Chronic lymphocytic leukemia (CLL), typically a monoclonal B cell malignancy, is frequently accompanied by various peripheral blood T cell abnormalities. These include T cell lymphocytosis, low T4-T8 ratios with inadequate helper activity for B cell proliferation and immunoglobulin synthesis, and depressed to absent natural killer activity and autologous mixed lymphocyte reactivity. In addition, variably decreased responses to mitogen-induced (phytohemagglutinin [PHA] or concanavalin A [Con A]) proliferation have been reported by several investigators. We have recently described a subgroup of CLL patients in whom only a minority of erythrocyte rosette positive (ER+) peripheral blood T cells reacted with monoclonal antibodies to T3, a 20- to 25-kd membrane antigen normally present on mature T cells and medullary thymocytes. In contrast to other CLL patients whose T3-ER+ ratios were more normal (≥0.6), T cells from this CLL subgroup exhibited markedly defective responses to PHA and Con A. The T3 antigen is now known to be required for natural killer activity and autologous mixed lymphocyte reactivity. Consequently, we have attempted to better define the mechanisms of abnormal T cell responsiveness to lectins in this group of CLL patients by enzymatically exposing additional membrane T3, studying the appearance of membrane activation antigens, measuring interleukin 2 (IL-2) production, and enhancing proliferative responses with exogenous normal IL-2.

**MATERIALS AND METHODS**

Eleven B-CLL patients and 11 normal donors, matched for age and sex, were studied. CLL patients were staged according to the method of Rai: stages 0 to 1, five patients; stage 2, four patients; and stage 4, two patients. No patient had received chemotherapy within four weeks of study.

**Preparation of ER+ cells.** T cells were isolated by virtue of their ability to rosette with sheep erythrocyte (SRBC). Peripheral blood mononuclear cells were prepared from heparinized venous blood by Ficoll-Hypaque centrifugation, resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS), and depleted of adherent cells by incubation in sterile plastic Petri dishes for one hour. Nonadherent cells were incubated with SRBC pretreated with AET (2-aminoiso-ethylisothyronium hydrobromide), and the ER+ cells were pelleted by centrifugation through Ficoll-Hypaque. ER+ lymphocytes were freed of SRBC by brief hypotonic lysis in distilled water. This procedure gave a purity 98% or greater for ER+ lymphocytes (measured by rerosetting with SRBC-AET).

**Treatment of ER+ lymphocytes with neuraminidase.** ER+ cells (2 × 10^6/mL) suspended in PBS were incubated with *Vibrio cholerae* neuraminidase (50 U/mL) (Behring Diagnostics, Somerville, NJ) at 37 °C for 30 minutes with shaking. The cells were then washed repeatedly with PBS to remove excess enzyme and resuspended in RPMI-10% FBS.

**Detection of T cell membrane antigens.** The following monoclonal antibodies were used to detect surface membrane antigens of ER+ lymphocytes: OKT3, TAC (anti-IL-2 receptor), OKT9 (anti-transferrin receptor), and 5/12 (anti-IA). (Monoclonal antibodies OKT3 and OKT9 were purchased from Ortho Laboratories, Raritan, NJ; TAC was generously provided by Dr Thomas Waldmann, National Institutes of Health; 5/12 was a generous gift of Dr Saldano Ferrone, New York Medical College, NY.) ER+ cells (1 × 10^6/mL) in RPMI-10% FBS were incubated (4 °C, 30 minutes) with 20 to 50 μL of an appropriate dilution of monoclonal antibody. The cells were then washed twice with RPMI-10% FBS and incubated for an additional hour with 100 μL of a 1:40 dilution of fluoresceinated goat F(ab)2, antimouse immunoglobulin (Dako, Burlingame, Calif.). After thorough washing, the cells were examined with a Zeiss fluorescence microscope equipped with epifluorescence filters.

**PHA-induced mitogenesis.** ER+ cells (2 × 10^6) isolated from controls or CLL patients were mixed in microtiter wells (Linbro, Hamden, Conn) with 1 μg/mL purified PHA (PHA-16, Burroughs Wellcome, Triangle Park, NC). After 72 hours of incubation (37 °C, 5% CO2), 2 μCi of tritiated thymidine (3[H]Tdr) (New England Nuclear, Boston) was added. After an additional 24-hour incubation, cells were harvested and radioactivity measured by liquid scintillation spectrometry. Results were calculated and expressed as mean ± standard deviation of the mean (SEM). In some studies, exogenous IL-2 was added simultaneously with PHA. The IL-2 was derived from normal, PHA-stimulated lymphocytes (see below), or recombinant IL-2 (generously provided by Cetus Corporation, Emeryville, Calif) was used. IL-2 concentrations ranging from 0.1% to 20% were used.
T CELL PROLIFERATION IN CLL

Fig 1. Relationship between T cell proliferation and T3 membrane antigen. The T3-ER ratios of CLL (O) (n = 17) or control (●) (n = 6) subject lymphocytes are plotted vs the [3H]Tdr incorporation of the same lymphocytes PHA-induced [3H]Tdr incorporation.

RESULTS

Relationship between CLL T cell proliferation and membrane T3 antigen. We have previously reported that CLL T cell populations containing low T3-ER ratios exhibit subnormal mitogenic responses to PHA. The present studies confirm our previous findings and demonstrate a high degree of correlation between T3-ER ratios and levels of [3H]Tdr incorporation (Fig 1). Ratios ≥0.6 were invariably associated with more normal proliferative responses. Patients with ratios ≤0.3 (n = 5) had significantly less proliferation than patients with ratios ≥0.6 (P < 0.5; n = 10).

In an effort to augment the defective responses, we treated CLL cell populations having low T3-ER ratios with neuraminidase, which, as we have previously shown, enhances their T3 antigen expression. Although neuraminidase treatment markedly increased the percentages of T3-positive cells from 38% to 78%, no significant improvement in PHA-induced [3H]Tdr incorporation resulted.

Appearance of membrane activation antigens in CLL and control T cells. Figure 2 summarizes the kinetics of appearance of various activation antigens after exposure of CLL and control T cells to PHA. During the first 48 hours, T9, TAC, and IA increased at similar rates in patient and control cells; thereafter, T9 and TAC gradually declined. In contrast, IA expression of control, but not of CLL, cells continued to increase between 48 and 72 hours. The rate and degree of expression of activation antigens bore no relationship to T3-ER ratios or to the magnitude of [3H]Tdr incorporation. The standard error of the mean for all points plotted in Fig 2 ranged from 4% to 10%.

IL 2 production by CLL and control T cells. CLL T cells were found to elaborate approximately 55% of control IL 2 activities in response to PHA stimulation. Table 1 summarizes the relationship of CLL ER lymphocytes to their membrane T3 presentation and PHA-induced IL 2 production and [3H]Tdr incorporation (Table 1). In general, the lower the T3-ER ratio, the lower the thymidine incorporation.

Fig 2. Sequential analysis of (A) CLL (n = 7) or (B) control (n = 7) ER lymphocyte membrane activation and post-PHA exposure. The mean percentage of each activation antigen (TAC = ○, IA = ●, transferrin = ▲ T9) is plotted over a 72-hour incubation period.
tion. IL 2 production was somewhat lower in ER' lymphocytes with <60% T3 reactive cells, but the difference was not statistically significant.

Enhancement of mitogenesis by exogenous IL 2. Because after PHA stimulation CLL T cells developed various activation antigens normally but produced diminished amounts of IL 2, we attempted to improve their mitogenic responsiveness by supplementing them with exogenous IL 2 (Fig 3). Addition of 10% IL 2 improved somewhat, but did not normalize, the proliferative responses of CLL T cells to PHA. The greatest degrees of improvement were seen in CLL patients exhibiting low T3-ER ratios. Addition of higher concentrations of IL 2, or repetitive supplementation with IL 2 over the 72-hour incubation period, failed to further enhance T cell proliferative responses.

Table 1. CLL ER' Lymphocytes in Relationship to Their T3 Membrane Antigen, IL2 Production, and PHA-induced [3H]Tdr Incorporation

<table>
<thead>
<tr>
<th>Group</th>
<th>T3 Positive (%)</th>
<th>IL 2 Production (µ/mL)</th>
<th>[3H]Tdr (cpm/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (0%-30%, n = 4)</td>
<td>13.5 ± 2.9</td>
<td>31.5 ± 27.8</td>
<td>2.9 ± 1.8 x 10^2</td>
</tr>
<tr>
<td>II (30%-60%, n = 4)</td>
<td>40.8 ± 1.0</td>
<td>32.8 ± 26.5</td>
<td>18.5 ± 1.4 x 10^3</td>
</tr>
<tr>
<td>III (&gt;60%, n = 6)</td>
<td>87.5 ± 3.6</td>
<td>45.8 ± 11.7</td>
<td>59.3 ± 6.8 x 10^3</td>
</tr>
<tr>
<td>Control (n = 11)</td>
<td>92.5 ± 2.6</td>
<td>64.6 ± 11.0</td>
<td>109.8 ± 15.8 x 10^3</td>
</tr>
</tbody>
</table>

CLL ER' lymphocyte groups were arbitrarily divided into three groups: I, ER' lymphocytes that were 0% to 30% reactive with OKT3; II, ER' lymphocytes 30% to 60% reactive with OKT3; and III, ER' lymphocytes >60% reactive with OKT3.

DISCUSSION

Activation of normal T cells may be arbitrarily conceptualized as occurring in three sequential stages: (1) engagement of T, the T cell antigen receptor intimately associated with T3; (2) development of multiple membrane activation antigens, including T9 (transferin receptor), TAC (the receptor for IL 2); the T cell growth factor, and IA20; (3) synthesis and elaboration of IL 2, which combines with TAC to trigger mitogenesis.21 In an effort to better understand the mechanisms of the defective proliferative responses of CLL T cells to PHA and other immunologic stimuli, we have examined each of the stages for possible abnormalities. We have previously reported that the peripheral blood of CLL patients showing exceedingly poor responses to PHA contain high percentages of ER' cells that poorly express T3 membrane antigen.13 T3 is known to be required for normal T cell proliferative responses and development of cytotoxic effector cell functions.15,16 The studies described confirm our previous findings and emphasize the close relationship between T3 expression and mitogenic responsiveness (Fig 1). Although treatment of these low T3-ER' cell populations with neuraminidase significantly improved their T3 expression, no parallel enhancement of mitogenic responsiveness occurred. The T3 antigen comprises several membrane glycoproteins containing different numbers of sialic acid residues.14 Because neuraminidase treatment exposes additional T3 antigenic sites without enhancing mitogenic responsiveness, additional yet undetected defects may be present within the CLL T3 complex. Alternatively, other abnormalities may be responsible for defective cell proliferation.

To investigate other possible abnormalities, we studied the appearance of various membrane activation antigens induced by PHA stimulation of CLL T cells. Surprisingly, the rates of appearance of T9, TAC, and IA were entirely normal, unrelated to the degree of T3 expression or to the mitogenic responsiveness of the individual cell populations. Although we detected significantly diminished IL 2 activity in PHA-CLL T cell conditioned media, no relationship was observed between the degree of abnormality in IL 2 elaboration, T3-ER' ratios, and mitogenic responsiveness. Moreover, only minimal improvement in the proliferative responses of these cells to PHA could be induced by addition of exogenous IL 2, despite the presence of apparently normal numbers of IL 2 receptors. These findings suggest that receptor binding of IL 2 may be defective or that even more distal defects in the mitogenic sequence may be present, i.e., in the generation of the recently described inducible cytoplasmic factor that initiates DNA replication.22
Thus it appears that CLL T cells exhibit multiple abnormalities that may relate to their diminished proliferative potential. Continued exploration of the molecular nature of these abnormalities should yield insights that may permit immunologic reconstitution of patients with this disorder.

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