In PBTL obtained from a healthy donor, a 56 kD band was detected by MOL 171. The *lyn* gene belongs to a non-receptor PTK gene family and encodes a protein of 56 kD in immunoblot assay.[17] The amino acid sequence of *lyn* kinase domain is highly homologous (75%) to that of *lck* kinase domain. MT-2 and RPMI1788 cells express $p56^{lyn}$ but not *lck* mRNA.[11, 18] Fig. 1b shows that MOL 171 does not bind to any protein bands in such lyn^+ lck^- cells in immunoblot assay. When MOL 171 was diluted

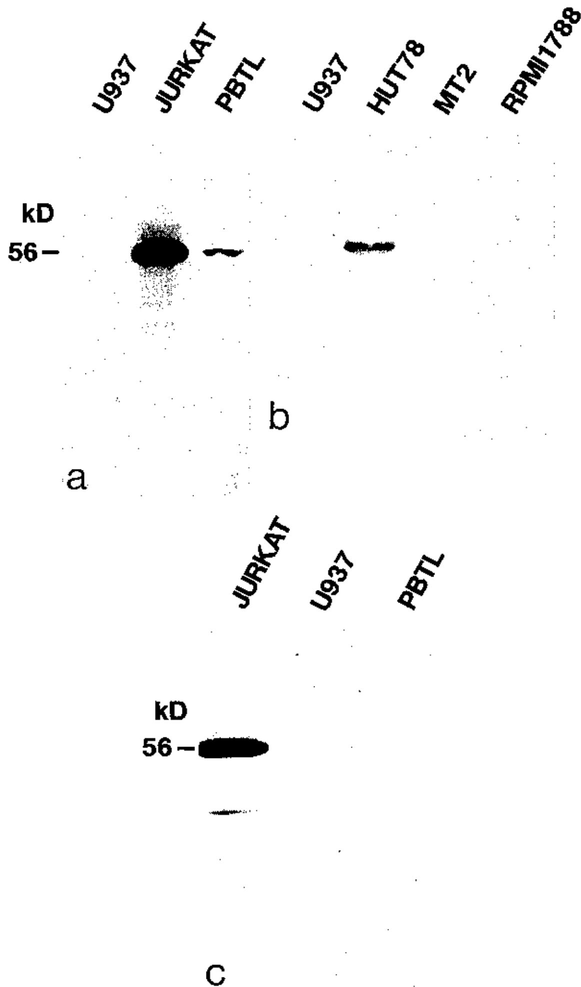

Fig. 1. Immunoblot analysis of p56^{lck} with anti-Lck monoclonal antibodies. Each lane was loaded with 100 μg of lysate prepared from various cell sources as indicated at the top of each lane. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane, and incubated with MOL 171 (a, b) or MOL 294 (c) for 1 h at room temperature. The bound antibodies were visualized by using peroxidase-conjugated anti-mouse IgG.

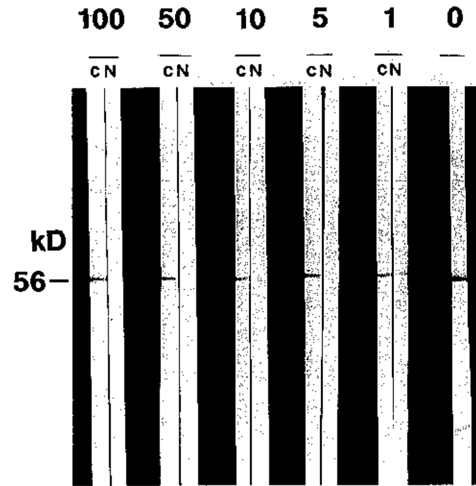

Fig. 2. Inhibition of the binding of MOL 171 to p56^{lck} by addition of N-SP. Nitrocellulose membrane strips transferred with ~50 μg of JURKAT cell lysate were incubated with 50 μg/ml of MOL 171 containing various amounts (μg) of N-SP (N) or C-SP (C) per ml as indicated at the top of each lane. The bound antibodies were visualized by using peroxidase-conjugated anti-mouse IgG.

serially, no 56 kD band could be detected any more at a concentration of less than 5 μg/ml (data not shown). To confirm further the specificity of MOL 171, the detection of p56^{lck} was performed by immunoblot analysis using 50 μg/ml MOL 171 preparations containing various amounts of N-SP, which was used as the antigen to raise MOL 171 antibody, or C-SP composed of 33 amino acids unrelated to the sequence of N-SP (Fig. 2). An amount of more than 5 μg/ml of N-SP completely blocked the binding of MOL 171 to p56^{lck}, whereas as much as 100 μg of C-SP could not inhibit the binding of MOL 171 to p56^{lck}.

MOL 294, another anti-Lck monoclonal antibody raised by C-SP, also detected a 56 kD protein band and a faint ~40 kD band in JURKAT but not in U937 or in PBTL (Fig. 1c). When more than 200 μg of cell lysate of PBTL was loaded on immunoblot assay, a faint band of 56 kD could be detected by MOL 294 (data not shown). Therefore it is considered that the binding affinity of MOL 294 to p56^{lck} is insufficient to detect p56^{lck} in PBTL sensitively by immunoblot assay.

Immunoblot analysis of HTLV-I-transformed T cell lines

The amounts of p56^{lck} in HTLV-I-transformed T cell lines were examined by immunoblot analysis using MOL 171 (Fig. 3). HTLV-I-negative, T cell leukemia lines (HUT78, JURKAT) expressed significant amounts of p56^{lck}, whereas HTLV-I-transformed T cell lines (NOBE, TOM-1, ILT-Su, KAN-Y) contained much less p56^{lck}, especially TOM-1 and ILT-Su. The relative levels of p56^{lck} in JURKAT, NOBE, TOM-1, ILT-Su and KAN-Y were 1.0, 0.6, 0.02, 0.1 and 0.5, respectively as determined by densitometric analysis. A notable finding is that a few protein products with molecular size larger than 56 kD, especially a 60 kD form, were detected in these HTLV-I-transformed T cell lines. When more than 5 μg/ml of N-SP was added to the MOL 171 antibody in immunoblot analysis using NOBE, the binding of MOL 171 to the 60 kD protein band, as well as that of MOL

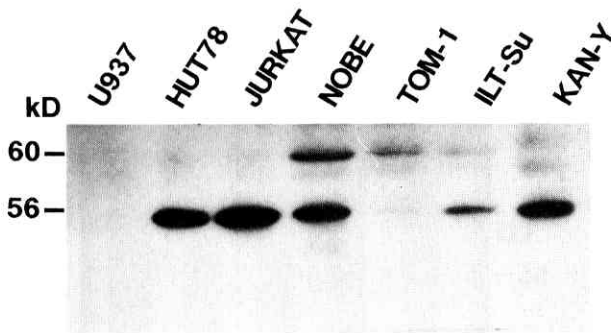

Fig. 3. Immunoblot analysis of HTLV-I-transformed T cell lines by MOL 171. The amounts of Lck proteins were examined in HTLV-I-positive T cell lines (NOBE, TOM-1, ILT-Su, KAN-Y), HTLV-I-negative T cell lines (HUT78, JURKAT) and a non T cell line (U937) by immunoblot analysis using MOL 171. The bound antibodies were visualized by autoradiography using ^{125}I-labeled anti-mouse IgG antibody.

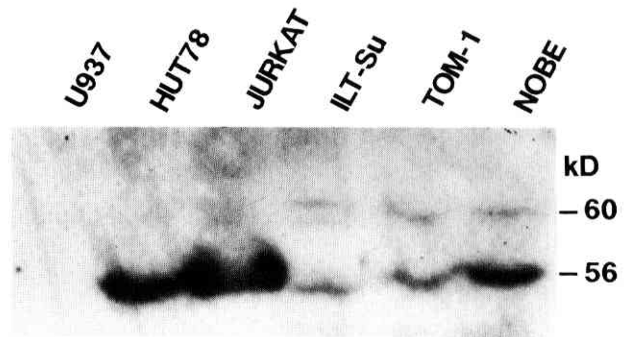

Fig. 5. Immunoblot analysis of HTLV-I-transformed T cell lines by MOL 294. HTLV-I-negative T cell lines (HUT78, JURKAT), HTLV-I-positive T cell lines (ILT-Su, TOM-1, NOBE) and a non T cell line (U937) were examined by immunoblot analysis using MOL 294. The bound antibodies were visualized by autoradiography using ^{125}I-labeled anti-mouse IgG antibody.

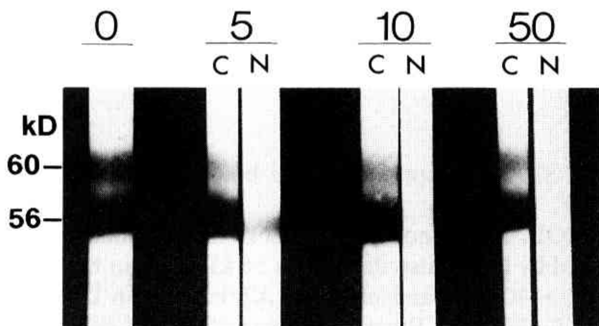

Fig. 4. Inhibition of the binding of MOL 171 to 60 kD protein band by addition of N-SP. Nitrocellulose membrane strips onto which ~50 μg of NOBE cell lysate had been transferred were incubated with 50 μg/ml of MOL 171 containing various amounts (μg) of N-SP (N) or C-SP (C) per ml as indicated at the top of each lane. The bound antibodies were visualized by peroxidase-conjugated anti-mouse IgG.

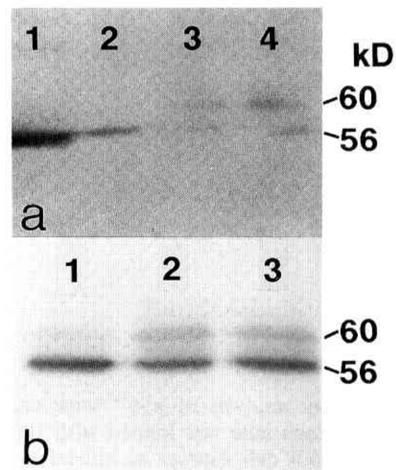

Fig. 6. Immunoblot analysis of cells stimulated with PMA by MOL 171. PBTL or JURKAT cells suspended in RPMI1640 at 5×10^5/ml were treated with PMA at 50 ng/ml for stimulation. Cells were sampled for immunoblot analysis before and after stimulation. a; 1, JURKAT. 2, PBTL before stimulation. 3, PBTL 15 min after stimulation. 4, PBTL 30 min after stimulation. b; 1, JURKAT 0 min after stimulation. 2, JURKAT 15 min after stimulation. 3, JURKAT 30 min after stimulation.

171 to the 56 kD band, was blocked (Fig. 4). Furthermore, MOL 294 antibody, recognizing the C-terminal region of p56^{lck}, also detected the 60 kD protein band in addition to the 56 kD band in those HTLV-I-transformed T cell lines (NOBE, TOM-1, ILT-Su) but not in HTLV-I-negative T cell lines (HUT78, JURKAT; Fig. 5). These results indicated that the 60 kD species, expressed predominantly in HTLV-I-transformed T cell lines, is another form of *lck* gene product and bears epitopes composed of N-SP and C-SP amino acids. Moreover in Fig. 5, the decrease in the amount of p56^{lck} was also recognized by MOL 294 as well as by MOL 171 in such HTLV-I-transformed T cell lines.

Detection of p60^{lck} by MOL 171 in PMA-stimulated cells
Marth *et al.* demonstrated the conversion of p56^{lck} to a 60 kD form, p60^{lck}, in cells stimulated with PMA.[19] To confirm further that MOL 171, which detects a 60 kD protein band in HTLV-I-transformed T cell lines, also recognizes p60^{lck}, cells were stimulated with PMA and

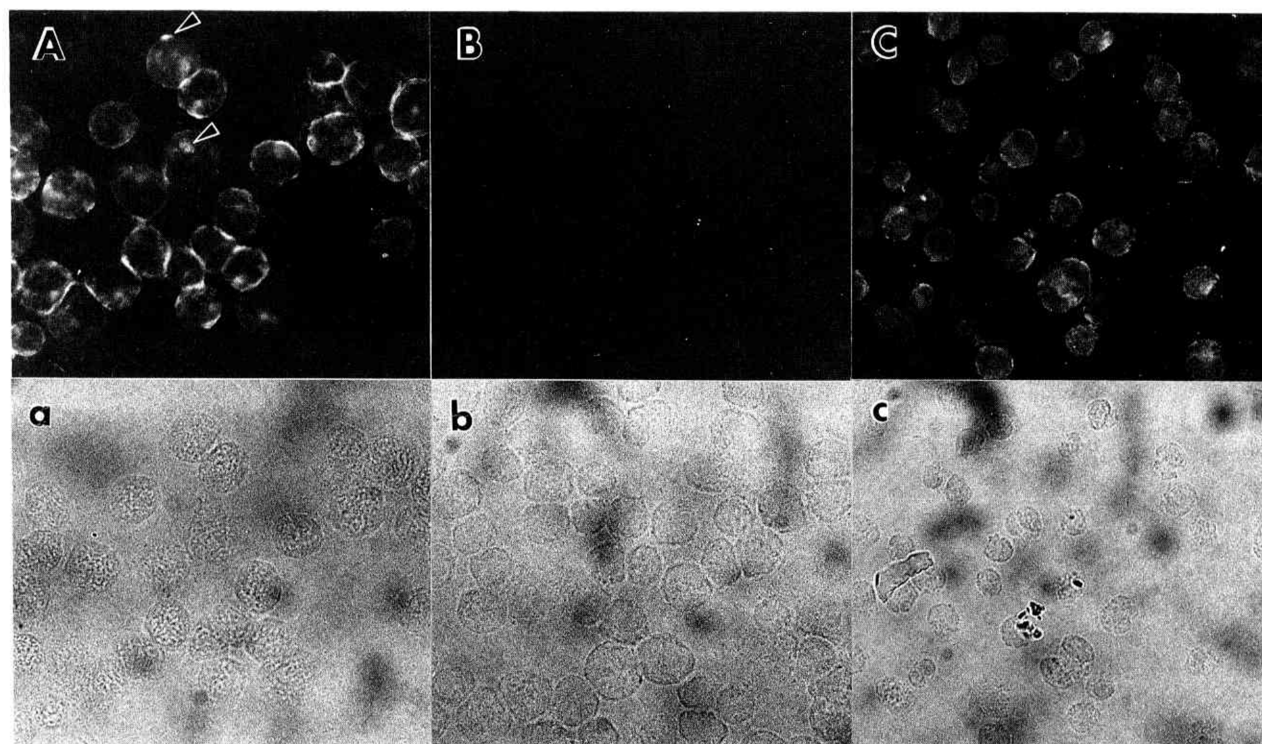

Fig. 7. Immunofluorescence of Lck proteins. JURKAT (A, a), U937 (B, b) and NOBE (C, c) cells were treated with acetone and incubated with 100 μg/ml of MOL 171 at 37°C for 1 h. After washing with PBS, the bound antibodies were visualized by using FITC-conjugated anti-mouse IgG antibody. Then the specimens were examined by fluorescence microscopy (A, B, C) and phase-contrast microscopy (a, b, c). Arrowheads indicate the patch formation of p56^{lck}.

examined by immunoblot analysis with MOL 171 (Fig. 6). The appearance of p60^{lck} accompanied with the decrease of p56^{lck} was clearly revealed by MOL 171 in PBTL (a) and JURKAT (b) after stimulation with PMA. These data support the hypothesis that a 60 kD form detected by MOL 171 in HTLV-I-transformed T cell lines is p60^{lck}, which is a modified form of p56^{lck}.

Immunofluorescence analysis The distribution of Lck proteins was examined by the immunofluorescence method using MOL 171 and FITC-conjugated anti-mouse IgG antibody in JURKAT, U937 and NOBE (Fig. 7). In viable cells, no significant membrane fluorescence could be detected by fluorescence microscopy (data not shown). When cells were treated with acetone to permeabilize cell surface membranes, a distinct membrane fluorescence was detected in JURKAT (A) and NOBE (C), both of which express Lck protein, but not in U937 (B), which expresses no Lck protein. Lck protein was not distributed homogenously but was localized to form vague patches at the membrane (indicated by arrows). Such an uneven distribution of Lck protein at the membrane was also observed after quick fixation of viable cells with formaldehyde followed by staining as described in "Materials and Methods" (data not shown), excluding the possibility of artifacts during the preparation of samples. However, no marked difference in the distribution of Lck protein was found between JURKAT expressing p56^{lck} and NOBE expressing p56^{lck} and p60^{lck}.

DISCUSSION

The genes encoding PTK consist of two distinct groups. Members of the first group are transmembrane proteins and are usually receptors for growth factors such as insulin, platelet-derived growth factor, epidermal growth factor and insulin-like growth factor 1.[20] The second group of PTK, represented by p60^{src}, is closely associated with the internal portion of the cell membrane owing to myristoylation at the amino terminal. The *lck* gene belongs to the second group and encodes 509 amino acids consisting of an N-terminal half where the amino acid sequence is unique among the members of the *src*-family and a C-terminal half where the amino acid sequence is homologous.

MOL 171 is an anti-Lck monoclonal antibody raised against N-SP, whose sequence corresponds to a part of the N-terminal amino acid sequence (71–95) deduced from the *lck* cDNA sequence. In immunoblot analysis, MOL 171 recognized a protein species of 56 kD in all the *lck* mRNA-expressing cells examined so far, such as PBTL and T cell tumor lines, but not the 56 kD protein band in *lck* mRNA-negative cell lines such as U937, MT-2 and RPMI1788. Yamanashi *et al.* reported that $p56^{lyn}$, a member of the membrane-bound PTK family which is highly homologous with $p56^{lck}$ in its kinase domain, is expressed abundantly in MT-2 and RPMI1788 cells.[17, 18] Therefore it is likely that MOL 171 is a highly specific monoclonal antibody recognizing $p56^{lck}$. The specificity of the epitope recognized by MOL 171 was confirmed by the inhibition of the binding of MOL 171 to the 56 kD band upon addition of N-SP (Fig. 2).

The amount of $p56^{lck}$ in HTLV-I-positive T cell lines was significantly less than that of $p56^{lck}$ in HTLV-I-negative T cell lines when examined by using MOL 171 and MOL 294. Moreover, another form of protein of 60 kD was detected in those HTLV-I-transformed T cell lines. N-SP specifically blocked the binding of MOL 171 to the 60 kD band as well as the 56 kD band. Furthermore, MOL 294, an anti-Lck monoclonal antibody raised against C-SP, also detected the 60 kD band in addition to the 56 kD band in HTLV-I-transformed T cell lines. These results support the hypothesis that the 60 kD form is another protein product of the *lck* gene. Marth *et al.* reported that the conversion of $p56^{lck}$ to a more slowly migrating form with the size of 60 kD ($p60^{lck}$) occurs in the T lymphocytes activated with mitogens or PMA.[19] They also found increased phosphorylation of $p56^{lck}$ in those T lymphocytes. In the present study, MOL 171 also detected the induction of $p60^{lck}$ in cells stimulated with PMA. Thus the 60 kD form found in HTLV-I-transformed T cell lines is compatible with $p60^{lck}$ reported by them. HTLV-I-transformed T cell lines exhibit "activated T cell" character, such as constitutive expression of IL-2Rα gene and downregulation of surface CD3 molecules.[21, 22] The accumulation of $p60^{lck}$ in HTLV-I-transformed T cell lines may reflect such an activated state of these T cell lines. The amount of *lck* mRNA accumulated in the cells was almost the same among those HTLV-I-negative and positive T cell lines, as reported before.[13]

In the previous study of HTLV-I-transformed T cell lines, we found that little or no *lck* mRNA is expressed in most IL-2-independent HTLV-I-transformed T cell lines such as MT-2, whereas it is expressed abundantly in IL-2-dependent HTLV-I-transformed T cell lines such as NOBE.[11] However, even in such IL-2-dependent cell lines expressing *lck* mRNA, the amount of $p56^{lck}$ is significantly less than that of $p56^{lck}$ in HTLV-I-negative T cell tumor lines.[13] $p56^{lck}$ is considered to be the functional protein product of *lck*, though its functional role remains unknown.[19, 23] Therefore the decrease of $p56^{lck}$ and the appearance of $p60^{lck}$ in IL-2-dependent HTLV-I-transformed T cell lines may induce functional abnormality of *lck* gene expression and the sequential events of HTLV-I-induced transformation.

When immunofluorescence analysis was performed using MOL 171, Lck proteins were detected predominantly at the cell surface membrane in the cells treated with acetone to permeabilize the surface membrane. In viable cells bearing intact surface membrane, on the other hand, no significant fluorescence was detected at the membrane. These results are consistent with the idea that $p56^{lck}$ is associated with the internal portion of the cell surface membrane owing to myristylation at its glycine residue 2.[24] No marked difference in the pattern of distribution of Lck proteins was observed in JURKAT with $p56^{lck}$ and in NOBE with $p56^{lck}$ plus $p60^{lck}$. Of note is the uneven distribution of Lck proteins at the membrane in those cells. While such localized distribution on the membrane has also been reported in the case of $p60^{v\text{-}src}$,[25] its biological significance remains unclear.

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