Accessory Receptors Regulate Coupling of the T-Cell Receptor Complex to Tyrosine Kinase Activation and Mobilization of Cytoplasmic Calcium in T-Lineage Acute Lymphoblastic Leukemia

By Jeffrey A. Ledbetter, Gary L. Schieven, Virginia M. Kuebelbeck, and Fatih M. Uckun

T-lineage acute lymphoblastic leukemia (T-ALL) cells have abundant cytoplasmic CD3/Ti but express low amounts on the cell surface and are deficient in CD3/Ti-mediated signal transduction. Nevertheless, plating T-ALL cells on dishes containing immobilized anti-CD3 monoclonal antibodies with a source of growth factors induced the expression of CD25 (interleukin-2 receptor α chain) and stimulated the formation of blast colonies in 12 of 14 cases studied. The proliferative response to CD3 ligation was modulated by the presence of antibodies to the CD2, CD4, or CD8 accessory T-cell receptors. The effect of these accessory receptors on signal transduction mediated by CD3/Ti was next investigated by monitoring cytoplasmic calcium concentration ([Ca^{2+}]i) and by measuring tyrosine phosphorylation after stimulation.

Crosslinking CD3, CD2, CD4, or CD6 alone did not induce cytoplasmic calcium mobilization in T-ALLs, but crosslinking the accessory receptors with CD3/Ti induced calcium responses in three of the T-ALLs and enhanced calcium responses in three of the T-ALL cell lines, including HPB-ALL, MOLT-4, and CEM. Crosslinking CD4 but not CD2 with CD3/Ti greatly enhanced tyrosine phosphorylation of multiple substrates in comparison with crosslinking either CD4 or CD3/Ti separately on both normal mature T cells and the CEM T-ALL cell line. Thus, CD4 regulates CD3/Ti signal transduction in T-ALL cells through the tyrosine phosphorylation of substrates whereas CD6 may regulate [Ca^{2+}]i signal transduction through a separate mechanism.

From the Oncogen Division of Bristol-Myers Squibb, Seattle, WA; and The Tumor Immunology Laboratory, Section of Cancer and Leukemia Biology, Departments of Therapeutic Radiology-Radiation Oncology, and the Bone Marrow Transplant Program, University of Minnesota Health Sciences Center, Minneapolis.

Submitted September 18, 1990; accepted November 9, 1990.

Supported in part by US Public Health Service Grants GM-42508, RO1-CA-42633, P01-CA-21737, and R29-CA-42111 awarded by the National Cancer Institute, DHHS, Bristol-Myers Special Research Grant No. 100-R220, and Special Grants awarded by the Minnesota Medical Foundation, Children’s Cancer Research Fund, Bone Marrow Transplant Research Fund, Graduate School, and Leukemia Task Force, University of Minnesota (F.M.U.); and in part by Oncogen Division of Bristol-Myers Squibb. F.M.U. is a Scholar of the Leukemia Society of America.

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
HH/2150 flow cytometer (Ortho, Westwood, MA) as previously described.\(^{14}\) The histograms were analyzed by programs that calculated the mean indo-1 violet/blue fluorescence ratio versus time. There are 100 data points on the X (time) axis on all flow cytometric data.

**Immunoblotting with antiphosphotyrosine.** Tyrosine phosphorylation was measured by immunoblotting with a purified rabbit antiphosphotyrosine antibody, prepared and purified as previously described.\(^{15}\) To prepare samples, 5 × 10^6 cells/mL sample were stimulated as indicated, rapidly pelleted by centrifugation, and lysed in 200 µL hot sodium dodecyl sulfate (SDS) sample buffer containing 50 µmol/L orthovanadate. Samples were boiled for 5 minutes, stored at −70°C until analysis on 10% SDS-polyacrylamide gels, and transferred to immobilon (Millipore Corp, Bedford, MA). Immunoblots were incubated for 3 hours with 0.5 µg/mL antiphosphotyrosine, followed by washing and development with 1 µCi/mL high specific activity Protein A (ICN Pharmaceuticals, Irvine, CA). Prestained high molecular weight markers (Bethesda Research Laboratory) were run on each gel, and migration positions are indicated by arrows. The rabbit antiphosphotyrosine antibody did not react with resting or activated T cells in the presence of 40 mmol/L of the hapten phenyl phosphate (data not shown).

**Cells.** Leukemic cells from bone marrow were obtained from patients at the bone marrow transplant unit at the University of Minnesota. T-ALL cell lines HPB-ALL, MOLT-4, HSB-2, JURKAT, PEER, HUT 78, and CEM were obtained from Dr Paul Martin, Fred Hutchinson Cancer Research Center, Seattle, WA. The phenotype and origin of the cell lines was described.\(^{16}\) The CEM cell line used here was a CD3+ subclone (CEM.6)\(^{17}\) isolated from the mosaic original CEM culture.\(^{17}\)

**T-ALL blast colony assays.** Thirty-five-millimeter petri dishes were coated with the indicated MoAb or MoAb homo/hetero-conjugates (10 µg/mL) by an overnight incubation at 37°C. Subsequently, antibody solutions were removed and cells (10^5/mL) in semisolid culture medium\(^{18}\) containing 0.9% methylcellulose, 30% FBS, 10% (vol/vol) phytohemagglutinin-lymphocyte conditioned medium (PHA-LCM), and 100 U/mL recombinant interleukin-2 (rIL-2; Cetus Corporation, Emeryville, CA) were plated at 1 × 10^6 cells/dish in duplicate dishes. After 7 days of culture at 37°C/5% PHA-LCM, blast colonies were enumerated under Zeiss inverted phase microscope of high optical resolution. In each case, leukemic T-cell precursors strongly expressed the early T-lineage surface antigens CD2, CD5, and CD7, but not the B-lineage antigens CD19 or CD24. They were strongly positive for nuclear TdT and cytoplasmic CD3. CD1, CD4, and CD8 were also detected on leukemic blasts from a number of patients, but surface CD3 was weakly expressed in only four of 14 patients studied. These immunophenotypic features are consistent with T-ALL.

**RESULTS**

To investigate the transmembrane signalling properties of T-ALL cells, bone marrow cells from 14 patients were loaded with the calcium-binding dye indo-1 and assayed for increases in cytoplasmic calcium concentration ([Ca^{2+}]). In each case, the cell phenotype was consistent with T-ALL because CD7, CD5, and CD2 were expressed in high frequency and B-lineage antigens CD19, CD10, and CD24 were not (Table 1). CD1, CD4, and CD8 were also seen in some patients, but CD25 (IL-2 receptor α chain) was negative and surface CD3 was weakly expressed in only four of the 14 patients studied.

The T-ALL cells were able to mount very weak or negative responses to optimal CD3 crosslinking with the IgM MoAb 38.1 when compared with normal mature T cells from peripheral blood (Table 2). Similarly, CD2 crosslinking with biotin-conjugated 9.6 followed by addition of avidin did not increase [Ca^{2+}]i in the T-ALL cells although mature T cells were highly responsive. Similar lack of responsiveness was seen after crosslinking of CD5, CD28, CD4, and CD8 (data not shown). Thus, the T-ALL cells appeared deficient in transmembrane signalling responses in comparison with mature T cells.

**Calcium mobilization by CD3/Ti-stimulation is regulated by CD4, CD8, or CD2 in T-ALL cells.** CD4, CD8, and CD2 are known to regulate [Ca^{2+}]i responses in mature T cells and have been implicated in the thymic selection processes. Therefore, we examined whether crosslinking accessory molecules to CD3/Ti could induce transmembrane signals in T-ALL cells. In three of the T-ALLs, responses could be obtained with MoAb heteroconjugates but not with anti-CD3 alone or anti-CD3 homoconjugates (Fig 1). Crosslinking...

---

**Table 1. Phenotype of Bone Marrow Cells From Patients With T-ALL (% cells positive)**

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD1</th>
<th>CD2</th>
<th>CD3</th>
<th>CD4</th>
<th>CD5</th>
<th>CD6</th>
<th>CD7</th>
<th>CD8</th>
<th>CD10</th>
<th>CD19</th>
<th>CD24</th>
<th>CD25</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>86</td>
<td>95</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>82</td>
<td>4</td>
<td>ND</td>
<td>80</td>
<td>94</td>
<td>ND</td>
<td>4</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>50</td>
<td>2</td>
<td>10</td>
<td>14</td>
<td>91</td>
<td>38</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>80</td>
<td>3</td>
<td>2</td>
<td>83</td>
<td>35</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>99</td>
<td>3</td>
<td>2</td>
<td>97</td>
<td>97</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>93</td>
<td>5</td>
<td>2</td>
<td>24</td>
<td>80</td>
<td>39</td>
<td>30</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>88</td>
<td>29</td>
<td>5</td>
<td>59</td>
<td>92</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>92</td>
<td>22</td>
<td>5</td>
<td>79</td>
<td>62</td>
<td>3</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>94</td>
<td>92</td>
<td>2</td>
<td>8</td>
<td>81</td>
<td>97</td>
<td>63</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>81</td>
<td>12</td>
<td>17</td>
<td>41</td>
<td>91</td>
<td>5</td>
<td>15</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>5</td>
<td>8</td>
<td>78</td>
<td>10</td>
<td>81</td>
<td>10</td>
<td>9</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>91</td>
<td>3</td>
<td>82</td>
<td>22</td>
<td>88</td>
<td>89</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>98</td>
<td>4</td>
<td>43</td>
<td>84</td>
<td>89</td>
<td>46</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>97</td>
<td>28</td>
<td>15</td>
<td>36</td>
<td>88</td>
<td>19</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Percent of cells positive was determined by indirect immunofluorescence with fluorescein-conjugated goat antimouse Ig (Tago, Burlington, CA) and flow cytometry. The MoAbs used included OKT6 (CD1), 9.6 (CD2), G19-4 (CD3), G17-2 (CD4), 10.2 (CD8), G3-7 (CD7), G10-1 (CD8), 24.1 (CD10), B43 (CD19), BA1 (CD24), and TAC (CD25).

Abbreviations: ND, not done.
Table 2. Mobilization of Cytoplasmic Calcium by Triggering CD3 on Leukemia T-Cell Precursors

<table>
<thead>
<tr>
<th></th>
<th>Anti-CD3 Stimulation (38.1)</th>
<th>Anti-CD2 Stimulation (biotin 0.6, then avidin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Ca²⁺]i Response (%) above basal</td>
<td>% Responding Cells</td>
</tr>
<tr>
<td></td>
<td>Mean Range</td>
<td>Mean Range</td>
</tr>
<tr>
<td>Leukemic T-cell precursors (n = 10)</td>
<td>25 0-88</td>
<td>13 6-39</td>
</tr>
<tr>
<td>Normal blood lymphocytes (n = 4)</td>
<td>&gt;1,000 670-1,400</td>
<td>82 71-89</td>
</tr>
</tbody>
</table>

Cytoplasmic calcium concentration ([Ca²⁺]i) was measured using indo-1 and a flow cytometer. The peak mean [Ca²⁺]i and percent responding cells were calculated as described. Cells responding beyond two standard deviations of the mean indo-1 ratio baseline cells are calculated as responding cells. Thus, 5% responding cells is a baseline.

Crosslinking of CD3XCD2 was most often effective, but CD3XCD4 crosslinking also enhanced responses in two cases. In contrast, the two T-ALLs that were able to respond to anti-CD3 did not show strongly enhanced responses to the MoAb heteroconjugates. As shown in Fig 2A, responses to CD3XCD2, CD3XCD4, and CD3XCD8 heteroconjugates were weaker than an equivalent concentration of anti-CD3 alone. The T-ALL shown in Fig 2B was similar except for some evidence of enhanced signal responses to CD3XCD2 but not to CD3XCD4 or CD3XCD8.

Some of the common T-cell ALL lines, including CD3+CEM, HPB-ALL, MOLT-4, JURKAT, HUT78, and PEER were similarly examined for CD2-, CD4-, or CD8-mediated regulation of CD3/Ti signalling (Fig 3). Three of the cell lines, including MOLT-4, CEM, and HPB-ALL, showed enhanced calcium mobilization when stimulated by crosslinking CD2, CD4, or CD8 with CD3 by MoAb heteroconjugates in comparison with maximal stimulation.

![Fig 1](image1.png)

**Fig 1.** Accessory-molecule dependence of [Ca²⁺]i responses to CD3 stimulation of some leukemic T-cell precursors. Cells were loaded with indo-1 and assayed for responses in [Ca²⁺]i by flow cytometry. The response to CD3 alone (IgM anti-CD3 MoAb 38.1, 1/500 dilution of strong ascites) was compared with heteroconjugates (CD3XCD2) (MoAbs G19-4X9.6, 2 μg/mL), (CD3XCD4) (MoAbs G19-4XG17-2, 1 μg/mL), and (CD3XCD8) (MoAbs G19-4x G19-1, 1 μg/mL). Data shown are from patients 2 (A), 11 (B), and 13 (C).

![Fig 2](image2.png)

**Fig 2.** Some leukemic T-ALLs respond optimally to CD3 alone. Cells were loaded with indo-1 and assayed for [Ca²⁺]i by flow cytometry. Cells were stimulated with CD3 MoAb G19-4 at 20 μg/mL and 1 μg/mL to heteroconjugates (CD3XCD4), (CD3XCD2), and (CD3XCD8) are also shown. Data represent results obtained with patients 1 (A) and 7 (B).
The responses to the CD3XCD2, CD3XCD4, and CD3XCD8 heteroconjugates by the CEM and MOLT-4 cell lines was further investigated to study the requirement for CD2, CD4, or CD8 interaction with CD3/Ti for signal augmentation. In Fig 4A, the CD3XCD2 heteroconjugate (1 μg/mL) gave strong signals, and the signal was inhibited when a 10-fold excess of the CD2 MoAb was added just before the heteroconjugate. The signals in response to CD3XCD4 or CD3XCD8 were similarly inhibited by CD4 or CD8 MoAb (Fig 4B and C). The calcium signals induced by crosslinking the accessory molecules with CD3/Ti could not be obtained by crosslinking the accessory molecules of CD3 alone. MOLT-4 was similar to the T-ALLs of Fig 1, because there was no response to optimal CD3 stimulation, but significant responses to CD3XCD2, CD3XCD4, and CD3XCD8 heteroconjugates. Two of the other cell lines, JURKAT and HUT78, responded maximally to CD3 alone and did not show enhanced responsiveness to CD3XCD2, CD3XCD4, or CD3XCD8 heteroconjugates. The PEER and HSB-2 cell lines were unlike the others because no calcium signals could be elicited from any of the stimulations attempted (Fig 3).

Fig 3. Transmembrane signalling patterns in leukemic T-cell lines. Cells were loaded with indo-1 and assayed for \([Ca^{2+}]_i\) with a flow cytometer. Stimulations were anti-CD3 alone (---) using 1/500 dilution of 38.1 ascites, (CD3XCD2) heteroconjugate (---) at 1 μg/mL, (CD3XCD4) heteroconjugate (---) at 1 μg/mL, and (CD3XCD8) heteroconjugate (---) at 1 μg/mL.

Fig 4. Enhanced signalling by heteroconjugates on CEM and MOLT-4 cells is dependent on the accessory molecule interaction with CD3/Ti. (A) The response to CD3XCD2 heteroconjugate (G19-4X9.6, 1 μg/mL) was compared either before (---) or after (-----) preincubation with 10 μg/mL CD2 MoAb 9.6. (B) The response to (CD3XCD4) heteroconjugate (19-4XG17-2, 1 μg/mL) was compared either before (---) or after (-----) preincubation with 10 μg/mL MoAb G17-2. (C) The response to (CD3XCD8) heteroconjugate (G19-4XG10-1, 1 μg/mL) was compared either before (---) or after (-----) preincubation with 10 μg/mL CD8 MoAb G10-1. (D) The responses to crosslinking individual antigens are shown, including 38.1 (IgM anti-CD3, 1/500 dilution of ascites), CD2 crosslinking with biotin-conjugated 9.6 at −5 minutes followed by avidin at 1.5 minutes; CD4 crosslinking with biotin-conjugated G17-2 at −5 minutes followed by avidin at 1.5 minutes; and CD8 crosslinking with biotin-conjugated G10-1 at −5 minutes followed by avidin at 1.5 minutes. Biotin-conjugated MoAbs were all used at 10 μg/mL followed by avidin at 40 μg/mL.
alone, because CD2, CD4, and CD8 did not signal at all in MOLT-4 and only CD2 could induce a weak response in CEM cells (Fig 4D).

**Induction of T-ALL proliferation and IL-2 receptor expression by CD3/Ti stimulation.** To further investigate CD3/Ti function on T-cell precursors, T-ALL cells were next cultured in methylcellulose on plates containing immobilized CD3 MoAb and given a source of growth factors (PHA-LCM). We chose to immobilize the MoAbs on plastic because these conditions are mitogenic for resting T cells isolated from peripheral blood. Several of the T-ALLs showed immediate and clear evidence of growth by colony formation, and the response was not restricted to a single anti-CD3 MoAb. Figure 5 shows examples of colony morphology of cells stimulated by immobilized CD3 MoAbs 64.1, G19-4, and UCHT-1. Each of these MoAbs was effective even though CD3Ki expression by the T-ALL cells was very low or even undetectable by flow cytometric analysis.

The stimulation of blast colony formation by the 14 T-ALLs studied here is shown in Table 3. Most of these

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. Stimulation</th>
<th>Anti-CD3</th>
<th>Anti-CD2</th>
<th>Anti-CD4</th>
<th>Anti-CD8</th>
<th>Anti-CD3xCD2</th>
<th>Anti-CD3xCD4</th>
<th>Anti-CD3xCD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>180</td>
<td>26</td>
<td>15</td>
<td>10</td>
<td>301</td>
<td>524</td>
<td>367</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>225</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>78</td>
<td>335</td>
<td>312</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>14</td>
<td>3</td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>415</td>
<td>645</td>
<td>119</td>
<td>444</td>
<td>457</td>
<td>38</td>
<td>464</td>
<td>971</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>13</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>344</td>
<td>1,392</td>
<td>104</td>
<td>0</td>
<td>416</td>
<td>884</td>
<td>946</td>
<td>1,114</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>424</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>68</td>
<td>59</td>
<td>410</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>101</td>
<td>110</td>
<td>202</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>52</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>20</td>
<td>48</td>
</tr>
<tr>
<td>13</td>
<td>80</td>
<td>348</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>116</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>80</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6</td>
<td>ND</td>
</tr>
</tbody>
</table>

Leukemia bone marrow from T-ALL patients was plated in vitro in the presence of 10% PHA lymphocyte-conditioned medium on dishes with immobilized anti-CD3 MoAB G19-4, anti-CD2 MoAb 9.6, anti-CD4 MoAb G17-2, anti-CD8 MoAb G10-1, MoAb heteroconjugates, or without antibody (no stimulation). Duplicate cultures were assayed for each stimulation, and means were shown. Blast colonies were counted on day 7 after plating.
showed marked responsiveness to immobilized anti-CD3 but not to anti-CD2, anti-CD4, or anti-CD8 alone. Indeed, the proliferation response to CD2, CD4, or CD8 was often lower than the control (no stimulation) cultures, reflecting inhibition of proliferation responses to the cytokine preparation (PHA-LCM). Crosslinking CD2, CD4, or CD8 to CD3 with immobilized heteroconjugates increased proliferation in several cases (Fig 6), but often proliferation was maximal after stimulation with anti-CD3 alone.

Colony cells had blast morphology with a basophilic cytoplasm, high nuclear to cytoplasmic ratio, highly irregular nuclear membrane, and prominent nucleoli. Colony blasts were found to be strongly positive for nuclear TdT, surface CD2, CD5, CD7, and, sometimes, CD1, as well as cytoplasmic CD3, but they were negative for surface CD3, consistent with T-ALL (Table 4). The immunophenotypic features of colony blasts from patients 1, 2, 11, and 13 corresponded to those of double positive CD4+ CD8+ immature thymocytes, while the immunophenotypic features of colony blasts from patients 4 and 8 corresponded to those of double negative CD4− CD8− immature thymocytes/prothymocytes. Colony blasts in patient 9 were CD1+ CD10+, a T-cell precursor phenotype not encountered in normal thymus or bone marrow. In patients 1, 9, and 13 the clonality of colony blasts was confirmed by Southern blot hybridization of genomic DNA with a T-cell receptor (TCR) probe (data not shown). Significantly, CD25 (IL-2 receptor α chain) was detected on cells from four of the seven cultures. No CD25 was detected before stimulation (Table 1), indicating that IL-2 receptor induction had occurred after CD3 stimulation.

**Regulation of tyrosine phosphorylation by CD4 association with CD3/CD2**. CD4 and CD8 are associated with the p56^Lck^ protein tyrosine kinase. To investigate whether the
enhancement of CD3/Ti signal transduction by association of CD3 with CD4 was related to activation of tyrosine phosphorylation, cells were lysed in SDS sample buffer, electrophoresed, transferred to immobilon membranes, and probed with an affinity-purified polyclonal antiphosphotyrosine antibody. In resting, purified T cells from peripheral blood, there were 15 proteins detected and their molecular weights are shown in Fig 7. The reactivity of the antiphosphotyrosine antibody was inhibited completely in the presence of 40 mmol/L of the hapten phenylphosphate, confirming the specificity of the antibody for tyrosine-phosphorylated proteins (data not shown).

Resting T cells were then stimulated either by crosslinking CD3 alone, crosslinking CD4 alone, or by crosslinking CD3 and CD4 together with a heteroconjugate of the CD3XCD4 antibodies. The effects of these stimulations on induction of new tyrosine phosphorylation are shown in Fig 8. CD3 stimulation alone strongly increased the tyrosine phosphorylation of 100-Kd, 122-Kd, and 137-Kd substrates, and also increased the tyrosine phosphorylation of the 75-Kd and 85-Kd substrates. CD4 crosslinking alone had only a weak effect on increasing the 100-Kd and 137-Kd substrates. However, the CD3XCD4 stimulation was significantly more potent than CD3 stimulation and, like CD3 stimulation, increased tyrosine phosphorylation of the 137-, 122-, 100-, 85-, and 75-Kd substrates. The 51-Kd and 55-Kd proteins also became strongly tyrosine phosphorylated after CD3XCD4 stimulation, but not after CD3 stimulation alone (Fig 8).

Activated T cells were next examined after stimulation by a similar protocol. T cells were cultured for 8 days in PHA without addition of exogenous IL-2. Tyrosine-phosphorylated proteins in these cells were the same as resting T cells except for a 65-Kd protein not detected previously (Fig 9). Crosslinking CD4 on these cells predominantly induced prolonged tyrosine phosphorylation of the 51- and 55-Kd proteins without strong effects on other substrates. CD3 crosslinking caused a transient increase in tyrosine phosphorylation in the 51-Kd and 55-Kd proteins as well as other proteins of 62, 65, 75, and 85 Kd and other substrates in the 100- to 137-Kd region not as well resolved in this experiment. In comparison with CD3, the CD3XCD4 stimulation more strongly increased tyrosine phosphorylation of the same proteins, and also prolonged the response so that it was still easily detectable by 20 minutes after stimulation.

The CD3+ CEM T-ALL cell line was also stimulated in

---

Table 4. Phenotype of ALL Blast Colonies Stimulated With Immobilized Anti-CD3

<table>
<thead>
<tr>
<th>Antigen</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>9</th>
<th>11</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1</td>
<td>45s/c</td>
<td>23</td>
<td>50</td>
<td>0</td>
<td>65s/c</td>
<td>25s</td>
<td>ND</td>
</tr>
<tr>
<td>CD2</td>
<td>95</td>
<td>94</td>
<td>85</td>
<td>95</td>
<td>100</td>
<td>40s/c</td>
<td>95s/c</td>
</tr>
<tr>
<td>CD3</td>
<td>75c</td>
<td>11s/80c</td>
<td>10s/90c</td>
<td>12s/95c</td>
<td>5s/90c</td>
<td>15s/75c</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>75</td>
<td>56</td>
<td>2</td>
<td>0</td>
<td>40</td>
<td>65</td>
<td>75</td>
</tr>
<tr>
<td>CD5</td>
<td>80</td>
<td>75</td>
<td>60</td>
<td>100</td>
<td>99</td>
<td>75</td>
<td>85</td>
</tr>
<tr>
<td>CD7</td>
<td>98</td>
<td>95</td>
<td>92</td>
<td>40s/c</td>
<td>98</td>
<td>55</td>
<td>25</td>
</tr>
<tr>
<td>CD8</td>
<td>25</td>
<td>67</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>CD10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>75s/c</td>
<td>0</td>
<td>10s/30c</td>
</tr>
<tr>
<td>CD19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD25</td>
<td>65s/c</td>
<td>0</td>
<td>25s/c</td>
<td>0</td>
<td>90s/c</td>
<td>100s/c</td>
<td>0</td>
</tr>
<tr>
<td>TdT</td>
<td>85</td>
<td>95</td>
<td>90</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

Percent of cells positive was determined by indirect immunofluorescence with a rhodamine-conjugated antimouse Ig second step and fluorescence microscopy. Blast colonies were stained with OKT6 (CD1), 9.6 (CD2), G19-4 (CD3), G17-2 (CD4), 617-2 (CD5), 10.2 (CD5), 63-7 (CD7), G10-1 (CD8), 24.1 (CD10), E43 (CD19), BAl (CD24), TAC (CD25), or TdT. Percent of positive cells staining in the cytoplasm (c) or on the cell surface (s) is shown for CD1, CD3, and CD25 antigens.
Fig 8. Changes in tyrosine phosphorylation of proteins in resting T cells after stimulation. Peripheral blood T cells, enriched by passage over a nylon wool column, were stimulated with 20 μg/mL anti-CD3 MoAb G19-4, with 20 μg/mL of a (CD3xCD4) heteroconjugate of (G19-4XG17-2), or with 20 μg/mL biotin-conjugated anti-CD4 MoAb G17-2 followed 5 minutes later by 80 μg/mL avidin. The 0 time point was before addition of G17-2, and the next time point was taken 2 minutes after addition of avidin.

Fig 9. Changes in tyrosine phosphorylation of proteins in T-cell blasts after stimulation. T cells were enriched from peripheral blood by passage over nylon wool and then activated with PHA plus rIL-2 (20 μL/mL). Cells were grown without further addition of IL-2 for 8 days. Stimulations were the same as described in the legend to Fig 8.

the same way and examined by antiphosphotyrosine immunoblotting. These cells were chosen because of the enhanced calcium mobilization observed by CD3xCD4 crosslinking compared with CD3 crosslinking alone (Fig 5). Either CD3 or CD4 crosslinking alone induced weak responses, whereas the specific aggregation of CD3 with CD4 generated a much stronger response on multiple substrates including a 35-Kd protein and a 21-Kd protein (Fig 9). The 55-Kd, 62-Kd, 65-Kd, 75-Kd, and 85-Kd proteins and other proteins between 100 Kd and 187 Kd were also very strongly phosphorylated. Therefore, the CEM T-ALL cell line was similar to normal T cells in enhanced activation of tyrosine kinase(s) by the association of CD3/Ti with CD4.

DISCUSSION

Previous studies of the phenotype of T-ALL cells have shown that CD3/Ti is abundant in the cytoplasm, but is not expressed on the cell surface.6-8 Our immunofluorescence data are consistent with this conclusion, because analysis with anti-CD3 MoAb and flow cytometry classified the T-ALLs studied here as surface CD3 negative or very low. In contrast, our assays of cell activation show that most T-ALLs express functional CD3/Ti; two of the 14 cases studied responded directly to anti-CD3 MoAbs by increases in [Ca++], while three others increased [Ca++] after crosslinking of CD2, CD4, or CD8 with CD3/Ti using MoAb heteroconjugates. Analysis of proliferation by T-ALLs showed that 12 of the cases increased colony formation when stimulated with immobilized anti-CD3 in the presence of growth factors, and the proliferating colonies showed specific induction of IL-2 receptor α chain (CD25). Together, these results indicate that most T-ALLs express functional CD3/Ti even when cell surface levels are too low to easily detect by immunofluorescence techniques.

Activation of T-ALLs with anti-CD3 was modulated by crosslinking CD3/Ti with CD2, CD4, or CD8. The effect of the accessory molecules was either enhancement or inhibi-
tion of the response to anti-CD3, whether assayed by proliferation or by increases in [Ca^{2+}]i. These differences could relate to differentiation stage, although no clear correlation with T-ALL classification was apparent. Notably, functional CD3/Ti regulated by accessory molecules was seen among the CD1-CD3- stage I (prothymocyte) T-ALLs as well as the CD1+CD3+ stage II (common thymocytic) and the three CD1+CD3+ stage III (mature) T-ALLs studied.

The upregulation or downregulation of CD3/Ti function by accessory molecules on T-ALLs may relate to positive or negative selection, processes that act on CD3/Ti at an early stage of differentiation in the normal thymus. The increase in [Ca^{2+}]i has been linked to apoptosis, a process of active cell death mediated by DNA fragmentation through activation of a calcium-regulated endonuclease. Although the [Ca^{2+}]i signal induces cell death, this response of thymocytes was reversed by simultaneous stimulation with phorbol myristate acetate (PMA) or IL-2, and can also be regulated by growth factors (erythropoietin) or stimulation of accessory receptors (CD40) in other cell systems.

There is strong functional evidence that the accessory molecules CD4 and CD8 regulate responses of immature thymocytes. CD4 is important in clonal deletion, because self-reactive T cells are not deleted when neonatal mice are treated with anti-CD4. In T transgenic animals, CD4 versus CD8 lineage selection is determined by the major histocompatibility complex (MHC) class I or class II restriction of the Tαβ receptor, thus implicating CD4 and CD8 in positive selection. Several of the T-ALLs examined here showed [Ca^{2+}]i responses that were highly regulated by CD3/Ti to increase T cell activation. The regulation of [Ca^{2+}]i signals by CD2 association with CD3/Ti on T-ALLs and on normal thymocytes is similar to results obtained with normal T cells. Taken together, the data presented here suggest that immature T cells with low levels of cell surface CD3/Ti are more dependent upon CD3/Ti associations with accessory molecules such as CD4, CD8, or CD2 for transmembrane signalling responses resulting in increases in [Ca^{2+}]i than are mature normal T cells.

The CD4 and CD8 receptors are specifically associated with the protein tyrosine kinase p56Lck. Tyrosine phosphorylation occurs before phospholipase C (PLC) activation in T cells and tyrosine kinase inhibitors prevent PLC activation by CD3/Ti. Therefore, we examined the effect of CD4 ligation on induction of tyrosine phosphorylation during activation of normal T cells and T-cell ALLs by immunoblotting with antiphosphotyrosine. Stimulation of CD3/Ti induces tyrosine phosphorylation of specific substrates in T cells. Similarly, CD4 crosslinking activates p56Lck, resulting in tyrosine phosphorylation of substrates in T cells and in immature thymocytes. However, there is no information about how CD4 interaction with CD3/Ti might regulate protein tyrosine kinase (PTK) activation in early T-cell ontogeny. Here we show in both normal mature T cells and in T-ALL cell lines that induction of tyrosine phosphorylation is optimized by crosslinking CD4 and CD3/Ti together. Crosslinking CD3/Ti alone or CD4 alone gave weak signals that were distinct. A 55-Kd protein increased in response to CD4 crosslinking, consistent with

![Fig 10. Changes in tyrosine phosphorylation of proteins in CD3\x2dCEM cells after stimulation. CEM cells directly from tissue culture were washed and resuspended to 5 x 10^6 cells/mL and then stimulated as described in the legend to Fig 8, except that CD4 stimulation used 20 μg/mL (CD4XCD4) homoconjugate (G17-2XG17-2).](from www.bloodjournal.org)
activation of p56\textsuperscript{ck} as previously reported.\textsuperscript{16,17} However, in our studies, substrates other than the 55-Kd protein were not strongly induced by CD4 crosslinking. CD3 ligation alone showed a weak and transient tyrosine phosphorylation of several substrates. The interaction of CD3/Ti and CD4 showed a synergistic induction of tyrosine phosphorylation in normal resting or activated T cells and in T-ALL cell lines CEM (Figs 8 through 10) and HPB-ALL (data not shown).

Because CD4 crosslinking activates p56\textsuperscript{ck} but CD3XCD4 association is required for optimal tyrosine phosphorylation during T cell activation, it will be important to determine whether p56\textsuperscript{ck} activity is also controlled by CD3/Ti. Alternatively, the substrates may be physically associated with CD3/Ti but not CD4, so that the CD3XCD4 interaction may regulate availability of substrates. In either case, the association of CD4 with CD3/Ti induced in normal T cells by recognition of antigen is shown here to regulate tyrosine phosphorylation responses by normal and malignant T cells.

How is the inositol phospholipid signalling pathway leading to mobilization of cytoplasmic calcium related to the activation of tyrosine kinase(s) in T cells? The upregulation of CD3/Ti [Ca\textsuperscript{2+}] signals by association with CD4 suggests that the pathway may be regulated by the activity of p56\textsuperscript{ck}. The inhibition of CD3/Ti [Ca\textsuperscript{2+}] signals by the CD45 protein tyrosine phosphatase\textsuperscript{6} is consistent with this model. It has been recently shown that PLC\textsubscript{II} activation in fibroblasts by platelet-derived growth factor (PDGF) or by epidermal growth factor (EGF) is associated with tyrosine phosphorylation of PLC\textsubscript{II}.\textsuperscript{6,18} A similar mechanism of PLC regulation involving tyrosine phosphorylation by p56\textsuperscript{ck} in T cells may occur. The association of CD2 with CD3/Ti also increases [Ca\textsuperscript{2+}] signals in normal T cells,\textsuperscript{19} in immature thymocytes,\textsuperscript{19} and in T-ALLs. However, CD2 is not associated with p56\textsuperscript{ck}, and although CD2 crosslinking induces tyrosine phosphorylation of some substrates,\textsuperscript{20} we have not seen enhanced tyrosine phosphorylation by (CD3XCD2) stimulation. Thus, the interaction of CD2 with CD3/Ti may upregulate PLC activation in T cells by another mechanism.

REFERENCES


14. Veillette A, Bookman MA, Horak EM, Bolen JB: The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56\textsuperscript{ck}. Cell 55:301, 1988

15. Rudd CE, Trevillian JM, Dasgupta JD, Wong LL, Schlossman SF: The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc Natl Acad Sci USA 85:5190, 1988


17. Barber EK, Dasgupta JD, Schlossman SF, Trevillian JM, Rudd CE: The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56\textsuperscript{ck}) that phosphorylates the CD3 complex. Proc Natl Acad Sci USA 86:3277, 1989


21. Finkel TH, McDuffie M, Kappler JW, Marrack P, Cambier...
TRANSMEMBRANE SIGNALLING IN T-ALL


60. Ledbetter JA, Tonks NK, Fischer EH, Clark EA: CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. Proc Natl Acad Sci USA 85:8628, 1988


Accessory receptors regulate coupling of the T-cell receptor complex to tyrosine kinase activation and mobilization of cytoplasmic calcium in T-lineage acute lymphoblastic leukemia

JA Ledbetter, GL Schieven, VM Kuebelbeck and FM Uckun