The results of lymphocyte functional studies performed with the peripheral blood mononuclear cells (MNCs) of patient Wa with a non-Sezary T-cell leukemia are described. The aggressive clinical course and the distinctive histologic, cytochemical, and cytogenetic features of this T-cell lymphoproliferative disorder are detailed in an accompanying report. Immunologic marker analysis revealed that 76% of the Wa MNC fraction formed rosettes with sheep erythrocytes (E-rosettes), and 13% showed Slg positivity by immunofluorescence. In vitro mitogenic responses of Wa cells measured by 3H-thymidine incorporation were extremely low, and responses to candida and streptococcal antigens were absent. Although a normal percentage of B cells was evident in the Wa MNC fraction, no Ig synthesis occurred in either pokeweed mitogen (PWM) stimulated or unstimulated cultures. In coculture experiments with normal allogeneic lymphoid populations, the Wa cells exhibited no suppressor or helper effects on the normal PWM-stimulated Ig synthesis. Importantly however, the Wa cells demonstrated suppressor effects on the normal mixed lymphocyte reaction (MLR) occurring between populations of allogeneic MNCs from two normal donors. The degree of MLR suppression increased with the presence of greater numbers of Wa cells, suggesting a dose-response relationship in these three-way MLRs. Control three-way MLRs that included two normal allogeneic lymphoid populations and other neoplastic T-cell suspensions, unrelated to Wa cells, did not show any MLR suppressor effects. Unlike the Con-A-induced MLR suppression mediated by a subset of normal T suppressor cells, the Wa suppression did not require preincubation with mitogen for the expression of this MLR suppressor activity. The absence of detectable suppressor effects by Wa cells on the proliferative responses of normal lymphocytes to Con-A and in the Ig synthesis of normal MNCs suggested that the Wa MLR suppression was a specific immune activity of the neoplastic cells rather than a nonspecific toxic effect. These data demonstrating MLR suppressor activity without suppressor effects on normal PWM-stimulated Ig synthesis have described a selective suppressor capacity possessed by the Wa cells and emphasize the usefulness of employing multiple assay systems in testing neoplastic T cells for putative T- and B-cell effector functions. Moreover, the results of this combined analysis of histology, cytochemistry, and immune activity of these neoplastic T cells should provide a basis for future comparisons of other T-cell lymphoproliferative disorders with definable lymphocyte function.

The aggressive clinical course, the distinctive morphological and cytochemical features, and the abnormal karyotype of the T-cell lymphoma of patient Wa has been described in an accompanying report. This article will present the results of an in vitro functional analysis of the neoplastic T lymphocytes taken from the peripheral blood during the terminal leukemic phase of the patient's illness. The cellular activities exhibited by the Wa T cells in two separate functional lymphocyte assays will be compared and correlated with the described morphological, cytochemical, and cytogenetic findings.

The results of in vitro functional studies of the neoplastic T-cell populations from some patients with T-cell leukemias and lymphomas have suggested that in certain individuals these neoplasms represent a proliferation of immunoregulatory clones retaining original helper or suppressor properties. The first example of neoplastic T cells with helper or suppressor activity was the functional analysis of Sezary lymphocytes reported by Broder and his colleagues. In this study, the Sezary lymphocytes were shown to express potent helper activity in pokeweed mitogen (PWM) stimulated B-cell Ig synthesis. More recently, a Sezary patient has been identified with characteristic Sezary cells that demonstrated potent suppressor rather than helper effects on normal PWM-stimulated Ig synthesis. Saxon et al. have published unique results that strongly suggest the expression of both helper and suppressor activity within the same clonal-related cell population of a non-Sezary T-cell leukemia.

This report presents the results of functional studies performed with leukemic T lymphocytes from patient Wa. However, unlike the previously reported cellular
studies that have emphasized a functional analysis surrounding the PWM stimulation of normal Ig synthesis, this investigation has incorporated an additional T-cell functional assay, the mixed lymphocyte reaction (MLR). The results of these combined functional tests have shown that the neoplastic Wa cells exerted suppressor activity on the MLR of normal allogeneic lymphocytes with, however, no demonstrable suppressor effects on the PWM stimulation of Ig synthesis. Nonspecific cytotoxic activity on normal lymphocytes by Wa cells in coculture was excluded by the absence of effects on both the Ig synthesis and the proliferative responses of control lymphocytes to mitogen.

**MATERIALS AND METHODS**

**Cell Isolation, Surface Marker Analysis, and Response to Antigens and Plant Lectins**

Peripheral blood obtained in preservative-free heparin from patient Wa and normal donors was allowed to sediment by gravity for 1 hr at 37°C. The leukocyte-rich plasma was centrifuged in a Ficol-Hypaque gradient, and the mononuclear cell (MNC) fraction was recovered at the plasma-gradient interface. Surface marker analysis and conventional lymphocyte stimulation assays were performed in the Immunology Core Laboratory of the University of Chicago Cancer Research Center. The MNC analyses included E-rosettes, EAC rosettes, surface Ig fluorescence, and peroxidase staining performed according to conventional procedures. Morphological identification of E-rosette-forming Wa lymphocytes was determined from a microscopic slide prepared in a cytocentrifuge (Shandon Southern Instruments, Sewickley, Pa.) and subsequently stained with Wright's stain. Plant lectin and antigenic stimulation of lymphocytes was performed in microtiter plates using previously determined optimal concentrations.

**In Vitro Ig Biosynthesis**

The mononuclear cell (MNC) fraction was washed 9 times in separate 15-mI volumes of sterile phosphate-buffered saline containing 1% fetal calf serum (FCS). Prior to culture, the washed cells were suspended in RPMI-1640 media containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FCS.

Patient and control MNCs at 0.5 × 10⁶ cells/ml in supplemented RPMI-1640 media were placed in 2-ml duplicate cultures with and without pokeweed mitogen (PWM) for 7 days at 37°C in 5% CO₂ with humidity. Duplicate 1 day cell cultures were also included for control Ig determinations. Patient and normal cell suspensions were cocultured for 7 days according to previously published procedures. In stimulated cultures, 4 µg of PWM (Sigma Chemical Co., St. Louis, Mo.) was added to each 2-ml culture. At the termination of the culture period, the cells were pelleted by centrifugation, and the culture supernatants were removed and stored at −20°C until assayed for Ig content.

The total Ig in the cell culture supernatants was taken as the arithmetic sum of the individual IgG and IgA measured in the supernatants. Quantitation of IgG and IgA was determined by a nanogram sensitive radiolabeled technique of indirect precipitation. This type of assay utilizes iodine-labeled human monoclonal F(ab')₂ fragments prepared from purified IgG and IgA myeloma proteins. IgG fractions of rabbit monoclonal human L-chain antisera, and goat antiserum to the Fc fragment of rabbit IgG. The preparation of the monoclonal F(ab')₂ fragments, rabbit anti-L-chain sera, and goat anti-rabbit Fcγ serum has been described previously. Absorption of the anti-L-chain sera was accomplished with a solid immunosorbent panel of Sepharose-coupled human Ig proteins of separate H- and L-chain classes. The anti-L-chain rabbit IgG fractions were diluted appropriately in borate-saline buffer, pH 8.0, containing 1% bovine serum albumin (BSA).

Binding specificity tests with monoclonal F(ab')₂ fragments and rabbit IgG specific for κ and λ chains were performed with a panel of purified monoclonal Ig protein inhibitors of the IgM, IgA, and IgG classes and of both L-chain types. In addition, cross-binding reactions, e.g., between anti-κ and anti-λ F(ab')₂ fragments, were conducted with the absorbed anti-L-chain preparations to insure monospecificity of the anti-L-chain IgG fractions.

In a typical analysis, duplicate 50-µl amounts of supernatant from each duplicate culture were tested for specific L-chain content by determining the binding inhibition in the L-chain assay. A standard curve of inhibition using known amounts of inhibitors was reestablished for each new set of culture supernatants.

**Mixed Lymphocyte Reaction (MLR)**

Quadruplicate MLR tests were performed in 300-µl microtiter trays (Costar) using 10⁶ cells from each of two control donors and varying number of cells from patient Wa (10⁶; 5 × 10⁶; 5 × 10⁷) in a 2- or 3-way mixed lymphocyte culture. Stimulating cells in some instances were treated with mitomycin-C, 50 µg/ml, for 30 min at 37°C, to make the reaction unidirectional. Following incubation for 5 days at 37°C, 1 µCi ³H-thymidine was added to the mixed lymphocyte cultures for 5 hr, after which the cells were harvested and counted in a liquid scintillation center.

**Con-A-Induced Suppression**

This technique has been described previously. Briefly, suppressor activity was induced by incubation of 10⁶ PB MNCs with 3 µg/ml Con-A. Control cells were placed in media with Con-A. After 96 hr of incubation, the Con-A-treated and untreated cells were washed and exposed to mitomycin-C (50 µg/ml for 30 min). The mitomycin-treated cells (10⁶) were cocultured with 10⁶ fresh normal MNCs in 3 µg/ml Con-A for 3 days. Tritiated thymidine was added for 5 hr prior to cell harvesting. In other studies, freshly isolated mitomycin-treated peripheral blood cells from Wa were cocultured with equal numbers of fresh PB lymphocytes from normal donors and 3 µg Con-A. Following 72 hr incubation, labeled thymidine incorporation into the cultured cells was determined.

**RESULTS**

The immunologic studies reported in this article were performed with Wa peripheral blood MNCs obtained at the time of an accelerated phase of the T-cell leukemia, prior to the administration of chemotherapy during the patient’s terminal hospital admission. The peripheral WBC was 44,000/cu mm with 84% lymphoid cells on peripheral smear. Unfortunately, repeat functional studies were prevented by the profound leukopenia following the chemotherapy and the subsequent rapid deterioration of the patient’s clinical status leading to her eventual death.
Surface Marker Analysis and Stimulation Responses of Wa Cells

Immunologic marker analysis of Wa MNCs revealed that 76% of the cells formed E-rosettes, and on microscopic examination, greater than 95% of the E-rosettes contained neoplastic lymphocytes. Surface Ig fluorescence was evident on 13% of the MNCs, 10% of cells formed EAC⁺ rosettes, and 3% were considered peroxidase-positive. Microscopic examination of the peroxidase-stained smear showed that 20% of the stained cells were of monocytic origin, while the remainder were from the granulocytic series.

The response of Wa cells to plant lectins (Con-A—924 cpm, PHA—1150 cpm, PWM—1450 cpm) was barely above background (873 cpm), while the responses to candida (510 cpm) and streptococcal (316 cpm) antigens were absent.

Immunoglobulin Synthesis by Wa Cells and Normal Peripheral Blood MNCs

The results of in vitro Ig synthesis with Wa cells, control PB MNCs, and respective cocultures of Wa and normal MNCs are presented in Table 1. The total Ig as reflected in the sum of λIg and λIg is graphed schematically in Fig. 1. Although a normal percentage of B cells was evident in the Wa cell suspension, no Wa Ig synthesis occurred in either unstimulated or PWM-stimulated cultures. In contrast, control MNCs from the normal donors demonstrated strong stimulation of Ig production under the same in vitro conditions. In coculture experiments with the control MNCs from two normals, the Wa cells exhibited no suppressor or helper effects on the normal PB lymphocyte Ig synthesis (Table 1, Fig. 1).

Mixed Lymphocyte Reactions With Wa Cells

The results of MLR studies with coculture experiments involving 2- and 3-way MLR cultures between controls and patient Wa are presented in Table 2. The Wa cells demonstrated suppression of the MLR between populations of allogeneic MNCs from normal donors (same cells used above in the Ig synthesis studies). The degree of MLR suppression increased with the presence of greater numbers of Wa cells, suggesting a dose–response type of relationship (Fig. 2). Since none of these cells were blocked with mitomycin, these coculture experiments represent reactions between three allogeneic cell populations.

In our experience, 3-way MLR cultures demonstrate at least comparable and usually greater thymidine incorporation than MLRs between any 2 of 3 donors (mean 306% ± 43%; range 80%–681%, n = 15), excluding crowding as an explanation for the suppression induced by Wa cells. In unidirectional MLRs, 10⁶ cells from patient Wa did not respond to 10⁴ mitomycin-treated stimulator cells from each normal donor (106 ± 19 cpm) nor did 10⁷ cells from the normal donors respond to 10⁶ mitomycin-treated cells from Wa (235 ± 75 cpm; 154 ± 17 cpm).

As a control neoplastic lymphoid population, we utilized neoplastic T cells from an unrelated lymphoblastic lymphoma patient, Fl. These cells did not respond to, or stimulate normal allogeneic cell populations. Unlike Wa cells, the Fl cells did not exhibit significant suppressor effects in the MLR of normal allogeneic lymphocytes (Table 2). We also studied cells from female patient Br with Sezary syndrome and found that her Sezary T cells augmented the MLR (Table 2).

Con-A-Stimulated Responses in Wa Cocultures

Con-A-stimulated cocultures of Wa cells and control lymphocytes were studied in order to determine if Wa cells possessed nonspecific cytotoxic effects on normal lymphocyte responses. These results are presented in Table 3 and demonstrate an absence of cytotoxic effects on Con-A-stimulated responses of normal lymphocytes. No interference with the Con-A
responses of normal lymphocytes in coculture with Wa cells was apparent. Similarly, cells of patients Wa that were preincubated 96 hr with Con-A and then treated with mitomycin did not exert a suppressor effect (data not shown).

DISCUSSION

Certain individual T-cell lymphomas and leukemias have been shown to possess immunoregulatory functional activity, suggesting that these tumors may represent the neoplastic expansion of immunoregulatory lymphoid clones. These malignant lymphoid cells with retained T-cell regulatory function should permit the subclassification of certain T-cell lymphomas according to functional characteristics and also provide a unique opportunity to study the particular expressed activity. For example, functional studies of the malignant T cells of Sezary syndrome by Broder et al. have demonstrated that Sezary lymphocytes possess helper activity for normal PWM-stimulated B-cell Ig synthesis. In a more recent case analysis, Hopper and Haren have identified an individual Sezary lymphocyte population that exhibited potent suppressor effects on normal PB lymphocyte Ig synthesis, indicating that Sezary lymphocyte populations are not solely derived from lymphocytes clones

Table 2. Influence of Leukemic Cells on Mixed Lymphocyte Culture Reactions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Control 1 (C₁) + Patient</th>
<th>Control 2 (C₂) + Patient</th>
<th>C₁ + C₂</th>
<th>Total (a)</th>
<th>C₁ + C₂ + patient (b)</th>
<th>b/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wa</td>
<td>209 ± 34</td>
<td>134 ± 18</td>
<td>7,581 ± 535</td>
<td>7,924</td>
<td>3,323 ± 227</td>
<td>42%</td>
</tr>
<tr>
<td>F1*</td>
<td>1,451 ± 189</td>
<td>2,605 ± 580</td>
<td>46,765 ± 2,253</td>
<td>50,534</td>
<td>41,772 ± 3,073</td>
<td>83%</td>
</tr>
<tr>
<td>Br†</td>
<td>912 ± 75</td>
<td>1,950 ± 704</td>
<td>2,992 ± 556</td>
<td>5,854</td>
<td>12,015 ± 1,346</td>
<td>187%</td>
</tr>
</tbody>
</table>

Data represent cpm ± SEM for quadruplicate cultures. Control donors (C₁ and C₂) used to determine effects of cells from the 3 patients are not the same in each experiment.

*F1, lymphoblastic lymphoma patient with a high percentage of neoplastic lymphocytes in peripheral blood mononuclear cell suspension.

†Br, patient with Sezary syndrome with a high percentage of Sezary cells in peripheral MNC suspension.
with helper function. In an elegant functional analysis, Broder et al. showed that neoplastic T cells from a certain infant with acute lymphocytic leukemia (ALL) with hypogammaglobulinemia exhibited suppressor effects on normal Ig synthesis only when normal T cells were also present in the coculture. These results suggested that the ALL blasts may have represented a progenitor suppressor cell or a prosuppressor T cell. Saxon et al. have recently reported on a human neoplastic cell population that exhibited both helper and suppressor effects on B-cell Ig synthesis. The monoclonality of this cell population was expressed by a distinct chromosomal abnormality present in the neoplastic cells. The authors concluded that neoplastic transformation must have occurred in a progenitor lymphocyte capable of further differentiation into both helper and suppressor cells.

It should be emphasized that these previously reported studies of neoplastic T cells utilized primarily a single functional assay, namely PWM-stimulated Ig synthesis, whereas the Wa cells were subjected to a coculture analysis in both this system and the MLR. Since these separate cellular assays measure effector activities of distinct T-cell subsets presumably also under separate immunoregulatory control, more information concerning the putative function(s) of neoplastic T cells should be obtained using multiple in vitro functional cell systems. The results of the Wa studies summarized below demonstrate this importance of using more than one cell assay system in a functional lymphocyte analysis.

The Wa MNC fraction used in this functional analysis essentially contained only lymphoid cells, as the cells were obtained during an accelerated preterminal phase of this T-cell leukemia. The peripheral WBC was 44,000/cu mm of which 84% were lymphocytes; marker analysis of the isolated MNC fraction showed that 76% formed E-rosettes (>95% appeared malignant on smear), 13% formed EAC^+ rosettes, 10% were SIg positive, and 3% of the MNCs exhibited peroxidase positivity. On microscopic examination, one-fifth of the peroxidase-positive cells were of monocytic origin; the remainder were clearly from the granulocytic series. In view of the high percentage of malignant cells evident on peripheral smear, with some showing marked immaturity, a significant portion of the 8%-10% MNCs unaccounted for in the marker analysis may have been malignant lymphoblasts lacking in membrane receptors for typical E-rosette formation.

Although the Wa MNC fraction contained a normal percentage of B cells, no Ig was produced in PWM-stimulated culture (Table 1, Fig. 1). Considering that virtually all the T cells were malignant cells, the cohort of normal T helper cells required for B-cell Ig synthesis was presumably lacking in the Wa MNC fraction. In coculture with control MNCs, the Wa cells exhibited no suppressor effects on the control PWM-stimulated Ig production (Table 1, Fig. 1).

Although Wa cells lacked suppressor effects on normal PB lymphocyte Ig synthesis, the Wa cells significantly suppressed a MLR between two allogeneic lymphocyte populations in coculture (Table 2, Fig. 2). The Wa suppression did not require preincubation with mitogens such as Con-A for the induction of suppressor activity. Furthermore, the absence of 3H-thymidine incorporation by Wa cells in culture alone or in coculture with normal lymphocytes blocked by mitomycin suggested that active proliferation was not necessary for the expression of this suppressor activity by Wa cells (Table 3). The absence of detectable cytotoxic effects by Wa cells on the responses of normal lymphocytes to Con-A (Table 3) and in the Ig

![Graph](image_url)
synthesis of normal PB lymphocytes (Table I, Fig. 1) suggested that the MLR suppression in the 3-way coculture experiments was not due to nonspecific toxic or cytolytic effects. In addition, suppression resulting simply from an obstructive presence of inert neoplastic cells preventing cell interaction in the MLR culture was excluded by the lack of MLR interference with cells from another T-cell leukemia, patient Fl, and the augmentation of the MLR by cells of patient Br. These results indicate that the neoplastic Wa cells without prior activation were capable of suppressing the proliferative responses of allogeneic control lymphocytes reacting in the MLR but, at the same time, lacked suppressor effects on the normal helper T cells activated by PWM to induce B-cell Ig synthesis. This selective suppression exhibited by the Wa cells suggests the possibility that T cells proliferating in response to allogeneic tissue antigens in the MLR and the PWM-activated helper cells involved in Ig synthesis are controlled by separate regulator cell populations. Alternatively, MLR-responsive T cells may possess greater sensitivity to suppressor effects than the PWM-activated helper cell. Also, considering the morphological immaturity of the Wa cells at the terminal leukemic phase, the Wa clone may have lost some suppressor function during neoplastic transformation and dedifferentiation.

Lymphocytes with autologous MLR-suppressor activity can be regularly induced in man and in the mouse by prior incubation with Con-A. Recently, Reinherz and Schlossmann have reported that in man, as in the mouse, this inducible suppressor lymphocyte is a T cell and belongs to a serologically identifiable subset of T cells with suppressor function. MLR-suppressor T cells not requiring prior activation with Con-A have been identified in a multiparous female suppressor T cells not requiring prior activation with subset of T cells with suppressor function. MLR-as in the mouse, this inducible suppressor lymphocyte activity can be regularly induced in man and in the some suppressor function during neoplastic transformation and augmentation of the MLR by cells of patient Br. Reinherz and Schlossmann have reported that in man, that elicit the MLR, but also for membrane structures type. Thus, it appears that the HLA-D locus codes highly specific for the MLR-responding cell haplotype but is not specific for the stimulating haplotype but is highly specific for the MLR-responding cell haplotype. Thus, it appears that the HLA-D locus codes not only for D antigens located primarily on B cells that elicit the MLR, but also for membrane structures on MLR-responding T cells recognized by these HLA-D-specific MLR-suppressor T cells. However, recently, a patient with a recurrent bladder tumor has been identified whose peripheral T cells suppressed MLR responses of individuals lacking HLA-D compatibility with the tumor patient. Regrettably, Wa MNCs were not saved initially nor obtained later for HLA typing during the patient’s subsequent phase of rapid clinical deterioration. Thus, lacking the HLA phenotype of patient WA, evidence for or against a genetic restriction of this MLR suppression is not unobtainable. The control MNCs used in the Ig synthesis and MLR analysis were from the same donors, and the separate studies were performed at the same time. Unfortunately, the patient’s cytopenic response to chemotherapy and rapid clinical demise precluded a repeat functional analysis of Wa cells for confirmatory evidence of the MLR-suppressor activity.

Histologically, the Wa cells possessed prominent azurophilic cytoplasmic granules. A recent morphological and ultrastructural analysis of normal human T cells separated into subpopulations based on membrane Fe receptors for IgM (Tm lymphocyte) and IgG (Tc-lymphocyte) revealed azurophilic cytoplasmic granules to represent a common cytoplasmic feature of the Tc lymphocyte. Suppressor cell activity has been identified and associated with the Tc cell fraction, and preliminary evidence has suggested that the release of suppressor substances into culture supernatants by Tc cells may correlate with the loss of detectable cytoplasmic granules. In this connection, the Wa cells demonstrate the parallel findings of prominent cytoplasmic granules and suppressor cell function.

The cytogenetic abnormalities of the Wa cells were numerous and complex. These findings are summarized and interpreted in an accompanying report. Importantly however, the Wa karotype bears some similarity to cytogenetic abnormalities noted in the T-cell helper-suppressor leukemia of a patient with ataxia telangiectasia reported by Saxon and coworkers. Certainly, more examples of combined functional and cytogenetic analyses will be required before possible correlations between karyotypic abnormalities and effector cellular functions of neoplastic lymphocytes can be discerned.

This functional analysis of the Wa cells illustrates the importance of testing lymphocyte function with multiple in vitro assay systems. The Wa cells lacked suppression in an analysis of PWM-stimulated Ig synthesis, but exhibited suppressor effects in the MLR. The MLR can often be used in a functional analysis of neoplastic T cells, since the stimulating cell antigens controlled by the HLA-D locus are primarily restricted to B cells and macrophages. Thus, the neoplastic T cells lacking HLA-D surface determinants provoke little stimulation in the 3-way MLR used in a test analysis. The use of multiple assay systems for testing B- and T-cell effector functions should lead to more frequent recognition of T-cell...
activity expressed by the neoplastic lymphocytes of certain patients with T-cell leukemias and lymphomas. Moreover, through a combined multidisciplinary approach that includes morphological, cytogenetic, and immunologic functional studies, a greater understanding of the various immunopathologic subtypes of T-cell leukemia and lymphoma should be achieved.

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Human T-cell lymphoma with suppressor effects on the mixed lymphocyte reaction (MLR). II. Functional in vitro lymphocyte analysis

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