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The human Lyt-3 molecule requires CD8 for cell surface expression

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We have previously identified a monoclonal antibody, T8/2T8-5H7, which clustered serologically with CD8 monoclonal antibodies, but lacked reactivity with L cell transfectants expressing the human CD8 molecule (Lyt-2 homologue). Based on these observations, we postulated that T8/2T8-5H7 might recognize the human Lyt-3 gene product. To test this hypothesis, we have isolated a full-length cDNA encoding the human Lyt-3 molecule and have characterized its product in additional transfection experiments. The results of these studies indicate that the human Lyt-3 cDNA encodes a product recognized by the antibody T8/2T8-5H7. Interestingly, the human Lyt-3 molecule cannot be expressed alone, but requires the human Lyt-2 homologue for efficient cell surface expression. A heterodimer composed of the human Lyt-2 and Lyt-3 molecules may have importance in T cell-target cell interactions.

Key words: CD8A/CD8B/human CD8 complex/human Lyt-3/T cell differentiation

Introduction

Thymus-derived lymphocytes (T cells) interact with their environment through a variety of specific cell surface proteins acquired in the course of their ontogenetic development. One such cell surface molecule is the CD8 antigen (T8, Tp32), which identifies cytotoxic/suppressor T cells which interact with Major Histocompatibility Complex (MHC) class I bearing targets (reviewed in Littman, 1987). The CD8 antigen is thought to play a role in the process of T cell mediated killing, as monoclonal antibodies (mAbs) which recognize the CD8 antigen block the formation of effector-target conjugates, the initial step in this process (Spits *et al.*, 1986). The role of CD8 in conjugate formation is thought to occur through recognition of and binding to the non-polymorphic determinants of the target cell MHC class I molecules (MacDonald *et al.*, 1982; Swain, 1983).

In man, CD8 molecules are expressed on the cell surface as disulfide-linked homodimers and multimers of a 32 kd protein on peripheral T cells, and as a disulfide-linked heterodimer with the CD1a antigen (T6, Tp45) on thymocytes (Snow and Terhorst, 1983; Snow *et al.*, 1985; Ledbetter *et al.*, 1985). The gene encoding the human CD8 molecule has been cloned (Kavathas *et al.*, 1984; Littman *et al.*, 1985) and transfection of this cDNA into a variety of lymphoid and non-lymphoid cells has allowed for the surface expression of human CD8 as recognized by well-characterized CD8 mAbs (DiSanto *et al.*, 1987; Hambor *et al.*, 1988; Ratnofsky *et al.*, 1988).

In contrast, rodents express the CD8 antigen as disulfide-linked heterodimers and multimers of two distinct polypeptide chains (Ledbetter *et al.*, 1981; Luescher *et al.*, 1984; Walker *et al.*, 1984). The first component (murine Lyt-2; rat OX-8 32 kd) is homologous to the cloned human CD8 cDNA (Johnson *et al.*, 1985; Johnson and Williams, 1986; Nakauchi *et al.*, 1985), while the second chain (murine Lyt-3; rat OX-8 37 kd), initially thought not to exist in humans, has only recently been shown to have a human homologue (Johnson, 1987). It is presently unknown whether the human Lyt-3 molecule is expressed on the cell surface.

In this report, we describe the isolation of a full-length cDNA clone for the human Lyt-3 molecule and demonstrate its cell surface expression in murine L cell transfectants and in human peripheral blood mononuclear cells (PBMC).

Results

We have previously characterized 40 mAbs recognizing the CD8 cluster of differentiation which were submitted to the *Third International Workshop on Leukocyte Differentiation.* These antibodies were tested for their reactivity with murine L cells expressing the human CD8 cDNA (LCD8 cells) in parallel with analyses of PBMC. The indirect immunofluorescence data are summarized in Figure 1 (panels A-F). LCD8 cells strongly express the CD8 antigen and exhibit a broad distribution of fluorescence intensity (panel B), as exemplified by their staining pattern with M236, a known CD8 mAb (DiSanto *et al.*, 1987). One of the 40 mAbs, T8/2T8-5H7, completely failed to bind to the LCD8 cells (panel C) while retaining a CD8-like pattern of expression on PBMC (as shown in panel F). This result was surprising since this antibody had been serologically clustered with other CD8 mAbs in the two previous international workshops (Haynes, 1986; McMichael and Gotch, 1987) and had also been shown to bind to $CD8^+$ cytotoxic T cell clones (unpublished data). We hypothesized that T8/2T8-5H7 might recognize a CD8 epitope that failed to develop in the L cells, perhaps due to a species-specific glycosylation difference (DiSanto *et al.*, 1987). Alternatively, we postulated that T8/2T8-5H7 might recognize another molecule with a distribution similar to CD8, such as the human Lyt-3 molecule.

The human Lyt-3 molecule is a homologue of the second chain of the rodent CD8 complex (murine Lyt-3; rat OX-8 37 kd) and has been recently identified at the genomic level (Johnson, 1987). To isolate a cDNA clone for the human Lyt-3 gene, we synthesized a 50 base oligonucleotide probe (50 mer) corresponding to the 5′ coding region of the genomic sequence. This probe detected a transcript of ~1.5 kb when hybridized to mRNA from the immature T cell line HPB-ALL (data not shown). A cDNA library from the HPB-

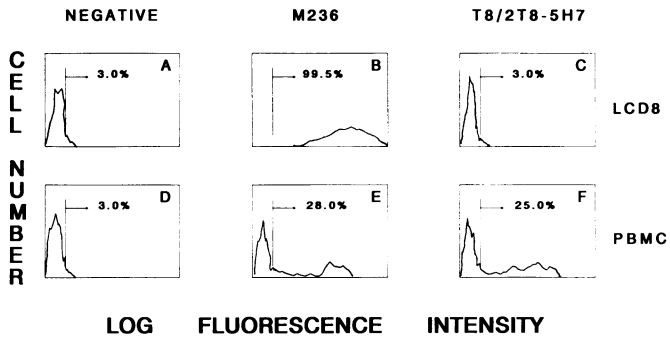

Fig. 1. Cytofluorographic profiles of indirect immunofluorescence analysis of L cells expressing the CD8 cDNA (**panels A–C**) and peripheral blood mononuclear cells (**panels D–F**). Panels A and D illustrate staining with negative control antibody P3X63. Panels B and E illustrate staining with CD8 antibody M236. Panels C and F illustrate staining with the antibody T8/2T8-5H7.

ALL cell line was prepared in the λgt10 vector (Huynh *et al.*, 1985). Using the 50 mer, 10^5 recombinant plaques were screened in order to isolate a recombinant phage containing a 1.4 kb insert encoding the human Lyt-3 cDNA. The sequence of this cDNA clone is depicted in Figure 2.

Comparison of the nucleotide sequence of the human Lyt-3 cDNA with the exons of the published genomic sequence showed them to be highly homologous, with three substitutions over 577 bases (99.5% overall homology). These substitutions result in two amino acid changes at residue 150 (Val to Ile) and residue 170 (Met to Ile). Although these changes may represent allelic differences in man, it should be noted that the rat homologue (OX-8 37 kd) contains isoleucine at both these residues (Johnson and Williams, 1986). The human Lyt-3 cDNA contains an open reading frame of 630 bp capable of encoding a polypeptide chain of 210 amino acids. Following removal of the 21 amino acid hydrophobic leader sequence, an unglycosylated polypeptide of 189 amino acids corresponding to a mol. wt of ~24,710 daltons is predicted. A single N-linked glycosylation site is present at position Asn-81. It is conceivable that glycosylation would increase the apparent mol. wt of the human Lyt-3 molecule by up to 10 kd, as suggested for the human CD8 molecule (Littman *et al.*, 1985) and the rodent Lyt-3 molecules (Luescher *et al.*, 1985).

The human Lyt-3 molecule has the structure of a transmembrane protein with a 118 amino acid extracellular domain having a single immunoglobulin-like variable region domain bounded by Cys-20 and Cys-95. This is followed by a 25 amino acid membrane-proximal domain having a high proportion of threonines (20%), prolines (16%) and lysines (20%). This domain is thought to contain the hinge-region and the trypsin-sensitivity sites of the molecule, similar to the murine Lyt-3 molecule (Blanc *et al.*, 1988; Panaccio *et al.*, 1987). Following a 30 amino acid hydrophobic transmembrane region is a 16 amino acid highly charged intracellular domain. It was originally believed that this region of the human Lyt-3 molecule consisted of a single exon and encoded only 13 amino acids (Johnson, 1987). Our sequence suggests that there must be at least one additional exon which encodes the terminal three intracellular amino acids (Phe-187 to Lys-189) of the human Lyt-3 molecule. Interestingly, one of these residues is a potential site for protein phosphorylation (Tyr-188). It is conceivable that this post-translational modification of the human Lyt-3 molecule may play a role in its function as has been suggested for the CD8 molecule (Acres *et al.*, 1987).

Expression of human Lyt-3 mRNA transcripts in a variety of hematopoietic cells and cell lines is depicted in Figure 3. A 1.5 kb message was detected in RNA derived from allogeneically stimulated PBMC and in the T cell leukemia cell line, HPB-ALL. No transcripts were detected in mRNA from other T cell lines or from myeloid or B cell lines. In addition, mRNA derived from leukemic cells expressing the phenotype and functional characteristics of mature NK cells also lacked human Lyt-3 transcripts (Lp, Figure 3). All of the cells and cell lines tested for the presence of human Lyt-3 transcripts expressed the 1.7 kb gamma actin transcript. In a parallel set of experiments, we have found that the antigen recognized by T8/2T8-5H7 is present on cells derived from an allogeneic mixed lymphocyte culture (MLC) and on HPB-ALL, but not on normal B cell lines or the T-cell line MOLT-4 (data not shown). Thus the binding of T8/2T8-5H7 and the expression of human Lyt-3 mRNA correlate very closely.

The 1.4 kb human Lyt-3 cDNA insert was subcloned into the mammalian cell expression vector pcEXV-3 (Miller and Germain, 1986). Clones were isolated in which the insert was present in the 'sense' (pLY3F) or 'antisense' (pLY3R) orientations with respect to the SV40 early region promoter. These expression plasmids were transfected into mouse L cells and LCD8 cells. After 3 days, the transfectants were screened for binding of the T8/2T8-5H7 antibody by a colony immunorosetting technique (DiSanto *et al.*, 1988). A series of phase-contrast photomicrographs of the transfected cells tested in these experiments are illustrated in Figure 4.

LCD8 cells were uniformly coated with beads (bead-rosettes) following binding of the CD8 mAb M236 (panel A). However, these cells did not form rosettes with the mAb T8/2T8-5H7 (panel B). Transfection of pLY3F ('sense' orientation) into LCD8 cells produced T8/2T8-5H7-positive cell clusters throughout the plate. Two representative clusters can be seen in panels C and D. Approximately 10–15% of the pLY3F transfected LCD8 cells formed bead-rosettes with T8/2T8-5H7. In parallel experiments, L cells were transfected with pLY3F and were screened for expression using T8/2T8-5H7 or the CD8 mAb M236. We repeatedly failed to detect binding of either T8/2T8-5H7 or M236 antibodies (panels E and F). Transfection of pLY3R ('antisense' orientation) did not result in T8/2T8-5H7 binding in either L cells or LCD8 cells (data not shown). Thus binding of the T8/2T8-5H7 antibody to the cell surface is dependent on the presence of the human Lyt-3 gene product. Furthermore, the human Lyt-3 molecule cannot be expressed alone, but additionally requires the presence of the human Lyt-2 molecule for efficient cell surface expression.

Discussion

The CD8 antigen has been hypothesized to interact with non-polymorphic determinants of MHC class I molecules during the course of T cell activation and effector function (reviewed in Littman, 1987). The interactions between CD8 and MHC class I molecules are thought to occur through the immunoglobulin-like domains present in both molecules (Littman *et al.*, 1985). By binding to MHC class I molecules, the CD8

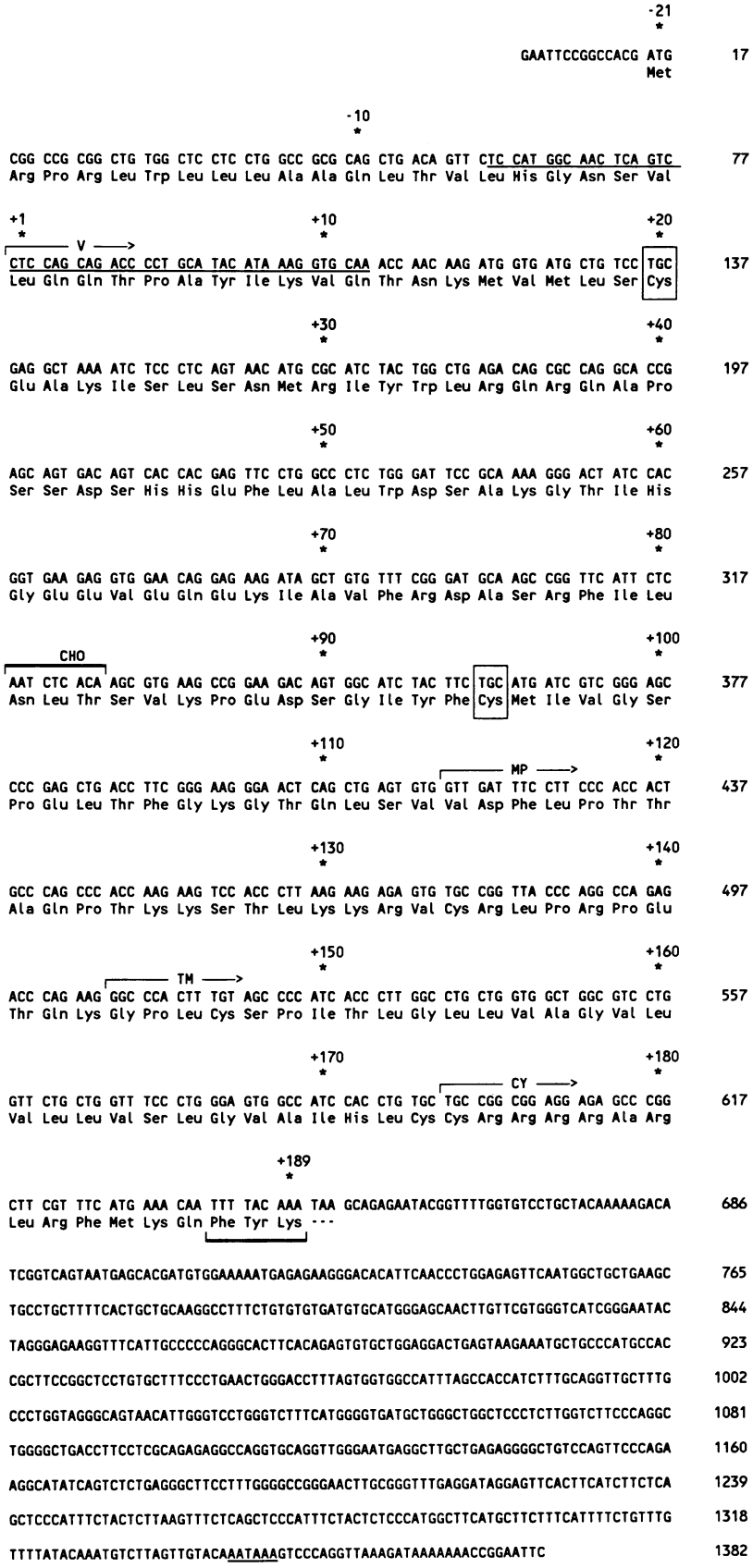

Fig. 2. Sequence of the human Lyt-3 cDNA and the predicted protein. The oligonucleotide probe used to isolate this clone was complementary to the 50 base sequence underlined at the N-terminus. Horizontal lines above the nucleotide sequence depict the variable (V), membrane-proximal (MP), transmembrane (TM) and cytoplasmic (CY) domains. The presumed intrachain cysteines are boxed. A single potential N-linked glycosylation site is marked CHO. The three previously unidentified cytoplasmic residues are double underlined. The consensus polyadenylation signal sequence (AATAAA) is underlined.

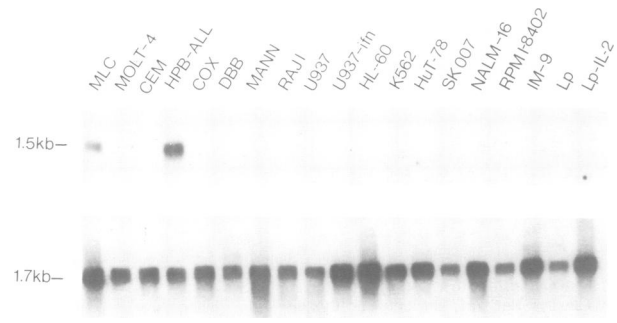

Fig. 3. Northern blot analysis using the human Lyt-3 cDNA as a probe (**upper panel**). MLC represents a bulk (uncloned) culture of allogeneically stimulated PBMC. Lp represents a patient with NK-leukemia. Lp-IL-2 RNA was derived from the same cells after culture in recombinant-IL-2 (50 U/ml; Amgen, Inc.). The same blot was rehybridized with a probe for gamma actin (**lower panel**).

antigen is believed to facilitate and stabilize T cell–target cell conjugate formation, which represents the initial step in T cell activation and cytotoxicity (Spits *et al.*, 1986).

The molecular structure of the CD8 antigen in man has been extensively studied (Kavathas *et al.*, 1984; Ledbetter *et al.*, 1985; Littman *et al.*, 1985; Snow and Terhorst, 1983; Snow *et al.*, 1985). The isolation and subsequent expression of a CD8 cDNA confirmed the idea that the human CD8 antigen was composed of a 32 kd protein which formed disulfide-linked homodimers and multimers on peripheral blood T cells (Littman *et al.*, 1985). This was in sharp contrast to the rodent CD8 complex, which clearly consisted of two distinct polypeptide chains expressed as a heterodimer on the cell surface (Ledbetter *et al.*, 1981; Luescher *et al.*, 1984; Walker *et al.*, 1984).

Recently, a human homologue for the gene encoding the second chain of the rodent CD8 complex (Lyt-3) has been described (Johnson, 1987). In this report, we describe the isolation of a human Lyt-3 cDNA. The striking similarity between the nucleotide sequence of this cDNA and the coding regions of the genomic sequence (>99.5%) strongly suggest that the cDNA is indeed derived from the previously described human Lyt-3 genomic locus. The only differences we have detected in the proposed amino acid sequence are in fact changes which make the human product more homologous to its rat counterpart (Johnson and Williams, 1986). The additional coding sequence present in the cDNA (15 amino acids in the leader sequence and 3 amino acids in the cytoplasmic domain) suggest that the genomic structure of the human Lyt-3 gene is considerably larger than previously appreciated (Johnson, 1987), and will contain at a minimum, two additional exons. Included in the human Lyt-3 cytoplasmic domain is a tyrosine residue, a known site for protein phosphorylation. Both the CD4 and the CD8 antigens are known to be phosphorylated following activation of thymocytes and peripheral blood T cells (Acres *et al.*, 1986, 1987; Blue *et al.*, 1987). It is possible that Lyt-3 is also phosphorylated as a consequence of T cell activation.

Although the human Lyt-3 gene was shown to be transcribed in thymocytes and some acute T-cell leukemias (Johnson, 1987), cell surface expression of a human Lyt-3 product has not been previously demonstrated. The present studies indicate that such a product is indeed expressed in man. This molecule can be detected by the mAb T8/2T8-5H7, a mAb which serologically exhibits a distribution very similar to classical CD8, yet which fails to bind L cell transfectants expressing the human CD8 cDNA. Interestingly, however, the binding of T8/2T8-5H7 to human Lyt-3 transfectants is strictly dependent on co-expression of the human Lyt-2 molecule. A similar phenomenon has recently been reported with murine Lyt-2 and Lyt-3 transfectants (Blanc *et al.*, 1988). Binding of T8/2T8-5H7 to human Lyt-3 transfectants cannot be detected (at least by rosetting techniques) in the absence of the human Lyt-2 homologue. It is conceivable that a small number of human Lyt-3 molecules reach the cell surface in the absence of CD8 but that their low levels of expression are not detectable with this methodology.

At the present time, we cannot ascertain whether T8/2T8-5H7 recognizes an epitope to which both CD8 and human Lyt-3 chains contribute or whether the epitope is dependent solely on the human Lyt-3 molecule, with CD8 necessary only to allow Lyt-3 to reach the cell surface. If T8/2T8-5H7 recognized an epitope formed by the association of human Lyt-2/Lyt-3, then this epitope must be spatially distinct from the Leu2a epitope, as T8/2T8-5H7 failed to block Leu2a binding (DiSanto *et al.*, 1987). Experiments are presently underway to characterize the epitope recognized by T8/2T8-5H7.

The human Lyt-3 transcript is present in thymocytes (Johnson, 1987), and in allogeneically stimulated PBMC (Figure 3). T8/2T8-5H7 has been demonstrated to bind to 50% of thymocytes, 25% of normal resting T cells, 25% of PHA-activated and allo-activated PBMC, and to mature $CD8^+$ cytotoxic T cell clones (Van Seventer *et al.*, 1987 and unpublished data). These findings indicate that the human Lyt-3 molecule is expressed on the T cell surface. The existence of a second cell surface protein in the human CD8 complex raises the possibility that a heterodimer of human Lyt-2/Lyt-3 may function in conjugate formation, or activation of thymocytes and mature T cells in man. Although functional data is still accumulating, T8/2T8-5H7 has been shown to block anti-CD3 induced proliferation of $CD8^+$ T cells, yet it failed to block the function of an allo-specific $CD8^+$ T cell clone (Van Seventer *et al.*, 1987).

In summary, the human T lymphocyte CD8 complex is composed of two molecules, the human Lyt-2 and Lyt-3 molecules (now referred to as CD8A and CD8B, respectively, as suggested by Spurr *et al.*, 1988). These molecules have the capacity to be expressed on the T cell surface as two distinct complexes, a homodimer of CD8A (CD8A/A) and a heterodimer of CD8A/B. The structures of these molecules have been conserved through evolution, and it remains to be seen if CD8A/A and CD8A/B subserve similar functions. Perhaps one complex is involved in binding to classical HLA-A, B and C molecules while the other interacts with non-classical or 'novel' class I molecules. Alternatively, these two complexes may transduce distinct signals from the cell surface to the cytoplasm, either through phosphorylation or via other yet undefined signalling mechanisms. The similarities and differences in the function of these two complexes in T cell physiology remains to be definitively established.

Materials and methods

Cells and antisera

L cells were maintained in minimum essential medium containing 10% fetal calf serum (FCS). L cells stably expressing the human CD8 antigen, LCD8,

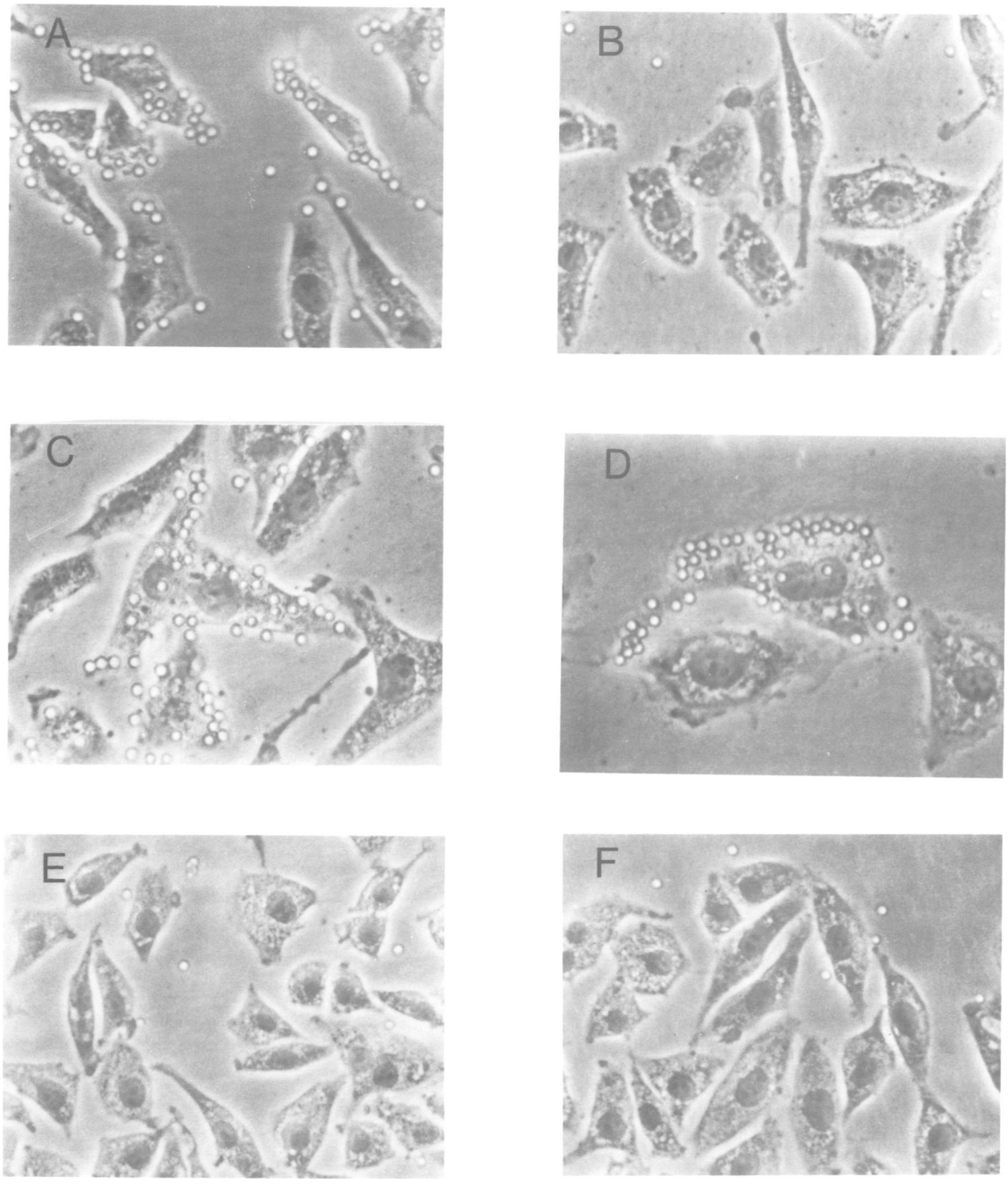

Fig. 4. Magnetic bead immunorosetting of L cell transfectants. (**A**) Rosetting of LCD8 cells with M236. (**B**) LCD8 cells rosetted with T8/2T8-5H7. There was no binding of the beads. (**C**) and (**D**) Rosetting of LCD8 cells transfected with human Lyt-3 cDNA using T8/2T8-5H7. Bead positive clusters were observed throughout the plate. (**E**) L cells rosetted with T8/2T8-5H7 following transfection with human Lyt-3 cDNA and (**F**) L cells rosetted with M236 following transfection with human Lyt-3 cDNA. There was no detectable binding of beads in either (E) or (F).

were constructed by transfecting a plasmid containing a full-length CD8 cDNA (Littman *et al.*, 1985) under the control of the Moloney Leukemia Virus long terminal repeat as described (DiSanto *et al.*, 1987). T-cell lines (MOLT-4, CEM, HPB-ALL, HuT-78, NALM-16 and RPMI 8402), an erythroleukemia cell line (K562), a Burkitt's lymphoma line (RAJI) and lines derived from patients with histiocytic lymphoma (U937) and promyelocytic leukemia (HL-60) were obtained from the American Type Culture Collection. The B lymphoblastoid cell lines, COX, DBB, IM-9 and MANN have been described elsewhere (Crumpton *et al.*, 1984; Schreuder and Degos, 1984). SK007 is a human plasma cell line. All hematopoietic cell lines were maintained in RPMI 1640 medium containing 10% FCS. PBMC were isolated on Ficoll-Hypaque gradients. Blood from a patient with NK-leukemia was obtained from Dr M.Andreeff, Memorial Hospital for Cancer and Allied Diseases, New York, NY.

A panel of 40 mAbs recognizing the human CD8 antigen was obtained through the *Third International Workshop on Leukocyte Differentiation.* Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG was obtained from Tago (Burlingame, CA).

Immunofluorescence

Cells were washed with phosphate buffered saline containing 2% FCS and 0.02% sodium azide and were incubated with saturating amounts of primary mAb for 30 min at 4°C. After two washes, cells were incubated with FITC conjugated goat anti-mouse IgG for 30 min. Cells were again washed twice before analysis on an EPICS C cytofluorograph.

Isolation of a human Lyt-3 cDNA

A cDNA library was constructed using polyadenylated [poly(A)$^+$] mRNA from the immature T cell line HPB-ALL. Full-length cDNA was synthesized

(Gubler and Hoffman, 1983), blunt-ended with Klenow enzyme, and tailed with *Eco*RI linkers. The cDNA was size fractionated using Ultrogel Aca34 (Watson and Jackson, 1985). cDNA >800 bp in length was used to construct a library in the phage vector λgt10 (Huynh *et al.*, 1985).

A 50 mer probe was constructed corresponding to the 5′ coding region of the genomic sequence of the human Lyt-3 gene (Johnson, 1987). This probe was synthesized at the Cornell University Department of Microbiology: 5′ TTGCACCTTTATGTAAGCAGGGGTCTGCTGGAGGACTGAGT-TGCCATGGA 3′. The probe was end-labeled using polynucleotide kinase and [γ-^{32}P]ATP (Maniatis *et al.*, 1982). Prehybridization and hybridization were performed at 48°C for 4 and 18 h, respectively, in buffer containing 6 × NET (1 × NET is 0.15 M NaCl, 1 mM EDTA and 15 mM Tris−HCl, pH 8.0), 0.1% SDS, 0.1% bovine serum albumin, 0.1% polyvinyl-pyrrolidone, 0.1% Ficoll-400 and 100 μg/ml sonicated salmon sperm DNA. Filters were washed twice in 6 × NET, 0.1% SDS at 55°C and were exposed to X-ray film for 6 h at −70°C. cDNA inserts of interest were subcloned into the plasmid vector, pUC18 (Yanisch-Perron *et al.*, 1985). Supercoiled plasmid DNA was sequenced (Chen and Seeberg, 1985) by the dideoxy method (Sanger *et al.*, 1977).

RNA hybridization

Total cellular RNA was prepared by standard techniques and the poly(A)$^+$ mRNA selected by oligo(dT) chromatography (Maniatis *et al.*, 1982). Poly(A)$^+$ RNA (1 μg) was electrophoresed through 1% agarose−formaldehyde gels and transferred to GeneScreen Plus membranes (Du Pont, NEN). Hybridizations with nick-translated probes were carried out for 16 h at 42°C in 1 M NaCl, 1% SDS, 10% dextran sulfate, 100 μg/ml sonicated salmon sperm DNA and 50% formamide. High stringency washes were at 60°C in 1% SDS and 0.2 × SSC (1 × SSC is 0.15 M NaCl and 15 mM NaCitrate, pH 8.0). Subsequent hybridization with a cDNA probe for gamma actin (Gunning *et al.*, 1983) was used to standardize the amounts of mRNA loaded per lane.

Transfection and immunomagnetic rosetting

Expression of the human Lyt-3 cDNA in mammalian cells utilized the plasmid vector pcEXV-3 (Miller and Germain, 1986). The 1.4 kb *Eco*RI fragment encoding the human Lyt-3 cDNA was cloned into the *Eco*RI site of pcEXV-3. Both orientations of the cDNA with respect to the SV40 promoter were obtained for use in transient expression in L cells or LCD8 cells. Transfections were carried out using the DEAE−dextran method as described (Selden, 1987). After 3 days, cells expressing human Lyt-3 were assayed by an immunorosetting technique (DiSanto *et al.*, 1988) using T8/2T8-5H7 and goat anti-mouse IgG coupled magnetic microspheres (Dynal M-450, Fort Lee, NJ).

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