Patients with non-Hodgkin’s B-cell lymphoma who received an antitumor vaccine of idiotypic Ig protein showed humoral and proliferative immune responses. Because immunity to some antigens, including tumor antigens and human pathogenic viruses, may be better correlated with the cytolytic cellular immune response, we evaluated 16 non-Hodgkin’s lymphoma patients immunized with autologous idiotypic Ig molecules for changes in tumor-specific cytotoxic T-lymphocyte precursor (CTLp) frequency using limiting dilution analysis. Eleven patients had a significant increase in tumor-specific CTLp. Eight of these 11 patients remain without evidence of disease or with stable minimal disease. In contrast, all five patients who did not have a significant change in tumor-specific CTLp have developed progressive disease. Patient vaccination with tumor-associated protein antigens can increase tumor-specific CTLp frequencies. The correlation of increased tumor-specific CTLp with freedom from progression is significant at P = .002. This study indicates that measurement of CTLp frequencies is relevant to the clinical evaluation of human tumor vaccines and suggests that cell-mediated cytolytic immune responses may be an important determinant of vaccine efficacy.

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MATERIALS AND METHODS

Patients. Patients diagnosed with follicular B-cell non-Hodgkin’s lymphoma were able to participate. All cases were reviewed by the Stanford Pathology Department and were classified according to the Working Formulation.7,8 Tumor cells were used in the development of idiotype-specific protein and were cryogenically stored as described.8 Chemotherapy was chosen by the referring physician. Patients were vaccinated after achieving a complete remission or minimal disease state. The vaccine consisted of 0.5 mg of idiotypic protein conjugated to 0.5 mg of keyhole limpet hemocyanin and was emulsified in an immunologic adjuvant containing 0.4 mg of threonyl-muramyl dipeptide for each treatment. This dose was divided between two subcutaneous sites. Each patient received a series of five immunizations beginning at least 2 months after completion of chemotherapy. Immunizations were administered on day 1 and week 2, 6, 10, and 20. The results described here are derived from the subset of patients in the trial for whom all of the necessary samples, including tumor cells, prevaccine, and multiple postvaccination peripheral blood lymphocytes (PBL), were available. This subset of patients was not otherwise selected. Patient identification numbers were assigned in chronological order based on date of enrollment.

Patient samples and cell lines. PBL were isolated by centrifugation of anticoagulated whole blood over lymphocyte separation medium (Organon Teknika, Durham, NC), suspended in 10% dimethyl sulfoxide (Mallincrodt Specialty Chemicals, Paris, KY) in heat-inactivated fetal calf serum (FCS; Hyclone, Ogden, UT), and cryopreserved in the vapor phase of liquid nitrogen until the time of the assay. Samples were obtained prevaccine, during the vaccination schedule, and at each subsequent follow-up visit for periods ranging from 6 to 18 months. Tumor specimens were obtained from excisional biopsies and handled as described.9 Autologous Epstein-Barr virus transformed lymphoblastoid cell lines (EBV-LCL) were established from PBL as described.9,10 K562 (ATCC CCL243), an erythroleukemia cell line lacking HLA class I and II antigen expression,
was used as a target for natural killer activity. Long-term CTL lines were established as described13 modified only by the use of irradiated, cryopreserved, tumor cells that had been in culture for less than 10 days in place of irradiated tumor cell lines as stimulators. All non-CTL lines and limiting dilution assays were maintained in RPMI 1640 (ICN Biomedicals Inc, Costa Mesa, CA) supplemented with 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Irvington Scientific, Santa Ana, CA). CTL lines were maintained in the media described above with the addition of 10% T-lymocyte–conditioned media.29

**Determination of cytotoxic T-lymphocyte precursor (CTLp) frequency.** Tumor specimens were thawed, placed into culture, and evaluated on days 3 through 7 post-thaw for 51Cr labeling as described.30 For each patient, an aliquot of each frozen PBL sample was thawed and placed into limiting dilution culture in 96-well plates at concentrations from 10,000 to 500,000 PBL/well with 5,000 irradiated (10,000 cGy) autologous tumor stimulators as described.31 After 6 days of culture, each well was assayed for cytolytic activity against 51Cr-labeled autologous tumor and control targets in a 4-hour chromium release assay.32 Autologous EBV-LCL, K562, or an isotype and histology matched unrelated non-Hodgkin’s lymphoma were used as control targets. Wells in which lysis of targets was greater than or equal to 10% above spontaneous release were considered positive. The CTLp frequency was calculated by the technique of Fazekas de St Groth.33 When sufficient prevaccine PBL were available, time points were divided into two individual assays.

**Monoclonal antibodies and fluorescence-activated cell sorter (FACS) analysis.** The following monoclonal antibodies were used in these studies: OKT3 and OKT8 (Ortho, Raritan, NJ); anti-CD4, anti–T-10R-1, anti-TCR α/β, (Becton Dickinson, Oxnard, CA); anti-CD11a, anti-CD18 (LFA-1) (TS1.18, TS1.22)34; anti-major histocompatibility complex (MHC) class I (PA2.6), anti-MHC class II (L243),35 anti-human Ig, anti-κ or -γ light chain; fluorescein isothiocyanate–conjugated F(ab)2 goat antimouse whole Ig; and isotype matched irrelevant mouse Ig (Caltag, San Francisco, CA). Cell surface expression of molecules binding these antibodies was evaluated on FACSscan (Becton Dickinson).

**Cell specificity panels and antibody inhibition assays.** Standard 4-hour chromium release assays were performed using long-term CTL lines as effectors.36 Target cells included autologous lymphoma, irrelevant lymphoma, autologous EBV-LCL, and K562. Specific lysis was calculated as [experimental release – spontaneous release/total release – spontaneous release] × 100. Antibody inhibition assays were performed with the antibody present throughout the standard CTL assay, and percent inhibition was determined as follows: 1 – (experimental specific cytolysis/control specific cytolysis) × 100.40

**Statistical evaluation.** Patient assays were performed in a blinded fashion with respect to clinical response and course. CTL frequencies for each time point were calculated by the technique of Fazekas de St Groth34 and evaluated using the x² test for fit to the Poisson distribution.41 Changes in CTLp frequency were considered significant only if 95% confidence intervals were nonoverlapping. Correlation of clinical course and maximal change in CTLp frequency was performed with CTLp as a dichotomous variable using the Wilcoxon Test.42 Nondichotomous variables were evaluated by the Wilcoxon Test.43 These statistical tests were performed using 4th Dimension v1.06 (ACI US Inc, Cupertino, CA) and Instat v2.0 (Graph Pad Software, San Diego, CA) on Apple Macintosh computers. Kaplan-Meier plots44 were developed using institutional software kindly provided by S Brain (Stanford University, Stanford, CA) with statistical significance derived by log-rank evaluation.45

**RESULTS**

**Patient characteristics.** Clinical and demographic characteristics of the 16 patients are summarized in Table 1. These patients are representative of the population of non-Hodgkin’s lymphoma patients enrolled in our clinical trial. There are six men and 10 women ranging from 23 to 59 years of age at the time of diagnosis. Patient 24 had stage IIA disease, and all remaining patients had either stage III or IV A disease. Eleven patients were in a clinical complete response, and 5 patients had minimal disease present at the time of vaccination. Treatment regimens were diverse (Table 1), and most of the patients received standard regimens for the treatment of low and intermediate grade non-Hodgkin’s lymphomas. Two patients (nos. 27 and 19) underwent transformation to intermediate grade histology that was effectively treated before vaccination. One patient (no. 27) had received antiidiotypic monoclonal antibody therapy; this patient and patient (19) underwent autologous bone marrow transplantation. The duration of disease ranged between 1 and 12.6 years, and time off therapy before initiation of vaccine therapy spanned 2 to 18 months. Seven patients remain in clinical complete response as of their most recent follow-up visit; one patient has stable disease; eight patients progressed, and one of those has died from disease. The correlation of clinical and demographic characteristics with freedom from progression are summarized in Table 2.

**CTLp frequencies.** CTLp frequencies were determined using each individual’s tumor and a control tumor or cell line as targets. Prevaccine values, maximal response, 95% confidence intervals, magnitude of response over prevaccine values, and time to maximal response for both tumor and control targets are summarized in Table 3. Prevaccine antitumor CTLp frequencies range from 0.3/10⁶ to 2.1/10⁶ PBL. Control targets consisted of autologous EBV-LCL for five patients; isotype, light chain, and histology matched unrelated tumor controls for two patients, and K562 (NK target) for nine patients. Neither patient with matched tumor controls demonstrated cytolytic activity to the unrelated tumor targets. Limited responses were evident with autologous EBV-LCL controls. The initial EBV-LCL CTLp response of patient 14 was the maximal value detected but was undetectable until week 6, and subsequent CTLp frequency ranged from 0.1/10⁶ to 0.3/10⁶ PBL. Patient 21 had an increase in autologous EBV-LCL CTLp frequency from undetectable in early samples to 0.1/10⁶ PBL at 11 weeks and reached a maximum at 54 weeks of 4.1/10⁶ PBL. The kinetics of this response were dramatically different from the antitumor response that reached its maximum at 8 weeks (Table 3). These two EBV-LCL responses were discordant with the antitumor CTLp responses. In contrast, patient 26 had a discordant increase in autologous EBV-LCL CTLp, providing evidence for lack of a tumor-specific CTLp response. Patient 25 demonstrated a discordant antitumor CTLp response and a cytolytic precursor response to K562, suggesting that the antitumor CTLp was due to natural killer cell activity. Patient 34, although demonstrating a 5.3-fold increase in tumor-specific CTLp, displayed overlapping 95% confidence intervals. This response was, therefore, considered not significant. The magnitude of the tumor-specific CTLp response with respect to disease status is plotted in Fig 1. For subsequent statistical evaluation, we established a threshold value of fivefold or greater increase in tumor-
Table 1. Characteristics of Patients Undergoing Idiotype Ig Vaccination for B-Cell Non-Hodgkin’s Lymphoma Who Were Evaluated for Antitumor Cytotoxic Responses

<table>
<thead>
<tr>
<th>Patient ID No.</th>
<th>Gender</th>
<th>Age at Diagnosis (yr)*</th>
<th>Histology</th>
<th>Idiotype Ig</th>
<th>Stage</th>
<th>Duration of Disease (mo)</th>
<th>Disease Status at Vaccination</th>
<th>Previous Treatment</th>
<th>Time Off Therapy to Vaccination (mo)</th>
<th>Disease Status at Last Follow-Up</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>F</td>
<td>42</td>
<td>FSC to FM</td>
<td>IgMα</td>
<td>IVA</td>
<td>26</td>
<td>NED</td>
<td>CVP x 10</td>
<td>12</td>
<td>NED</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>36</td>
<td>FSC to F  and DLC</td>
<td>IgMα</td>
<td>IVA</td>
<td>152</td>
<td>NED</td>
<td>CVP x 7, CHOP x 3</td>
<td>4</td>
<td>NED</td>
</tr>
<tr>
<td>22</td>
<td>F</td>
<td>42</td>
<td>FSC</td>
<td>IgMα</td>
<td>IVA</td>
<td>29</td>
<td>NED</td>
<td>CVP x 7</td>
<td>18</td>
<td>NED</td>
</tr>
<tr>
<td>27</td>
<td>F</td>
<td>23</td>
<td>FSC to F  and DLC</td>
<td>IgMα</td>
<td>IVA</td>
<td>65</td>
<td>NED</td>
<td>CVP x 8, splenectomy</td>
<td>Vaccination initiated before ABMT, 0</td>
<td>NED</td>
</tr>
<tr>
<td>30</td>
<td>F</td>
<td>45</td>
<td>FSC</td>
<td>IgGα</td>
<td>IIIA</td>
<td>28</td>
<td>NED</td>
<td>CVP x 9</td>
<td>2.5</td>
<td>NED</td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>25</td>
<td>FSC to FM</td>
<td>IgGα</td>
<td>IIIA</td>
<td>15</td>
<td>NED</td>
<td>Fludarabine x 9</td>
<td>3.5</td>
<td>NED</td>
</tr>
<tr>
<td>33</td>
<td>M</td>
<td>36</td>
<td>FSC to F  and DLC</td>
<td>IgMα</td>
<td>IIIA</td>
<td>13</td>
<td>NED</td>
<td>pC-P x 3, CVP x 2, pChl x 2</td>
<td>2.5</td>
<td>NED</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>43</td>
<td>FSC to FM</td>
<td>IgMα</td>
<td>IIIA</td>
<td>88</td>
<td>Disease present × 8 mo, pC-P x 6, pP x 1,</td>
<td>pC-P x 2</td>
<td>3</td>
<td>Stable disease</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>59</td>
<td>FM</td>
<td>IgMα</td>
<td>IIIA</td>
<td>21</td>
<td>NED</td>
<td>pC-P x 8</td>
<td>10</td>
<td>Progression</td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>32</td>
<td>FSC</td>
<td>IgMα</td>
<td>IVA</td>
<td>29</td>
<td>NED</td>
<td>pC-P x 8</td>
<td>6</td>
<td>Progression</td>
</tr>
<tr>
<td>28</td>
<td>F</td>
<td>33</td>
<td>FSC</td>
<td>IgMα</td>
<td>IVA</td>
<td>13</td>
<td>NED</td>
<td>CVP x 8</td>
<td>3</td>
<td>Progression</td>
</tr>
<tr>
<td>34</td>
<td>F</td>
<td>40</td>
<td>FSC to FM</td>
<td>IgGα</td>
<td>IIIA</td>
<td>16</td>
<td>Disease present</td>
<td>CHOP x 6</td>
<td>15</td>
<td>Progression</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>43</td>
<td>FM</td>
<td>IgGα</td>
<td>IVA</td>
<td>19</td>
<td>Disease present</td>
<td>CVP x 10</td>
<td>2</td>
<td>Progression</td>
</tr>
<tr>
<td>29</td>
<td>M</td>
<td>53</td>
<td>FSC</td>
<td>IgMα</td>
<td>IVA</td>
<td>10</td>
<td>Disease present</td>
<td>CVP x 10</td>
<td>4</td>
<td>Progression</td>
</tr>
<tr>
<td>25</td>
<td>M</td>
<td>33</td>
<td>FSC</td>
<td>IgGα</td>
<td>IVA</td>
<td>15</td>
<td>Disease present</td>
<td>CVP x 10</td>
<td>4</td>
<td>Progression</td>
</tr>
<tr>
<td>26</td>
<td>F</td>
<td>38</td>
<td>FSC</td>
<td>IgMα</td>
<td>IVA</td>
<td>53</td>
<td>Disease present</td>
<td>pC-P x 12</td>
<td>6</td>
<td>Progression</td>
</tr>
</tbody>
</table>

Abbreviations: FSC, follicular small cleaved cell lymphoma; FLc, follicular large cell lymphoma; FM, follicular mixed small and large cell lymphoma; DLC, diffuse large cell lymphoma; NED, no evidence of disease; CVP, cyclophosphamide, vincristine, prednisone; CHOP, cyclophosphamide, adriamycin, vincristine, prednisone; BCEPP, bleomycin, cyclophosphamide, VP-16, procarbazine, prednisone; ABMT, autologous bone marrow transplantation; BAEP, bleomycin, adriamycin, VP-16, prednisone; DHAP, cisplatin, Ara-C, dexamethasone; pC-P, chlorambucil and prednisone (5-day pulse); pP, chlorambucil (5-day pulse); pChl, chlorambucil daily; AWD, alive with disease; DOD, dead of disease.

Table 2. Characteristics of Patients According to Their Clinical Response to Antitumor Vaccination for B-Cell Non-Hodgkin’s Lymphoma

<table>
<thead>
<tr>
<th>Tumor-specific CTLp response (&gt;5x increase/ &lt;5x increase)</th>
<th>Sustained Clinical Response (n = 8)</th>
<th>Progressive Disease (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease status at vaccination (present/absent)</td>
<td>8/0</td>
<td>3/5</td>
<td>.0256</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>3/5</td>
<td>3/5</td>
<td>1.0000</td>
</tr>
<tr>
<td>Age at diagnosis (yr)*</td>
<td>36.50 ± 8.37</td>
<td>41.38 ± 9.92</td>
<td>.6454†</td>
</tr>
<tr>
<td>Duration of disease before vaccination (mo)*</td>
<td>52.1 ± 47.9</td>
<td>22.0 ± 13.78</td>
<td>.1048†</td>
</tr>
<tr>
<td>Time off treatment before vaccination (mo)*</td>
<td>5.69 ± 6.06</td>
<td>6.13 ± 4.39</td>
<td>.5054†</td>
</tr>
<tr>
<td>Stage (&lt;I/IV)</td>
<td>4/4</td>
<td>2/6</td>
<td>.6084</td>
</tr>
<tr>
<td>Idiotype Ig</td>
<td>2/6</td>
<td>3/5</td>
<td>1.0000</td>
</tr>
<tr>
<td>Light chain (κ/λ)</td>
<td>4/4</td>
<td>2/6</td>
<td>.6084</td>
</tr>
</tbody>
</table>

P values for frequency counts derived by two-tailed Fisher Exact test; others (1) by two-tailed, Wilcoxon rank sum Test. Abbreviations used are identical to those of Table 1.

* Values are means ± SD.

Specific CTLp frequency over prevaccine values as a significant change.

A greater than fivefold increase in tumor-specific CTLp correlates with the likelihood of progression free survival as a dichotomous variable with P = .0256 (Table 2). Kaplan-Meier analysis demonstrated a significant (P = .002) correlation of freedom from progression with an increased tumor-specific CTLp response (Fig 2). Exclusion of the two patients treated with bone marrow transplantation or inclusion of all tumor-specific responding patients in the Kaplan-Meier analysis maintained statistical significance. Individual patients demonstrated different patterns of CTLp responses. Two patients (nos. 22 and 32) showed steady increases in the tumor-specific CTLp well beyond the termination of the vaccine procedure (Fig 3B, C, and D). The nonspecific response for patient 25 (Fig 3B and C) and the insignificant response of patient 34 are also shown (Fig 3D).

Generation of CTL lines. Cytolytic T-cell lines were established and sustained in culture for periods ranging from 24 to 36 weeks from patients 14, 19, 21, 22, and 24. All of these lines were tumor-specific; cytolytic (Fig 4); and CD3+.
CD8+, CD4+, α/β T-cell receptor (TCR)+ by FACS analysis (Fig 5). Recognition of tumor was MHC class I restricted as demonstrated by antibody inhibition in standard chromium release assays (Fig 6). In no case were the CTL MHC unrestricted or γ/δ TCR+.25,46

**DISCUSSION**

Although vaccination is a proven approach for prophylaxis against numerous infectious agents,47 its utility for tumor immunotherapy is still under investigation. By analogy with viral vaccines, interest in tumor vaccines first focused on the induction of neutralizing antibodies.48-51 Antitumor humoral and delayed-type hypersensitivity (DTH) responses have been shown to correlate with protection to a subsequent tumor challenge in animal models4,5,15,21 and with responses to established tumors.5,53 Although humoral, proliferative, and DTH responses have been evaluated in many clinical studies,6,8,13,52-61 these responses have rarely been correlated with clinical outcome in human trials.8,53-56 For tumors,14,15 as for certain infectious agents,16,62-69 it appears that cell-mediated cytotoxicity may be more relevant than humoral responses.12,13 Antibodies recognize antigens in the form of whole molecules, whereas T cells typically recognize antigens in the form of small peptides combined with MHC molecules. This understanding of antigen presentation has aided the recent identification of a number of tumor associated antigens (MAGE, MART, Melan-A, Tyrosinase, gp75, MUC-1, p53, p21WAF1, Her-2/neu) that are recognized by CTL.25,18,70-73

Recently, several studies have suggested that providing in vivo exogenous antigen by vaccination can elicit antigen-specific CTL.12,13,59-61,74-76 Because CTL are important in tumor rejection in numerous animal models,12,13,21,22 it seemed likely that induction of CTL responses in vivo may be an important determinant for the efficacy of human tumor vaccines.

The B-cell non-Hodgkin’s lymphomas represent a particularly attractive target for vaccine therapy because each tumor expresses specific determinants encoded by variable region sequences of the rearranged Ig gene. However, the indolent natural history of low-grade non-Hodgkin’s lymphomas along with the well-documented capacity for spontaneous

<table>
<thead>
<tr>
<th>Table 3. Patient Prevaccine, Baseline, and Maximal Cytotoxic Precursor Frequencies</th>
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<tr>
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</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>19</td>
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<tr>
<td>16</td>
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<tr>
<td>21</td>
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</tbody>
</table>

Values in parentheses are upper and lower 95% confidence interval. Values for CTLp in K562 control assays represent NKp frequencies rather than cytotoxic T-lymphocytes precursor frequencies.

Abbreviation: NS, not statistically significant.

**Fig 1.** Distribution of change, from prevaccine values, in tumor-specific CTLp frequency for patients with progression-free survival (stable minimal disease or no evidence of disease) or progressive disease. (•) Patient 34, who did not have significant changes in CD8+ cytotoxic precursors. (□) Patients 25 and 26, who did not have a tumor-specific responses.
regression\textsuperscript{7} require large numbers of patients with extensive follow-up for definitive conclusions regarding efficacy of any immunotherapy. Immunologic responses are candidate surrogate end points that can guide development of immunotherapies. We have previously documented that patients with B-cell lymphoma can be induced to make humoral and proliferative responses to the idiotype on their tumor.\textsuperscript{5,7} We recently reported that such immune responses were strongly correlated with freedom from progression of disease.\textsuperscript{8} The present report shows that CTL precursors are also induced by vaccination and they too show a strong correlation with freedom from progression. The significant correlation of an increase in tumor-specific CTLp and freedom from progression reported here provides additional evidence that vaccination with protein antigens can induce CTL immune responses\textsuperscript{15,59-61,69,74-76} and supports the role of CTL in the antitumor immune response.

Vaccination with idiotypic Ig and immunologic adjuvant attempts to induce a response to a "self-antigen." This immunization may elicit highly specific immune responses or nonspecific responses, including natural killer activity, shared B-cell reactivity, or unexpected tumor cell reactivity. Therefore, several target cell controls were possible for these studies with each one evaluating different aspects of immune reactivity. Limited by the numbers of PBL for each patient at each time point, we elected to test only one "alternative" (control) target cell for each patient. Selection of the most appropriate control target cell is not obvious and was performed randomly. We included natural killer cell targets (K562) (9 patients); autologous nontumor B cells (EBV-LCL) (5 patients); and unrelated, histology, and isotype matched non-Hodgkin's lymphomas (2 patients). The response to EBV-LCL is complicated by the endogenous anti-EBV cytolytic immune response (both CTL and natural killer),\textsuperscript{78} which varies with reactivation status of latent EBV infection that is present in the vast majority of adults in this country. Unrelated but histology and isotype matched lymphoma targets may lack critical HLA alleles or express different populations of accessory molecules. We were, as expected, able to demonstrate natural killer (3 patients), EBV-LCL cytolytic (3 patients), and tumor (15 patients) reactivity in patients undergoing this vaccination procedure. However, only increases in tumor-specific responses correlate with freedom from progression.

Therefore, several target cell controls were possible for these studies with each one evaluating different aspects of immune reactivity. Limited by the numbers of PBL for each patient at each time point, we elected to test only one "alternative" (control) target cell for each patient. Selection of the most appropriate control target cell is not obvious and was performed randomly. We included natural killer cell targets (K562) (9 patients); autologous nontumor B cells (EBV-LCL) (5 patients); and unrelated, histology, and isotype matched non-Hodgkin's lymphomas (2 patients). The response to EBV-LCL is complicated by the endogenous anti-EBV cytolytic immune response (both CTL and natural killer),\textsuperscript{78} which varies with reactivation status of latent EBV infection that is present in the vast majority of adults in this country. Unrelated but histology and isotype matched lymphoma targets may lack critical HLA alleles or express different populations of accessory molecules. We were, as expected, able to demonstrate natural killer (3 patients), EBV-LCL cytolytic (3 patients), and tumor (15 patients) reactivity in patients undergoing this vaccination procedure. However, only increases in tumor-specific responses correlate with freedom from progression.

**Fig 2.** Kaplan-Meier plots and log rank P value for freedom from progression v time from initiation of vaccination schedule in patients with greater than fivefold or less than or equal to fivefold increase in tumor-specific CTLp. Individual tick marks represent censored observations, i.e., patients with progression-free survival.
Fig 4. Standard 4-hour $^{51}$Cr release assay of long-term, autologous tumor, CTL line from patient 21, with (□) autologous tumor, (◆) autologous EBV-LCL, and (■) K562 targets.

The patterns of responses seen in this set of patients is of significant interest. Our calculated CTLp frequencies may be an underestimation of the true frequency because we elected not to add T-cell growth factors to the limiting dilution cultures to minimize the generation and amplification of nonspecific (natural killer) cytolytic activity. Nevertheless, patient 25 demonstrated natural killer and antitumor activity similar to that reported by other groups. Of note, this patient developed progressive disease as did patient 26, who also had a nonspecific increase in cytolytic activity. The absence of cytolytic activity toward the two irrelevant matched tumor controls (patients 15 and 16) and the majority of autologous EBV-LCLs suggests that the tumor-specific responses measured are tumor antigen specific. This conclusion is supported by the tumor specificity of the long-term CTL lines established from five of these patients along with previously reported long-term CTL lines. It is of note that patients 22 and 32 have sustained increases in tumor-specific CTLp and remain free from progressive disease. Khazaie et al suggest that sustained antitumor immune responses may be due to "dormant" tumor cells that provide continued antigenic stimulation. The long-term maintenance of antitumor immune responses in these two patients, as demonstrated by persistent elevations of tumor-specific CTLp, may be a result of such low-grade antigenic stimulation due to minute and undetectable tumor. The persistent immune response may, in turn, maintain the tumor load at an undetect-
Mean Fluorescence

Fig 5. FACS plots of a long-term CTL line established from patient 22. Thin lines in both panels represent irrelevant antibody, E12 (endothelial cell specific), negative control. Experimental plots are labeled adjacent to the apex of each respective plot.

Figure 6. Antibody inhibition of tumor cytolysis by a long-term tumor-specific CTL at an effector to target ratio of 15:1 in a 4-hour 51Cr release assay.

Antibodies

Fig 6. Antibody inhibition of tumor cytolysis by a long-term tumor-specific CTL at an effector to target ratio of 15:1 in a 4-hour 51Cr release assay.

able level. The growth or initial development of a tumor from a neoplastic clone may well represent disruption of this delicate balance.

Limiting dilution analyses to evaluate CTL precursor frequencies have been used extensively in the study of antiviral and other cell-mediated immune responses. Although CTLp frequencies have been measured less often in the study of human tumor responses, in large part due to the very low frequency of autologous antitumor CTLp, several reports are of note. Coulie et al expanded graded numbers of PBL in the presence of autologous tumor, IL-4, and IL-2 for approximately 4 weeks before performing limiting dilution cytolytic analysis and used cold target inhibition with K562 to quench natural killer activity. Using this technique of expansion, they calculated tumor-specific CTLp frequencies comparable with allogeneic CTLp frequencies in seven patients but did not report clinical correlation. Mazocchi et al performed similar experiments expanding PBL for several weeks before examining cytolytic precursors for both CTL and natural killer cells in peripheral blood and tumor invaded lymph nodes. They showed that there was no difference in CTLp frequency between PBL and tumor-invaded lymph nodes in six of nine patients, a higher CTLp frequency in tumor-invaded lymph nodes in two of nine patients, and higher CTLp frequency in PBL in one of nine patients. This group did not report clinical correlation with these CTLp frequencies. It is not possible to compare the numerical values for CTLp frequencies reported in our study, using traditional limiting dilution analysis on PBL, with those of Coulie or Mazocchi given the expansion phases used by both. Additionally, we previously showed for non-Hodgkin's lymphomas that tumor-specific CTL can be generated within a 4-week interval from autologous or naive donor peripheral blood mononuclear cells. Mitchell et al evaluated the induction of CTL precursors that recognize allogeneic tumor cells, the source of the antimelanoma vaccine, in a fashion similar to that which we used. They demonstrated a correlation of these CTL precursors with patient's clinical course.
and showed that, in some cases, established allogeneic CTL lines could kill autologous melanoma cells. Our study, however, is the first human study to both directly evaluate changes in autologous tumor-specific CTLp frequency induced during an immunomodulatory therapy and correlate these changes with patient’s clinical course.

We determined that idiotypic Ig vaccination can elicit changes in tumor-specific CTLp frequency and that these changes show a significant correlation with clinical course. Our study suggests that evaluation of changes in the CTL compartment can be important in the assessment of patients undergoing immunomodulatory therapies. Such evaluation may provide insight into the nature of tumor-associated antigens and the relative efficacy of various vaccines. Our findings suggest that new vaccine strategies, including cytokine-augmented cellular, protein, peptide, and/or polynucleotide (naked DNA) vaccination, which optimize the development of antigen-specific CTL responses, may result in improved clinical outcomes.

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