The expression of cytoplasmic CD3 (CyCD3) was analyzed in 45 leukemias, five thymus cell samples, five peripheral blood (PB) samples, and ten cell lines. All T cell acute lymphoblastic leukemias (T-ALL) that did not express surface membrane CD3 (SmCD3) appeared to express CyCD3. Furthermore, the majority of SmCD3+ T-ALL also expressed CyCD3. Analogous results were obtained with thymus cell samples in that about 95% of the thymocytes expressed CyCD3 whereas 60% to 75% of the thymocytes also expressed SmCD3. In normal peripheral blood only prominent SmCD3 expression was found. These data indicate that immature T cells express CyCD3 only, that the combined expression of CyCD3 and SmCD3 is characteristic for intermediate differentiation stages, and that mature T cells express prominent SmCD3. All (precursor) B cell leukemias, acute myeloid leukemias, and non–T cell lines tested did not express CyCD3. On the basis of these data, we conclude that CyCD3 expression is restricted to the T cell lineage and can be used as a diagnostic marker for immature SmCD3+ T cell malignancies. Therefore, we evaluated which fixative is optimal for CyCD3 staining, and we determined by immunofluorescence staining and Western blotting which anti-CyCD3 monoclonal antibody (MoAb) can be used for the detection of CyCD3. In our opinion, acid ethanol was the best fixative for the cytocentrifuge preparations. Furthermore, we demonstrated that CyCD3 can be easily detected by use of MoAbs raised against denatured CD3 chains such as those of the SP series (SP-6, SP-10, SP-64, and SP-78). In addition we tested 22 anti-CD3 MoAbs of the Oxford CD3 panel that were raised against native SmCD3, and it appeared that only four (UCHT1, VIT-3b, G19-41 and SK7/Leu-4) of them were able to detect CyCD3. In Western blot analysis all four MoAbs recognized the CD3-ε chain only.

**T CELL DIFFERENTIATION and T cell ontogeny can be studied by analysis of thymus subpopulations or, alternatively, by analysis of T cell leukemias because the latter can be regarded as the malignant counterparts of cells in the various T cell differentiation stages.** Such studies revealed that the immunologic phenotype of T cells gradually changes during differentiation, finally resulting in mature T cells that express the CD3 antigen on their cell surface.

The human CD3 antigen consists of at least three protein chains (CD3-γ, CD3-δ, and CD3-ε), the encoding genes of which have been cloned recently. These CD3 chains are closely associated with the antigen-specific receptor of T cells, the so-called T cell receptor (TcR). To date three different TcR chains have been described: TcR-α, TcR-β, and TcR-γ. Like the immunoglobulin genes, the TcR genes rearrange during T cell differentiation to enable receptor diversity. The majority of the mature T cells express a TcR that is composed of disulfide-linked TcR-α and TcR-β chains. The TcR-γ chain is expressed by a subpopulation of thymocytes and a small population of blood lymphocytes that can display non–major histocompatibility complex–restricted cytotoxicity.

In the TcR-CD3 complex on the cell membrane of mature T cells the CD3 antigen probably plays a role in transduction of signals from the TcR to the cytoplasm.

Link et al reported cytoplasmic expression of CD3 (CyCD3) by immature T cell leukemias as detected in frozen tissue sections and cytocentrifuge preparations, whereas no CD3 expression could be detected on the cell surface of these immature T cells by immunofluorescence (IF) staining in suspensions. At that time they could not explain their findings; however, recent studies indicate that CD3 expression on the cell surface is dependent on the presence of the TcR and that CD3 mRNA is already present in immature T cells. Therefore, CyCD3 can be expected in immature T cells that do not yet express the CD3 antigen on the cell surface membrane (SmCD3). Such CyCD3 expression has recently been reported by Furlay et al and Campana et al.

In this study we evaluated the expression of CyCD3 during early T cell differentiation and proved its application as a diagnostic marker for immature T cell malignancies. Therefore, we determined by Western blotting and IF staining techniques which anti-CD3 monoclonal antibodies (MoAb) can be used for the detection of CyCD3.

**MATERIALS AND METHODS**

**Cell Samples**

Leukemic cell samples. Bone marrow (BM) and peripheral blood (PB) samples were obtained from 45 leukemia patients at diagnosis. These comprised 19 T cell acute lymphoblastic leukemias (T-ALL), one T cell chronic lymphocytic leukemia (T-CLL), 12...
precursor B-ALL (null ALL, common ALL, or pre-B-ALL), seven B-CLL, and six acute myeloid leukemias (AML).

Normal PB samples. Five PB samples were obtained from five healthy adult volunteers.

Thymus samples. Five thymus samples were obtained from five children (<2 years old) undergoing cardiac surgery. These samples were minced with scissors in RPMI 1640 medium containing 15% fetal calf serum (FCS) and were flushed through a nylon gauze filter with 100-μm openings (Stokvis and Smits Textielmi, Haarlem, The Netherlands).

All human cell samples were obtained with the approval of the Committee of Medical Ethics of the Academic Hospital, Rotterdam.

Cell lines. For control experiments and for testing the various anti-CD3 MoAbs, we used the T cell lines Molt4, Hg, 8102, CEM, and HSB; the precursor B cell lines BY173 and SMS-SB; and the Burkitt's lymphoma-derived cell lines ROS-1, ROS-15, and ROS-17. The cell lines were cultured in RPMI 1640 medium supplemented with 15% FCS and antibiotics.

Immunologic Marker Analysis

Mononuclear cells (MNC) from BM and PB were isolated by Ficoll-Paque (density, 1.077 g/mL; Pharmacia, Uppsala, Sweden) density centrifugation. All washings were performed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), pH 7.8.

Routine immunologic marker analysis. Routine immunologic marker analysis of the cell samples was performed by determining the nuclear membrane expression of terminal deoxynucleotidyl transferase (TdT), surface membrane expression of HLA-DR and various T cell markers, B cell markers, and myelomonocytic markers.

Positivity for TdT was tested on cytocentrifuge preparations (Cytofix, Nordic Immunological Laboratories, Tiltberg, The Netherlands) by using a rabbit anti-TdT antiserum and a fluorescein isothiocyanate (FITC)-conjugated, goat antirabbit Ig, second-step antiserum (Supertechs, Bethesda, MD). Expression of SmIg was tested by incubation of cells in suspension with FITC or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antihuman Ig, second-step antiserum (Supertechs, Bethesda, MD). Expression of SmIg was tested by incubation of cells in suspension with FITC or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antihuman Ig, second-step antiserum (Supertechs, Bethesda, MD).

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Western blotting. Immunofluorescence purified CD3 (0.1 nmol) from human tonsils was loaded into a 10-cm well and electrophoresed in a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. As was demonstrated previously, CD3-γ, CD3-δ, and CD3-ε chains can be separated from each other in SDS-polyacrylamide gel electrophoresis. The separated polypeptides were electrophoretically transferred at 4°C to a nitrocellulose filter (Schleicher and Schuell, Dassel, FRG) at 300 V for two hours in a Tris-glycine-methanol buffer. The filter was blocked overnight at 4°C with 2% (wt/vol) BSA, 5% (vol/wt) skim milk powder, 0.05% Tween 20, and 0.02% Na azide in PBS. Reactivity of the various anti-CD3 MoAbs with the transferred CD3 polypeptides was studied by incubating strips of the nitrocellulose filter overnight at 4°C with the MoAb at a dilution of 1:50 in PBS containing 2% (vol/wt) BSA and 0.02% Na azide. After washing three times in PBS and 0.05% Tween 20, the strips were incubated for 1.5 hours with a biotinylated rabbit antihuman Ig antiserum (Vector Laboratories, Burlingame, CA) and washed as described earlier. The strips were finally incubated for one hour with horseradish peroxidase-conjugated avidin (Vector). After washing, bound antibody was visualized with diaminobenzidine (0.5 mg/100 mL) and hydrogen peroxide (0.06%) in 10 mmol/L Tris-HCl buffer, pH 7.4.

Detection of CyCD3 protein chains. For the detection of CyCD3 protein chains, cytocentrifuge preparations were made, air-dried, fixed, and subsequently incubated with anti-Cy3-γ, anti-Cy3-δ, or anti-Cy3-ε antibodies. Some fixatives for fixation of the cytocentrifuge preparations were tested: methanol, ethanol, acetone, acid ethanol (ethanol with 5% [vol/vol] acetic acid), and formol acetone (acetone with 1% [vol/vol] formaldehyde).

The following anti-CD3 protein chain antibodies were used: a rat anti-CD3-γ polyclonal antiserum, the anti-CD3-3 MoAbs SP-64 and SP-78, and the anti-CD3-ε MoAbs SP-6 and SP-10. An FITC-conjugated, goat antirat Ig antiserum (Nordic) was used as a second-step reagent in the case of the rat antiserum and an FITC-conjugated, goat antimouse Ig antiserum in the case of the MoAbs.

Double IF staining for a surface membrane marker and an intracellular marker. Double IF stainings were performed for SmIg and CyCD3 as well as for T cell membrane markers and TdT. For SmIg/CyCD3 double IF stainings, the cells in suspension were incubated with a TRITC-conjugated, antihuman Ig antiserum. Subsequently, cytocentrifuge preparations of these labeled cells were subjected to indirect IF staining with an anti-Cy3-δ or anti-Cy3-ε MoAb and an FITC-conjugated, goat antimouse Ig antiserum as a second-step reagent. For T cell marker/TdT double IF stainings, the cells in suspension were incubated with an anti-T cell marker MoAb and a TRITC-conjugated, goat antimouse Ig antiserum.
CYTOPLASMIC EXPRESSION OF THE CD3 ANTIGEN

Fig 1. Double IF staining for SmIg and CyCD3 (SP-64) on a mixture of the Burkitt’s lymphoma-derived cell line ROS-1 and the T cell line Molt4. (A) Phase-contrast morphology. (B) SmIg+ cells (TRITC labeled). (C) CyCD3+ cells (FITC labeled). The three micrographs represent the same field. All SmIg+ cells are negative for CyCD3.

RESULTS

Detection of CyCD3 Protein Chains

The MoAbs SP-6, SP-10, SP-64, and SP-78 were used to determine which fixative should be used for the detection of CD3 protein chains in the cytoplasm of the T cell lines Molt4, H9, 8102, CEM, and HSB. Fixation of the cytocentrifuge preparations with acid ethanol (ethanol with 5% [vol/vol] acetic acid) for 15 minutes at –20°C appeared to result in the most prominent CyCD3 staining. It should be remarked that only cytocentrifuge preparations with a good cytomorphology as determined by phase-contrast microscopy resulted in optimal CyCD3 staining. Therefore, all cytocentrifuge preparations were checked for their quality before they were subjected to the CyCD3 staining procedure. Each of the four MoAbs of the SP series was able to detect CyCD3 chains in the five T cell lines tested. Also, surface membrane-like CD3 staining was observed in the cytocentrifuge preparations of the T cell lines H9 and CEM (see the next section).

To exclude nonspecific staining by the anti-CD3 chain MoAb, an SmIg/CyCD3 double IF staining was performed on a 1:1 mixture of the T cell line Molt4 and the Burkitt’s lymphoma-derived cell line ROS-1. In these experiments it appeared that all SmIg+ cells were negative for CyCD3 and vice versa, thereby indicating that CyCD3 staining can be easily used for evaluation at the single-cell level (Fig 1).

CD3 Staining Patterns in Cytocentrifuge Preparations

The typical CyCD3 staining is a nondiffuse, irregular, filamentous staining that is most prominent at the position of nuclear clefts. This often results in a dotlike staining when a large nuclear cleft is present (see Figs 1, 2, and 3). Such information can be obtained only if the fluorescence microscope is equipped with optimally adjusted phase-contrast facilities and if the cells are studied at various focus levels.

As was indicated earlier, the cells of the T cell lines H9 and CEM also expressed surface membrane-like CD3 staining in addition to the typical CyCD3 staining. This was confirmed by CyCD3 staining of these cells in suspension. The SmCD3 staining pattern in cytocentrifuge preparations is dependent on the focus level and varies from ringlike (Fig 4), when the focus level is just above the object glass, to a more diffuse staining, when the focus level is somewhat higher (Fig 2). It should be remarked, however, that a weak CyCD3 staining may be undetectable in our assays when the SmCD3 staining is very bright.

Expression of CyCD3 by Human Thymocytes

Five thymocyte cell samples from children (<2 years old) were evaluated for the expression of CyCD3 by using the MoAbs of the SP series and the rat anti-CD3-γ antiserum. The majority (>95%) of the thymocytes were positive for the typical CyCD3 staining, and in addition, surface membrane-like staining was also seen on a major part of the thymocytes. The intensity of the CyCD3 and SmCD3 stainings varied from cell to cell. Three different types of CD3 staining patterns could be recognized in the thymus cell samples: about 20% to 30% of the thymocytes expressed only CyCD3, 60% to 75% expressed both CyCD3 and SmCD3, and about 5% of all thymocytes expressed prominent levels of SmCD3, whereas no typical CyCD3 staining was detectable in the latter cells (Fig 2). These findings indicate that the majority of SmCD3+ thymocytes also contain variable amounts of CD3 protein chains in the cytoplasm.

Fig 2. Staining for CD3 (SP-10) on a cytocentrifuge preparation of a thymus cell sample. (A) Phase-contrast morphology. (B) CD3-positive cells. This figure illustrates different CD3 staining patterns: the majority of the thymocytes express both CyCD3 and SmCD3, whereas one cell (arrow) mainly expresses SmCD3.
Expression of CyCD3 by T-ALL Cells

Nineteen T-ALL cell samples were analyzed for the expression of CyCD3-δ (SP-64 and SP-78) and CyCD3-α (SP-6 and SP-10) as well as the expression of TdT and the surface membrane expression of the T cell markers CD1, CD2, CD3, CD4, CD5, CD7, and CD8; the B cell markers CD10, CD19, CD20; and the myeloid markers CD13, CD14, CD15, and CD33. The results of the immunologic marker analysis are summarized in Tables 1 and 2. All T-ALL samples expressed TdT. If the percentage of TdT cells was lower than 60%, double IF stainings for a T cell marker and TdT were performed to determine the immunologic phenotype of the TdT+ cells (Table 1).

Eleven T-ALL samples did not express SmCD3 whereas eight T-ALL samples were positive for SmCD3. One SmCD3+ T-ALL sample had the phenotype of the putative prothymocyte (TdT+/HLA-DR+/CD7+/CD2+/CD5+/CD1−/CD3−), and six T-ALL samples appeared to be examples of immature thymocytic T-ALL (TdT+/CD7+/CD2+/CD5+/CD1−/CD3−). All eleven SmCD3+ T-ALL samples did express CyCD3, including the patient with prothymocytic T-ALL (Fig 3). Also seven of the eight T-ALL samples that expressed SmCD3, as determined by labeling of the cells in suspension, expressed CyCD3. These data probably indicate that all T-ALL samples, whether they are immature or mature, express either CyCD3, SmCD3, or both.

In addition, it should be noted that in five of the nine SmCD3+ T-ALL samples almost no TcR-αβ (WT31)+ cells were detected, and if they were detected, it was demonstrated by double IF staining that the TdT+ cells were negative for TcR-αβ (WT31). In six SmCD3+ T-ALL a large discrepancy of about 60% was found between the percentages of SmCD3 positivity and TcR-αβ (WT31) positivity. This may indicate that the T-ALL cells in these cell samples express the TcR-γ protein chain.22–24

Analysis of 45 Leukemias and Ten Cell Lines for CyCD3 Expression

The results of the immunologic marker analysis of the 45 leukemias and the ten cell lines are summarized in Table 2.

All 19 T-ALL samples were positive for TdT and the CD7 antigen. In addition, they all expressed CyCD3 and/or SmCD3 as detected in cytocentriuge preparations using anti-CyCD3-δ and anti-CyCD3-α MoAbs (see the previous section and Tables 1 and 2).

The T-CLL sample tested was negative for TdT but expressed the T cell markers CD2, CD3, CD5, and CD7 on the cell surface. The SmCD3 expression was confirmed by the surface membrane-like CD3 staining in cytocentriuge preparations (Table 2).

Three of the five T cell lines were positive for TdT and mainly expressed CyCD3, whereas the other two T cell lines expressed both CyCD3 and SmCD3.

All 12 precursor B-ALL samples were positive for TdT, HLA-DR, and the B cell antigen CD19. Eleven of the 12 precursor B-ALL samples also expressed the CD10 antigen (common ALL antigen). These precursor B-ALL samples did not express CyCD3 or SmCD3. This is illustrated in Fig 4, which shows CD3 staining on a cytocentriuge preparation of BM cells from a patient with a precursor B-ALL: the leukemic blasts were negative for CD3, whereas a few normal T lymphocytes (about 3% of the total MNC population) expressed SmCD3. The B-CLL samples tested were positive for HLA-DR, the B cell antigen CD19, and the T cell antigen CD5, and in addition they weakly expressed SmIg. The B-CLL samples did not express CyCD3 or SmCD3. The two precursor B cell lines and the three Burkitt's lymphoma–derived cell lines were positive for HLA-DR and the B cell antigen CD19, but they did not express CyCD3 or SmCD3.

The six AML samples expressed HLA-DR and the myeloid antigen CD33. Three of these AML samples also
observed. One SmCD3 T-ALL sample did not express CyCD3 (see Table 1). The percentages of T lymphocytes varied from about 50% to 65%, whereas the percentage of B lymphocytes (CD19+ or CD20+) varied from about 5% to 10% and the monocytic cells (CD14+) from about 10% to 20%.

The use of the anti-CD3 chain MoAbs in cytotoxic preparations of PB samples resulted in surface membrane-like CD3 staining only. The typical CyCD3 staining could not be detected. The percentage of cells positive for SmCD3-δ and SmCD3-ε staining in the cytotoxic preparations were comparable with the percentages of SmCD3+ cells as found by staining of the PB cells in suspension (Table 2).

Table 1. Immunologic Marker Analysis (Including the Detection of CyCD3 Chains) of 19 T-ALL Cell Samples

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<th>Immunologic Marker</th>
<th>(BM)</th>
<th>(BM)</th>
<th>(PB)</th>
<th>(BM)</th>
<th>(BM)</th>
<th>(BM)</th>
<th>(BM)</th>
<th>(BM)</th>
<th>(BM)</th>
<th>(BM)</th>
<th>(BM)</th>
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<td>83</td>
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<td>100</td>
<td>85</td>
<td>86</td>
<td>99</td>
<td>70</td>
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<td>63</td>
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<td>96†</td>
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<tr>
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<td>99</td>
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<td>98</td>
<td>80</td>
<td>73</td>
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<td>94†</td>
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<td>86</td>
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The figures represent percent positivity per MNC. The figures in parentheses represent percent positivity per TdT+ cells as detected by double IF staining; such T cell marker/TdT double IF staining were primarily performed if the percentage of TdT+ cells was less than 25%.

*Cytoplasmic staining as determined in cytotoxic preparations.
†Surface membrane staining as determined in cytotoxic preparations.
§Surface membrane staining as determined by analysis of cells in suspension.

Table 2. Immunologic Marker Analysis (Including the Detection of CyCD3 Chains) of 45 Leukemias, Ten Cell Lines, and Five Normal Samples

<table>
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<tr>
<th>Immunologic Marker</th>
<th>TdT</th>
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<th>CyCD3-δ (SP-64)</th>
<th>CyCD3-ε (SP-64)</th>
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<th>CD5 (Leu-1)</th>
<th>CD7 (3A1)</th>
<th>CD10 (VIL-1)</th>
<th>CD13 (My7)</th>
<th>CD14 (My4)</th>
<th>CD15 (VIM-D5)</th>
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<tr>
<td>Normal PB</td>
<td>&lt;0.1</td>
<td>26</td>
<td>9</td>
<td>67</td>
<td>9</td>
<td>66</td>
<td>109</td>
<td>60</td>
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<td>68</td>
<td>56</td>
<td>74</td>
<td>54</td>
<td>11</td>
</tr>
</tbody>
</table>

The figures represent the number of positive cells or cell lines per total number of samples tested. A leukemia or cell line was considered to be positive if more than 25% of the MNC were positive. The normal PB samples, percentages of positive cells ± SD are given.

Aberration: NT, not tested.

*In the SmCD3-δ T-ALL, mainly cytoplasmic staining for CD3 chains was found, whereas in seven of the eight SmCD3-ε T-ALL samples both cytoplasmic and surface membrane-like staining was observed. One SmCD3-δ T-ALL sample did not express CyCD3 (see Table 1).
†The CD13-ε T-ALL is the prothymocytic T-ALL of patient 1 (see Table 1 and Campana et al8 and Van Dongen et al2). The CD13-ε T-ALL is the immune thymic T-ALL of patient 2 (see Table 1).
‡No CyCD3 staining was detected; only SMCD3 staining was found.
§In the five T cell lines, mainly cytoplasmic staining for CD3 was found (see Table 3).
These percentages represent surface membrane-like CD3+ cells in the cytotoxic preparations; no typical cytoplasmic staining was detected.
Reactivity of the Anti-CD3 MoAbs of the Oxford Panel in IF Techniques and Western Blotting

In addition to the rat anti-CD3-γ antiserum, the anti-CD3-γ MoAbs SP-64 and SP-78, and the anti-CD3-ε MoAbs SP-6 and SP-10, 22 anti-CD3 MoAbs of the Oxford CD3 panel were evaluated for their capability to detect CyCD3 in cytocentrifuge preparations of the T cell lines Molt4, H9, and 8102. In addition, these MoAbs were tested for their reactivity with CD3 chains in Western blotting.

Detection of CyCD3 by the use of anti-CD3 MoAbs of the Oxford panel. The rat anti-CD3-γ antiserum and the MoAbs of the SP series could detect CyCD3 chains in the three T cell lines (Table 3).

The MoAbs of the Oxford panel were first optimally titrated by testing their reactivity with SmCD3 on normal PB lymphocytes in suspension. Subsequently the MoAbs were tested for their capacity to detect CyCD3 in the T cell lines Molt4, H9, and 8102. Two different titers were used for this purpose, i.e., the titer that was optimal for the detection of SmCD3 as well as a fourfold-lower titer. It appeared that four anti-CD3 MoAbs (UCHT1, VIT-3b, G19-4.1, and SK7/Leu-4) were able to detect CyCD3 in all three T cell lines and that a fifth MoAb, T3(2ADA), gave surface membrane-like staining in cytocentrifuge preparations of H9 and 8102. These five MoAbs did not show reactivity in cytocentrifuge preparations of B cell lines. The observation that Molt4 cells do not express SmCD3 may explain the absence of surface membrane-like staining in cytocentrifuge preparations stained with the MoAb T3(2ADA); however, the surface membrane-like staining of the MoAb T3(2ADA) in cytocentrifuge preparations of the SmCD3- cell line 8102 remains unexplained. The MoAb VIT-3 caused a weak CyCD3 staining in cytocentrifuge preparations of Molt4 but did not react with H9 and 8102. Lowering of the titer of this MoAb did not result in a better staining. The MoAb T3/2T8-2F4 gave a nonspecific filamentous staining in all three T cell lines. The same nonspecific staining was also found in PB monocytes, as determined by phase-contrast facilities, as well as in B cell lines. This filamentous staining was also described by Lobach et al and resembles the filamentous staining of the anti-CD3 MoAb T10B9 as described by Campana et al.

These results indicate that the MoAbs UCHT1, VIT-3b, G19-4.1, and SK7/Leu-4 are able to detect CyCD3 in all three T cell lines tested and that the MoAb T3(2ADA) only gives surface membrane-like CD3 staining in cytocentrifuge preparations.

Reactivity with CyCD3 chains in Western blotting. Antibodies were screened in Western blots against an immunofluorescence purified preparation of CD3 from human tonsils. An anti-CD3-γ polyclonal antiserum was used to determine the location of the CD3-γ, CD3-δ, and CD3-ε chains in the filter strips. As could be expected, the rat anti-CD3-γ antiserum reacted with the CD3-γ band, whereas MoAbs SP-64 and SP-78 reacted with the CD3-δ band and MoAbs SP-6 and SP-10 with the CD3-ε band. All anti-CD3 MoAbs of the Oxford panel were tested at a titer of 1:50. Five MoAbs, (UCHT1, VIT-3b, G19-4.1, T3(2ADA), and SK7/Leu-4) showed reactivity with the CD3-ε band and an 18-kd degradation product of this band, whereas all other MoAbs showed no reactivity (Fig 5).

Comparison of the data of the IF staining with the data of the Western blotting indicates that the five anti-CD3 MoAbs of the Oxford panel that are able to give CyCD3 and/or surface membrane-like CD3 staining in cytocentrifuge preparations are the same MoAbs that react with the CD3-ε chains in Western blots (Table 3, Fig 5).

DISCUSSION

Expression of the CD3 antigen by a leukemia or non-Hodgkin's lymphoma proves the T cell origin of such a malignancy because the CD3 antigen is a T cell-specific marker. However, many T-ALL do not express the CD3 antigen on their surface membrane. Link et al demonstrated CD3 expression in the cytoplasm of some immature T
CYTOPLASMIC EXPRESSION OF THE CD3 ANTIGEN

Fig 5. Western blot analysis of the anti-CD3 MoAbs of the Oxford CD3 panel. The reactivity of a polyclonal anti-CD3-\(\gamma\) \(\delta\) \(\epsilon\) antiserum, shown in the first filter strip, indicates the localization of the CD3-\(\gamma\), CD3-\(\delta\), and CD3-\(\epsilon\) bands. Only the MoAbs UCHTI, VIT-3b, G19-4.1, T3(2ADA), and SK7/Leu-4 show reactivity in the Western blot filter strips. They all recognize the CD3-\(\epsilon\) band. The 18-kd band migrating ahead of the CD3-\(\epsilon\) band (*) is a specific degradation product of the CD3-\(\epsilon\) polypeptide.\(^{30}\)

Cell malignancies that did not express SmCD3\(^{29}\) Such CD3 expression in immature T cells has recently also been described by Furley et al\(^{33}\) and Campana et al.\(^{35}\) Cytoplasmic expression of antigens preceding the surface membrane expression has also been described for other proteins such as the B cell antigen CD22.\(^{43}\) Another well-known example is the weak cytoplasmic expression of \(\mu\) heavy chains in pre-B cells that precedes the SmIg expression on B lymphocytes.\(^{44,45}\)

Our study indicates that CD3 expression already occurs in immature stages of T cell differentiation because the majority of thymocytes and all immature T-ALL express CD3 (Table 1, Figs 2 and 3). Even the most immature T-ALL, ie, the prothymocytic T-ALL, weakly expressed CD3 (Table 1). Expression of the CD3 antigen in the cytoplasm of immature SmCD3\(^{-}\) T cells is in line with the recent finding that CD3-\(\delta\) and CD3-\(\epsilon\) gene transcripts occur in all T-ALL.\(^{33,34}\) The expression of the CD3 antigen on the surface membrane is dependent on the presence of the TcR.\(^{30-32}\) The processes involved in the production of the TcR protein chains such as rearrangement and transcription of the TcR genes take place during thymic differentiation, finally resulting in the expression of the TcR-CD3 complex at the cell surface.\(^{19,33,34}\) In our study, the majority of thymocytes and most SmCD3\(^{+}\) T-ALL appeared to express CyCD3 as well as SmCD3 (Fig 2 and Table 1). The mature T lymphocytes in PB and BM and a minor population of thymocytes (about 5%) expressed prominent levels of SmCD3, whereas the typical CyCD3 staining was not detectable in these cells (Table 2 and Figs 2 and 4). It should be remarked, however, that a weak CyCD3 staining may be undetectable in our assays when the SmCD3 staining is very bright. Thus, immature SmCD3\(^{-}\) T cells express CyCD3 only, the combined expression of CyCD3 and SmCD3 is characteristic for intermediate differentiation stages, and SmCD3\(^{+}\) cells represent mature T cells. It might well be that the latter cells display a low CyCD3 expression because a continuous CD3 chain biosynthesis should compensate the SmCD3 turnover.

Whether the precursor T cells in BM, ie, the prothymocytes, also express CyCD3 has to be further investigated. On the basis of double and triple immunostainings we have previously postulated that a minor population of the TdT\(^{+}\) cells in BM represent prothymocytes that express the TdT\(^{+}/\)HLA-DR\(^{+}/\)CD7\(^{+}/\)CD3\(^{+}/\)CD5\(^{-}/\)CD11\(^{-}/\)CD3\(^{-}\) phenotype.\(^{42,46}\) Recently, Campana et al performed double IF stainings for CyCD3 and TdT but could not detect double-positive cells in BM.\(^{35}\) Although we did not perform such double IF stainings on BM samples, we demonstrated that the prothymocytic T-ALL sample tested weakly expressed CyCD3, and in addition, we recently showed that such leukemic cells contain CD3-\(\delta\) and CD3-\(\epsilon\) gene transcripts.\(^{34}\)

The expression of the various T cell antigens, CyCD3, and the TcR-CD3 complex as well as the presence of CD3 transcripts during T cell differentiation are summarized in Fig 6.

To exclude the possibility that non-T cells expressed CyCD3, we analyzed 12 precursor B-ALL samples, seven B-CLL samples, five (precursor) B cell lines, and six AML samples in addition to 19 T-ALL samples, one T-CLL sample, and five T cell lines. Only the T cell leukemias and T cell lines appeared to express CyCD3 and/or SmCD3. All other non-T cell leukemia samples and non-T cell lines were negative for the CD3 antigen. Also the CD7\(^{+}\) AML samples did not express CyCD3 (Table 2). These data and those of Campana et al\(^{35}\) indicate that CyCD3 expression is restricted to the T cell lineage and can therefore be used as diagnostic marker for immature SmCD3\(^{-}\) T cell malignancies.

For the detection of CyCD3, Link et al used the MoAb Leu-4 on acetone-fixed tissue sections or cytocentrifuge preparations.\(^{29}\) Furley et al used MoAbs of the SP series but did not mention their fixation method,\(^{33}\) and Campana et al used the MoAbs UCHTI and OKT3 on acetone-fixed cytocentrifuge preparations.\(^{35}\) Because the data discussed earlier indicated that CyCD3 is an important diagnostic marker, we
evaluated which fixation method is optimal for the CyCD3 staining, and we determined which anti-CD3 MoAbs can be used for the detection of CyCD3.

In our hands acid ethanol appeared to be the best fixative to fixate the cytocentrifuge preparations and to denature the CD3 chains. Our data and those of Furley et al\(^38\) clearly demonstrate that CyCD3 expression can readily be detected by MoAbs of the SP series\(^38\); however, the MoAbs SP-6 and SP-10 (anti-CD3-\(\varepsilon\)) and the MoAbs SP-64 and SP-78 (anti-CD3-\(\delta\)) do not recognize SmCD3 on cells in suspension,\(^38\) and in addition, they are not widely available. Therefore, we wished to analyze the reactivity of anti-CD3 MoAbs that are raised against native SmCD3. For this purpose we were allowed to use 22 anti-CD3 MoAbs of the Oxford CD3 panel, kindly provided by professor A.M. McMichael. These MoAbs were tested for their capacity to detect CyCD3 in acid ethanol-fixed cytocentrifuge preparations of the T cell lines Molt4, H9, and 8102 as well as for their reactivity with CD3 chains in Western blotting.

Only five (UCHT1, VIT-3b, G19-4.1, T3(2ADA), and SK7/Leu-4) of 22 anti-CD3 MoAbs of the Oxford CD3 panel could detect CD3 chains in cytocentrifuge preparations. Western blotting showed that the same five MoAbs recognize the CD3-\(\varepsilon\) chain. All other anti-CD3 MoAbs showed no reactivity with the CD3 chains in Western blotting. Four (UCHT1, VIT-3b, G19-4.1, and SK7/Leu-4) of the five mentioned MoAbs recognized both CyCD3 and SmCD3 in five T cell lines Molt4, H9, and 8102 as well as for their reactivity with CD3 chains in Western blotting. The lack of reactivity of the majority of the anti-CD3 MoAbs with denatured CD3 chains in cytocentrifuge preparations and Western blots may be due to the possibility that they recognize epitopes on the CD3 antigen that are composed of two or more chains or epitopes that change during denaturation. In view of these speculations, it is interesting to find that all five anti-CD3 MoAbs of the Oxford panel that do detect CD3 in cytocentrifuge preparations recognize the CD3-\(\varepsilon\) chain. This may be connected with the fact that CD3-\(\varepsilon\) chains, in contrast to CD3-\(\gamma\) and CD3-\(\delta\) chains, are not glycosylated.\(^11,13\) Another explanation may be that some epitopes on the CD3-\(\varepsilon\) chains are less prone to denaturation-induced changes.

Our results as a whole are consistent with CyCD3 being expressed by immature cells during T cell differentiation but not by precursor B cells and early myeloid cells. Thus CyCD3 can be used as a diagnostic marker for SmCD3- T cell malignancies. Evaluation of different anti-CD3 MoAbs to be used for the detection of CyCD3 in cytocentrifuge preparations demonstrated that CyCD3 can be detected not only by the use of MoAbs raised against denatured CD3 chains such as those of the SP series but also by a minority of anti-CD3 MoAbs that are raised against SmCD3, eg, UCHT1, VIT-3b, G19-4.1, and SK7/Leu-4.

Shortly before submission of this manuscript a report from Miro et al was published in which they described the detection of CyCD3 in five T-ALL cell samples by flow cytometry.\(^47\)

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Cytoplasmic expression of the CD3 antigen as a diagnostic marker for immature T-cell malignancies

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