Induction of Immunoglobulin Secretion in Follicular Non-Hodgkin's Lymphomas: Role of Immunoregulatory T Cells

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B cell neoplasms are clonal expansions of B lymphocytes thought to be frozen at various points along the normal B cell differentiation pathway. We studied cell suspensions from lymph nodes involved by follicular (nodular) non-Hodgkin’s lymphoma to determine the capacity of the malignant B cells to secrete immunoglobulin (Ig). Neoplastic B cells from all 14 follicular lymphomas secreted monoclonal immunoglobulin in culture when appropriate signals were provided. In most cases, maximal Ig secretion occurred when autologous T cells were removed by E rosette depletion, replaced with allogeneic normal T cells, and the cultures were exposed to 12-O-tetradecanoylphorbol-13-acetate. Autologous T cells exerted a suppressor effect on Ig secretion in 8/8 cases studied, diminishing the response of the malignant B cells to allogeneic T cells. This suppressor effect did not correlate with the percentage of cells staining with anti-Leu-2a or with “helper-suppressor” (Leu-3a-Leu-2a) ratios of the lymph node T cells. Our findings demonstrate that the arrested differentiation of most follicular lymphomas is reversible and implicates T cell-mediated host immunoregulatory mechanism affecting Ig secretion in vivo. An additional contribution of our results is the demonstration of a cell culture system for synthesis of sufficient monoclonal Ig for use as an immunogen in production of anti-idiotypic antibodies.

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NEOPLASMS of B lymphocytes are thought to represent proliferations of single clones of lymphocytes frozen at various points along the normal pathway of B lymphocyte differentiation. The cause of this differentiation arrest could be an intrinsic defect of the malignant B cell itself, the effect of abnormal host immunoregulatory factors, or possibly both. The malignant cells in multiple myeloma and Waldenström’s macroglobulinemia spontaneously secrete abundant monoclonal immunoglobulin (Ig), functionally mirroring normal terminally differentiated B (plasma) cells. The malignant cells of most B cell non-Hodgkin’s malignant lymphomas appear to be less mature, as they synthesize Ig and incorporate it into their cell surface membranes but do not secrete a monoclonal serum protein in vivo at levels sufficient for detection by routine serum protein electrophoresis. Monoclonal serum proteins are detectable in only 0.6% of patients with follicular (nodular) lymphomas, an incidence similar to that in the normal population. It has been reported that malignant B cells from most patients with chronic lymphocytic leukemia (CLL) export a small amount of monoclonal Ig in vivo when more sensitive and specific methods than routine serum protein electrophoresis are used for detection. Spontaneous secretion of small amounts of monoclonal Ig by cells placed in culture has also been reported in follicular non-Hodgkin’s lymphomas.

To determine whether malignant B cells derived from the “nonsecretory” B cell neoplasms were intrinsically capable of further differentiation, many laboratories have attempted to induce synthesis and secretion of Ig by the neoplastic B cells in vitro. It appears that some neoplastic B cell clones do retain the ability to differentiate when appropriate external signals are provided. Induction of differentiation has been demonstrated in precursor B (acute lymphoblastic) leukemia cells after exposure to 12-O-tetradecanoylphorbol 13-acetate (TPA), a phorbol ester known to promote in vitro differentiation of other types of human leukemic cells. Mitogen-induced differentiation, usually requiring the addition of normal allogeneic T cells, has been accomplished in vitro in isolated cases of B-CLL. In vitro differentiation and enhancement of Ig production has also been reported after exposure of B-CLL cells to TPA. In vitro induction of differentiation and Ig secretion in a series of cases of B-CLL when the cells were treated with TPA.

The purpose of this study was to determine if follicular (nodular) non-Hodgkin’s B cell lymphomas retained the capacity to differentiate and secrete Ig and to identify the external signals needed to induce Ig secretion. We found that in all follicular lymphomas studied, the malignant B cells could secrete monoclonal Ig in vitro culture. In most of the cases studied, the induction of maximal Ig secretion required the removal of the autologous T cells present in the lymph node biopsy specimens, provision of allogeneic normal peripheral blood T cells, and exposure of the cultures to TPA. The in vitro culture systems described can consistently generate sufficient idiotypic Ig for use as an immunogen in the ultimate production of anti-idiotypic antibodies. These methods can therefore serve as an alternative strategy to previous, more cumbersome approaches for rescue of idiotypic protein synthesized by malignant lymphoma B cells, such as limited cytosol papain digestion and hybridization of human B lymphoma cells to murine myeloma cells to obtain an idiotype-secreting hybridoma.

MATERIALS AND METHODS

Cells and cultures. Cells from biopsies of involved lymph nodes from 14 patients with a diagnosis of follicular (nodular) lymphoma were studied, including five with small cleaved cell type, seven with mixed small cleaved and large cell type, and two with large cell lymphoma, as classified according to the National Cancer Institute Working Formulation. In none of the patients studied was a monoclonal serum Ig detectable by routine serum protein electrophoresis. Cell suspensions were prepared from lymph nodes, viable frozen in a programmed cell freezer, and stored in a liquid nitrogen freezer, as previously described. In all lymph node cell suspensions selected for study, the B cell population was composed almost

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exclusively of neoplastic B cells, as determined by immunostaining procedures (described later). All cultures contained a B cell concentration of 10^6 cells per milliliter in RPMI 1640 (GIBCO, Grand Island, NY) with 10% fetal calf serum. The allogeneic normal T cells were isolated by an E rosette separation procedure (see later) from the peripheral blood leukapheresis specimen of a single normal donor and were chosen because they had previously been found to provide help for Ig secretion by normal peripheral blood B cells cultured in the presence of 1.6 x 10^-4 mol/L TPA (Sigma Chemical Co, St Louis) (data not shown). The T cells were also viably frozen and stored in a liquid nitrogen freezer before use. All cultures were placed in a humidified 37°C incubator with 5% CO₂ for seven days.

Preparation of purified T and B lymphocytes. Populations enriched for T and B lymphocytes were prepared by a method based on the formation of rosettes by T cells with sheep erythrocytes treated with 2-aminoethylisothiouronium bromide hydrobromide (AET; Sigma). Cell suspensions from lymph nodes involved by follicular lymphoma, and peripheral blood mononuclear cells from the normal donor, were adjusted to a concentration of 2.5 x 10^6 cells per milliliter and mixed with an equal volume of 5% AET-treated sheep erythrocytes. The mixture was centrifuged for ten minutes at 400 g and maintained on ice for one hour. After rosette formation, the cell pellets were gently resuspended and centrifuged over a Ficoll-Hypaque gradient at 400 g for 30 minutes, to separate the rosetted T cells from the nonrosetted cells. The T cells were recovered from the pellet after removal of the sheep erythrocytes by ammonium chloride lysis on ice for five minutes. The allogeneic normal T cell preparation contained 97% T cells and less than 1% B cells by immunostaining and fluorescence-activated cell sorter (FACS) analysis (described later) after one E rosette depletion procedure. The follicular lymphoma B cell preparations, from the interface, contained 2% or fewer T cells. Monocytes were not removed and constituted 1% to 15% of the interface cells by alpha-naphthyl-butyrate esterase staining. The T cell preparations from lymph nodes containing follicular lymphoma were composed of 80% to 90% T cells and a small number of neoplastic B cells after two E rosette separations. The small number of cells obtained from lymph node biopsies limited the performance of additional purification procedures on the T cell fractions. Viabilities of the neoplastic B cell fractions generally exceeded 75%.

Immunostaining. Surface Ig isotypes were identified by staining with monoclonal anti-immunoglobulin antibodies against gamma, alpha, delta, kappa, and lambda chains (Becton Dickinson Monoclonal Antibodies, Mountain View, Calif) and μ chain (Bethesda Research Laboratories, Gaithersburg, Md), and fluorescein-conjugated, chain-specific, affinity-purified F(ab')2 fragments of goat IgG anti-human gamma, μ, delta, alpha, kappa, and lambda chains (Tago, Burlingame, Calif). Immunofluorescence staining was determined on a FACS II (Becton Dickinson, Sunnyvale, Calif), as previously described. The composition of the unfractionated cell suspensions and the purified B and T cell preparations was determined by staining with the following antibodies: Lyt 3, an antibody that recognizes the sheep erythrocyte receptor (New England Nuclear, Boston) anti-Leu-3a and anti-Leu-2a, antibodies associated with “helper” and “suppressor” T cell subsets, respectively (Becton Dickinson) and B1, a monoclonal antibody specific for B cells (Coulter Corp, Hialeah, Fls).

Immunoperoxidase staining of cytocentrifuge preparations for cytoplasmic Ig was done by the avidin-biotin-peroxidase complex (ABC) method, as follows. The slides were fixed in acetone (24°C, five minutes) and incubated for ten minutes with 1% normal goat serum; 60 minutes with biotinylated, chain-specific, goat anti-human IgM, IgG, kappa, or lambda (Tago); and 30 minutes with the ABC solution. The color was developed with diaminobenzidine HCl (Sigma) in the presence of hydrogen peroxide. All washes were done in 0.05 mol/L, pH 7.6 Tris buffer.

Quantification of immunoglobulin secretion. An enzyme-linked immunosorbent assay (ELISA) was used for detection of secreted immunoglobulin in culture supernatants. Culture supernatants (200 μL) were added to 96-well NUNC immunoplates previously coated with 200 μL of chain-specific, goat anti-human IgM, IgG, kappa, or lambda (Tago) at a concentration of 1.5 μg/mL. Standard solutions containing known quantities of the various human heavy and light chains were also added to each plate. After incubation at room temperature for two hours, the ELISA was completed using the ABC technique, with development of color by addition of O-phenylenediamine (Aldrich Chemical Co, Milwaukee) in the presence of hydrogen peroxide. The reaction was terminated by addition of 8N sulfuric acid, and optical densities were determined at 492 nm using a Titertek Multiscan Photometer (Flow Labs, McLean, Va). The concentrations of Ig present in culture supernatants were determined by use of a standard curve. This plate ELISA method detected as little as 10 ng/mL Ig.

RESULTS

Ig secretion by unFractionated follicular lymphoma cell preparations. The baseline levels of Ig secretion by the unstimulated and unFractionated follicular lymphoma cell cultures and the effect of TPA stimulation on these same cell preparations were determined. UnFractionated cell suspensions from lymph node biopsies of 14 follicular lymphoma patients, adjusted to provide a B cell concentration of 10^6/ per milliliter, were incubated in the presence or absence of TPA (10^-4 mol/L). After seven days in culture, supernatants were removed and secretion of IgM, IgG, and kappa and lambda light chains were measured using the plate ELISA method (Fig 1A). In five of the 14 cases, lymphoma cells did not secrete detectable Ig. Low levels of secreted Ig (30 to 70 ng/mL) were detected in six cases, and in five of these six cases, Ig secretion occurred or was enhanced only when TPA was present. In one lymphoma (Case 11), a low level of spontaneous Ig secretion was detected in the unstimulated cultures, and TPA stimulation increased the level of secretion more than fourfold. Cells from two patients (Cases 7 and 13) spontaneously secreted large amounts of Ig into culture supernatants; this secretion was little affected by exposure of the cultures to TPA. In all cultures in which Ig secretion was detected, the Ig produced was of the same heavy and light chain type as the cell surface Ig detected on the malignant B cells.

Ig secretion by E rosette-depleted follicular lymphoma cell preparations. To determine the effect of removal of the autologous T cells on Ig secretion by the follicular lymphoma B cells, we removed T cells by E rosette depletion, leaving no more than 2% T cells (Lyt 3 positive). The B cell concentration in all cultures was adjusted to 10^6 per milliliter, and the cells were cultured in the presence or absence of TPA for seven days. The removal of autologous T cells had minimal effects on levels of Ig secretion by the follicular lymphoma B cells (Fig 1B). Cell cultures from ten patients still secreted little or no detectable Ig. Three cultures in which low levels of secretion had been detected before were now negative. The three unFractionated cultures that had secreted large
amounts of Ig continued to secrete comparable amounts of Ig when autologous T cells were depleted.

**Allogeneic normal T cells allow Ig secretion by follicular lymphoma B cells.** We next questioned whether allogeneic normal T cells might be able to "help" the neoplastic B cells to secrete Ig. B cells were isolated from the involved tissues from all 14 follicular lymphoma patients and cultured for seven days with normal allogeneic peripheral blood T cells at a 1:1 T cell-B cell ratio, and cultured with or without TPA (Fig 2). Neoplastic B cells from all five cases that had failed to secrete any Ig in prior experiments now secreted Ig (160 to 640 ng/mL). In the six cases that had secreted at low levels, Ig secretion was markedly enhanced (2.3 to 7.0 times baseline). In ten lymphomas, maximal secretion occurred in the presence of both allogeneic T cells and TPA, but in two cases (6 and 10), exposure to TPA was not necessary to achieve secretion. The two follicular lymphomas (Cases 7 and 13) that secreted high levels of monoclonal Ig spontaneously in vitro in the presence or absence of autologous T cells, were unaffected by the addition of allogeneic T cells. The Ig secreted by the follicular lymphoma cells was always the same isotype as the lymphoma cell-associated monoclonal Ig identified by immunostaining procedures. As a control, the T cells used in these experiments were cultured for seven days with and without TPA, and no significant Ig secretion was detected in the culture supernatants. Thus these follicular lymphoma B cells appeared to be intrinsically capable of Ig secretion if sufficient helper T cell activity was provided.

**Immunoperoxidase staining for surface and cytoplasmic Ig** done on the first and seventh days of culture confirmed the monoclonality of the B cell populations (single light chain) (Fig 3). The staining pattern changed during the culture period, in that the malignant B cells demonstrated predominantly cell surface staining on day 1 but by day 7 of culture with allogeneic T cells had lost the surface Ig staining and developed abundant cytoplasmic Ig (Fig 3). The amount of cytoplasm increased, but the neoplastic B cells did not develop a true plasmacytic appearance.

**Functional role of autologous T cells.** Because it appeared that autologous T cells from some lymph nodes containing follicular lymphoma either failed to help or actively suppressed Ig secretion by follicular lymphoma B cells, we examined the functional activity of the T cells present in the lymph node biopsy specimens. (1) In eight of the patients, T cells were isolated from involved lymph nodes by E rosette separation, added back to autologous malignant B cells at a 1:1 T cell-B cell ratio, and cultured with or without TPA for seven days. (2) Parallel cultures contained the identical neoplastic B cell preparations and normal allogeneic peripheral blood T cells at a 1:1 T cell-B cell ratio. (3) Finally, both autologous lymph node and allogeneic peripheral blood T cells were added to neoplastic B cells at a 1:1 T cell-B cell ratio. The addition of concentrated autologous T cells increased levels of Ig secretion somewhat over those seen in the unFractionated cultures in 4/8 lymphomas, but Ig secretion averaged only 33 (± 17)% of the levels obtained when allogeneic peripheral blood T cells were added (Fig 4). When allogeneic and autologous T cells were added simultaneously to the same B cell cultures, the amount of Ig secreted by the neoplastic B cells was 42 (± 12)% of that seen when allogeneic T cells were added alone. The inhibitory effect of the autologous T cells implicates suppressor effector cells within the autologous T cell populations of lymph nodes involved by follicular lymphoma.
Fig 3. Immunoperoxidase staining of cultured follicular lymphoma cells (case 11). (A) Staining of the purified B cell preparation with anti-kappa antibody on day 1 shows positive staining of virtually all cells, with a predominantly surface membrane pattern, which appears as a thick dark rim. The cell nuclei are only lightly counterstained so as not to obscure the anti-Ig staining (Original magnification x 600). (B) The same B cell preparation stained with anti-lambda antibody shows no surface or cytoplasmic staining, confirming the monoclonality of the B cell population (Original magnification x 600). (C) The same B cells, cultured in the presence of allogeneic T cells and TPA for seven days and stained with anti-kappa antibody, contain an increased amount of cytoplasm and abundant cytoplasmic Ig (Original magnification x 600).

Effect of irradiation on the functional activity of autologous T cells. To further investigate the functional role of the T cells from the follicular lymphomas, T cells from five lymph nodes containing follicular lymphoma were irradiated to 1,500 R, which selectively abrogates suppressor T cell activity. The T cells were added back to autologous B lymphoma cultures at a 1:1 T cell-B cell ratio. Ig secretion was increased in all cases studied (130% to 270%) over control cultures containing nonirradiated T cells. This enhancement of Ig secretion indicates that a radiosensitive, presumably suppressor T cell population is functionally active in the lymph nodes containing follicular lymphoma.

Phenotypic markers and helper-suppressor ratios of autologous T cells. The phenotypic surface markers of T cell fractions from all 14 follicular lymphomas were examined next to determine if Ig secretion correlated with percentages of total admixed T cells, T cell subsets, or helper-suppressor T cell ratios. The unfractionated follicular lymphoma cell preparations contained from 11% to 69% E rosette- and Lyt 3-positive (T) cells that were morphologically normal when examined in Wright-stained cytocentrifuge preparations. The percentages of cells staining for anti-Leu-3a and anti-Leu-2a are shown in Table 1, along with the calculated helper-suppressor T cell ratios and levels of Ig secretion by the unfractionated cell preparations. Immunoglobulin secretion did not correlate with the percentages of total T cells, Leu-3a-positive cells, or Leu-2a-positive cells. The helper-suppressor T cell ratios varied widely and also failed to show any correlation with Ig secretion. Furthermore, there was no association between the histologic subtype of the lymphoma and its capacity for Ig secretion (data not shown).

DISCUSSION

We have described induction of Ig secretion in vitro by neoplastic B cells from patients with follicular lymphomas. By manipulation of culture conditions, we found that virtually all follicular lymphomas could be induced to secrete monoclonal Ig when the appropriate external signals were provided. These results demonstrate that the arrest of differentiation seen in the follicular lymphoma B cells is reversible.
and provide a correlate to previously published reports describing reversal of differentiation arrest in other types of B cell malignancy.18,19,21

To explore the immunoregulatory mechanisms operative in follicular lymphomas, we focused on the autologous lymph node T cells in this disease. The role played by T cells is of particular interest in follicular lymphomas, because involved lymph nodes usually contain 30% to 40% T cells, present both outside and within the neoplastic nodules.38 In the present study, most follicular lymphoma B cells, cultured alone or with autologous T cells, secreted little or no Ig, even in the presence of TPA. Induction of Ig secretion required T cell help, and this “helper” effect could be provided by allogeneic peripheral blood T cells. Furthermore, autologous lymph node T cells appeared to contribute a suppressor effect in some cases, since their presence diminished the responsiveness of the neoplastic B cells to allogeneic T cells. Coculture of neoplastic B cells with both allogeneic normal and autologous T cells produced levels of Ig secretion lower than those obtained in the cultures of neoplastic B cells with allogeneic T cells alone. Irradiation of autologous T cell fractions to selectively abrogate suppressor T cell activity increased the T cells alone. Irradiation of autologous T cell fractions to obtain in the cultures of neoplastic B cells with allogeneic normal lymphocytes and tissues involved by malignancy in humans with malignant lymphomas and other types of malignancies.49 The functional activity of peripheral blood T lymphocytes in patients with B cell lymphomas may be quite different from the activity of the T cells isolated from involved lymph nodes.

Spontaneous clinical regression is not uncommon in follicular lymphomas,29,51 and indeed it has been postulated that such regressions may occur as a consequence of immunoregulation in vivo.72 The demonstration in the present study that follicular lymphoma B cells are responsive to immune regulation in vitro further implies that therapeutic strategies exploiting immunoregulatory mechanisms might be successful in this group of patients who usually present with a disseminated disease incurable with current therapeutic regimens. In one therapeutic approach, a patient with a follicular lymphoma was treated with monoclonal antibody against the idiotypic Ig expressed by his lymphoma cells and has sustained a complete remission for more than two years.53 It has been speculated that the clinical response seen in this patient may have been mediated through triggering of a host immunoregulatory circuit, possibly involving generation of idiotypic reactivity.73,74 It has been postulated that the clinical response seen in this patient may have been mediated through triggering of a host immunoregulatory circuit, possibly involving generation of idiotypic reactivity.73,74 The capacity of some follicular lymphomas to respond to suppressor T cell activity demonstrated in the current study may be an important factor in their potential responsiveness to anti-idiotypic antibody therapy. Additional studies are needed in other patients to confirm the therapeutic value of anti-idiotypic antibody treatment.

The ability to stimulate secretion of monoclonal Ig in vitro in nearly all cases of follicular lymphoma has important practical applications. Rescue of idiotypic Ig secretion from the lymphoma cells for subsequent immunization has been an inefficient and laborious task, requiring either production of mouse-human hybridomas25,26 or direct extraction of Ig from lymphoma cells by proteolytic enzyme digestion.22–24 We have found that Ig secreted by follicular lymphoma cells in vitro can be purified by affinity chromatography and used

### Table 1. T Cell Surface Marker Phenotypes in Follicular Lymphomas: Correlation With Ig Secretion by Unfractionated Cells

<table>
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<tr>
<th>Case No.</th>
<th>Leu-3a (%)</th>
<th>Leu-2a (%)</th>
<th>H:S Ratio</th>
<th>Ig Secretion* (ng/mL)</th>
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<td>1</td>
<td>19</td>
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Mean ± SD: 24 ± 10 12 ± 5 2.5:1 135 ± 260

H:S, helper-suppressor.

†Spontaneous secretion when the lymphoma cells were placed in culture.
FOLLICULAR LYMPHOMA

as an immunogen to produce monoclonal anti-idiotype antibodies (for an example of case 13, see reference 55). Anti-idiotype antibodies may be useful for monitoring and therapy of B cell lymphoma patients and should prove valuable in dissecting the mechanisms in the host governing differentiation, and ultimately proliferation, of malignant B cells.

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Induction of immunoglobulin secretion in follicular non-Hodgkin's lymphomas: role of immunoregulatory T cells

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