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Interleukin 6 dependence of spontaneous *in vitro* differentiation of B cells from patients with IgM gammapathy

(lymphoid proliferation/B-cell maturation/monoclonal IgM)

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ABSTRACT **Blood B cells from eight patients with clonal lymphoid disorders characterized by monoclonal IgM secretion (four with malignant plasmacytic proliferation typical of Waldenström macroglobulinemia and four without overt lymphoid neoplasia) were found to spontaneously differentiate *in vitro* into plasma cells. In all instances, monoclonal plasma cells (8–45% of the cells) were generated from extensively purified B cells or T-cell-depleted peripheral blood mononuclear cells after a 7-day culture period, with a corresponding high rate of IgM secretion into the culture medium. This differentiation occurred in the absence of any cell proliferation process as measured by [^{3}H]thymidine uptake at day 2 or 4. Normal B cells did not differentiate under the same experimental conditions. Detection of interleukin 6 (IL-6) bioactivity in all patients' B-cell culture supernatants as well as of IL-6 mRNA in freshly prepared, uncultured B cells in the two cases studied by *in situ* hybridization suggested that IL-6 secretion by B cells may play a role in this process. Moreover, in the four patients without overt lymphoid proliferation, B-cell differentiation was significantly inhibited (60–80%) in the presence of anti-IL-6 antibodies. In contrast, anti-IL-6 antibodies did not preclude the differentiation into plasma cells of B cells from the four patients with bona fide Waldenström macroglobulinemia. These results suggest a two-step pathogenesis for such human lymphoplasmacytic clonal proliferations, the initial stage being characterized by an IL-6-dependent autocrine differentiation pathway.**

Interleukin 6 (IL-6) has pleiotropic effects on cells from various lineages. Alone or in synergy with other lymphokines, IL-6 triggers the production of acute-phase proteins by hepatocytes (1), the proliferation of T cells (2, 3), the differentiation of cytotoxic T cells (4), and the proliferation and differentiation of hematopoietic precursors (5). IL-6 has two distinct effects on B cells, since it drives activated B cells to terminal plasma-cell differentiation, as originally described (6), and also behaves as a murine plasmacytoma or hybridoma growth factor (7, 8). The latter effect may be significant in the development and/or maintenance of malignant plasma cells in human multiple myeloma (9). Here we provide evidence that IL-6 may also have a key role in the development of lymphoid disorders characterized by secretion of monoclonal IgM.

MATERIALS AND METHODS

Patients. The eight patients studied had a serum monoclonal IgM. Four suffered from a malignant lymphoplasmacytic proliferation typical of Waldenström macroglobulinemia, whereas the other four showed no evidence of overt lymphoid neoplasia. The latter patients had peripheral neuropathy and their monoclonal IgM exhibited an autoantibody activity directed to myelin-associated glycoprotein (MAG) (reviewed in ref. 10).

Preparation of Lymphoid Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll/Hypaque gradient centrifugation. T-cell depletion of PBMCs was achieved by rosetting twice with *S*-(2-aminoethyl)isothiouronium bromide-treated sheep erythrocytes. Purified B cells were obtained after initial monocyte elimination with 5 mM L-leucine methyl ester (Sigma) added to PBMCs for 40 min in serum-free medium (11). This preparation contained <1% monocytes or T cells. In one set of experiments, purified B cells were further depleted of potentially residual monocytes by complement-dependent cytotoxicity using CD11 monoclonal antibody (mAb).

Cell Culture. T-cell-depleted PBMCs or purified B cells were cultured in tissue culture tubes at 10^6 cells per ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. In some experiments, anti-IL-6 antibodies [rabbit polyclonal IgG (Genzyme) or purified goat antibodies or mouse mAb anti-BSF-2 166 (ref. 12); generous gifts from T. Hirano and T. Kishimoto] were added at various times of culture. Controls included irrelevant rabbit, goat, or mouse IgG. Culture supernatants were filtered and stored at −20°C until use.

Evaluation of B-Cell Differentiation. Cytoplasmic immunoglobulin (cIg)-containing cells were detected by direct immunofluorescence on fixed cells, using $F(ab')_2$ fragments from either polyvalent goat IgG to human immunoglobulins or monospecific rabbit IgG to human immunoglobulin chains according to published methods (13). IgM in cell culture supernatants was measured by an immunocapture ELISA (14).

IL-6 Bioassay. The presence of IL-6 was evaluated using the IL-6-dependent 7TD1 hybridoma cell line, obtained through the courtesy of J. Van Snick (Ludwig Institute for Cancer Research, Brussels). Cells (10^3) were cultured for 48 hr in the presence of triplicate serial dilutions of cell supernatant in 96-well microtiter plates. Cell proliferation was estimated by [^{3}H]thymidine incorporation during the last 6 hr of culture. Recombinant IL-6 (Genzyme) was used as an internal standard in all assays. One reference unit of IL-6 was defined by the half-maximal proliferation of 7TD1 cells. It corresponded reproducibly to 2 pg of recombinant IL-6.

Detection of IL-6 mRNA by *in Situ* Hybridization. The technique used was slightly modified from published methods

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Abbreviations: IL, interleukin; cIg, cytoplasmic immunoglobulin; mAb, monoclonal antibody; MAG, myelin-associated glycoprotein; PBMC, peripheral blood mononuclear cell.

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(15, 16). In brief, cells were centrifuged onto glass slides and immediately fixed in 4% paraformaldehyde. Preparations were then transferred into a solution of 70% ethanol and stored at 4°C until used. The IL-6 probe (ref. 17; a gift from C. Vaquero and J. Sanceau) used in the present studies was a 700-base-pair *Xmn* I–*Ban* I insert of human IL-6 cDNA cloned into the pGEM-4 plasmid (Promega) by standard methods (18). As negative controls the sense IL-6 probe and control template were used (19). Run-off transcripts of appropriate linearized plasmids were synthesized using T7 RNA polymerase according to the supplier's instructions (Promega), with 60 μCi of [α-[^{35}S]thio]UTP (1200 Ci/mmol, Amersham; 1 Ci = 37 GBq), in order to prepare antisense IL-6, sense IL-6, and λ RNA probes. The fragment length of RNA probes was adjusted to a mass average of 100–150 bases by limited alkaline hydrolysis. RNA probes were ethanol-precipitated with yeast tRNA and salmon sperm DNA and washed with 70% ethanol, and the dried pellets were resuspended in the hybridization buffer to give final concentrations of 50% deionized formamide, 0.3 M NaCl, 20 mM Tris·HCl (pH 8), 0.5 mM EDTA, 1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 10% dextran sulfate, 20 mM dithiothreitol, 500 μg of yeast tRNA per ml, 1 mg of salmon sperm DNA per ml, and 0.25 μg of RNA probe per ml. Finally, the slides were postfixed in 2% paraformaldehyde/1% glutaraldehyde, dehydrated in ethanol, and air-dried. The hybridization was performed at 50°C for 18 hr. The slides were washed twice in 50% formamide/2× SSC/10 mM dithiothreitol (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) at 52°C, then treated with RNase A (10 μg/ml in 2× SSC) for 10 min at 37°C, dehydrated in ethanol, dried, and coated with NTB2 emulsion (Kodak) for autoradiography. After 10 days of exposure, the slides were developed in Dektol (Kodak), fixed in Unifix (Kodak), and stained with hematoxylin. The grain count distribution on 10^2 cells was estimated under light microscopy (×400).

RESULTS

B Cells from Patients with Serum Monoclonal IgM Differentiate Spontaneously *in Vitro*. T-cell-depleted PBMCs from eight patients with serum monoclonal IgM were cultured in medium alone for 7 days. Study of cIg-containing cells showed in all instances the generation of morphologically typical plasma cells. This spontaneous B-cell differentiation process occurred in cultures from all patients with or without overt lymphoid proliferation. Cultures from six patients contained an unexpectedly high percentage of plasma cells (20–45%), whereas in the other two cases plasma cells

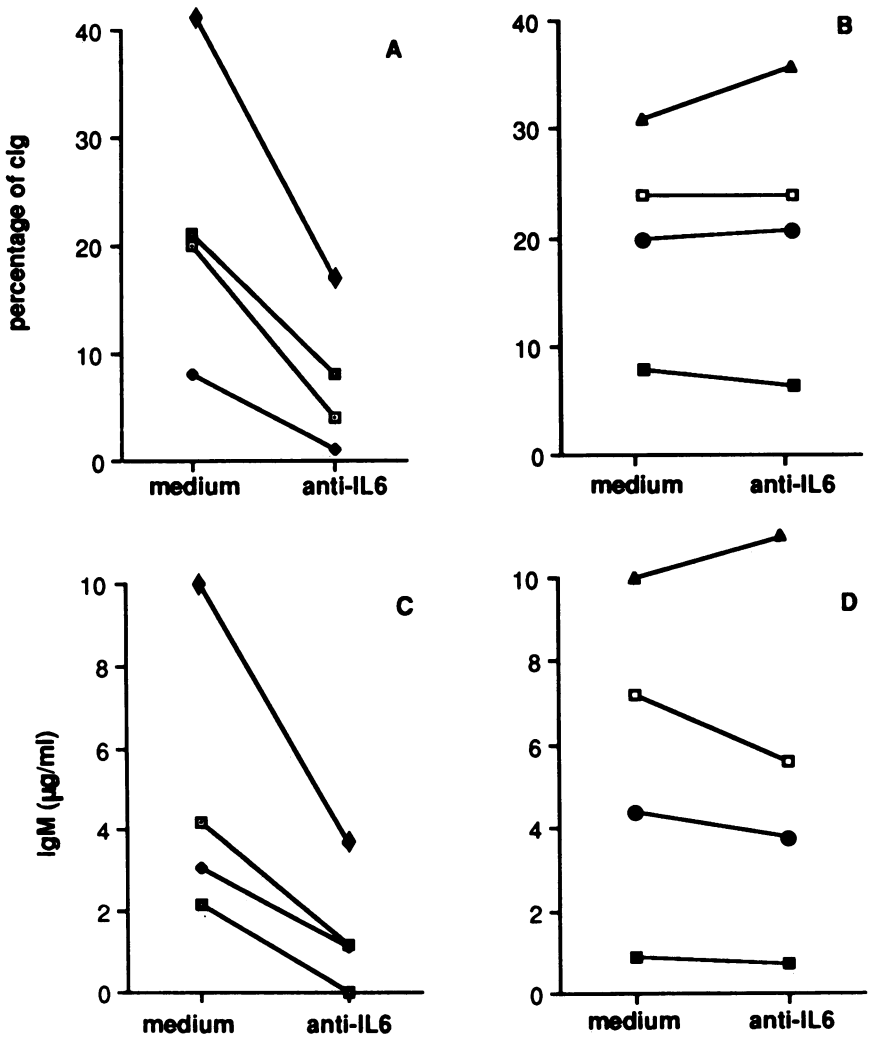

FIG. 1. Spontaneous differentiation of B cells from patients with monoclonal IgM. T-cell-depleted (▣, ●, □, ■) or purified B cells (▲, ■, ◆, ◆) were cultured for 7 days in medium alone or with anti-IL-6; percentage of cIg-positive cells was determined by direct immunofluorescence (*A* and *B*) and of IgM secretion (μg/ml) was measured by ELISA (*C* and *D*). (*A* and *C*) Patients without lymphoid proliferation. (*B* and *D*) Patients with Waldenström macroglobulinemia. The results obtained in the presence of either rabbit (10–30 μg/ml), goat (1:5000 final dilution), or mouse anti-BSF-2 166 (1:1000 final dilution) anti-IL-6 antibodies are shown at right in each panel. Control IgG did not significantly alter plasma-cell differentiation.

accounted for 8% of the cells. When purified B cells from four of these patients were similarly studied, the generation of plasma cells was unaffected. Representative results are shown in Fig. 1 for patients without (*A*) or with (*B*) overt lymphoid proliferation. Comparable results were obtained in several experiments and on different cell samples. High-rate IgM secretion by patients' T-cell-depleted PBMCs or purified B cells at day 7 of culture was confirmed by the measurement of supernatant IgM (Fig. 1 *C* and *D*).

Before culture, cIg-containing cells accounted for <1% of the cell preparations. In our experimental conditions, T-cell-depleted PBMCs or purified B cells from healthy individuals did not differentiate (<1% plasma cells). When studied with monospecific antibodies to heavy and light chains, >99% plasma cells in the patients' cultures were stained with anti-μ reagents and displayed a single light-chain isotype. In three patients whose IgM was directed to MAG, quantitative ELISAs showed that most if not all of the secreted IgM possessed this antibody activity.

This differentiation of B cells occurred early in the culture period, since as early as day 3 the percentage of cIg-containing cells represented around half of that observed at day 7. Importantly, the differentiation event occurred without a detectable increase in [^{3}H]thymidine incorporation (<500 cpm) measured at days 2 and 4 in the three cases studied.

IL-6 Dependence of Spontaneous B-Cell Differentiation. To investigate the possible role of IL-6 in the spontaneous differentiation of B cells, we assayed the culture supernatants for IL-6 activity and studied the effects of addition of anti-IL-6 antibodies to the culture medium.

Supernatants from purified B cells were collected at days 1 and 3 and serial dilutions were assayed for the ability to induce proliferation of the IL-6-dependent 7TD1 cell line. In all patients' samples studied, bioactive IL-6 (40–200 units/ml) was detected, whereas no or barely detectable IL-6 activity was found in supernatant from normal blood B cells (Fig. 2). In one experiment, purified B cells were submitted to a further step of monocyte depletion, by complement-dependent cytotoxicity with CD11 mAb; the level of IL-6 in the culture supernatant was not reduced by this treatment.

Various anti-IL6 antibodies were added to T-cell-depleted PBMCs (four cases) or purified B cells (four cases) at the onset of cultures to study their effects on plasma-cell generation. A 60–82% decrease in the number of cIg-containing cells was noted when cells (one T-cell-depleted preparation and three purified B-cell preparations) from the four patients without overt malignant proliferation were studied (Fig. 1*A*). On the other hand, no inhibition of B-cell differentiation occurred with cells (three T-cell-depleted preparations and one purified B-cell preparation) obtained from the four patients with Waldenström macroglobulinemia (Fig. 1*B*). These results were corroborated in another set of experiments, by the measurement of IgM concentration in the culture supernatants collected at day 7 (Fig. 1 *C* and *D*).

As could be expected from the kinetics of plasma-cell generation (see above), the addition of rabbit anti-IL-6 antibodies at day 3 or 6 had only marginal inhibitory effects. Finally, addition of recombinant IL-6 (10 ng/ml) at the onset of the culture period did not enhance the development of plasma cells either in patients' (three cases) or in normal donors' (two cases) cell preparations.

Detection of IL-6 mRNA. Purified uncultured B cells were prepared from a normal donor and two patients without overt lymphoid proliferation. *In situ* hybridization was performed using the antisense IL-6 probe, with both the λ probe and the sense IL-6 probe as controls. A low number of silver grains per cell (<15) was found when the three cell preparations were hybridized with control probes or when normal B cells were hybridized with the antisense IL-6 probe. In contrast, 30–50% of B lymphocytes from both patients were significantly labeled by the antisense IL-6 probe (25–40 grains per cell) (Fig. 3).

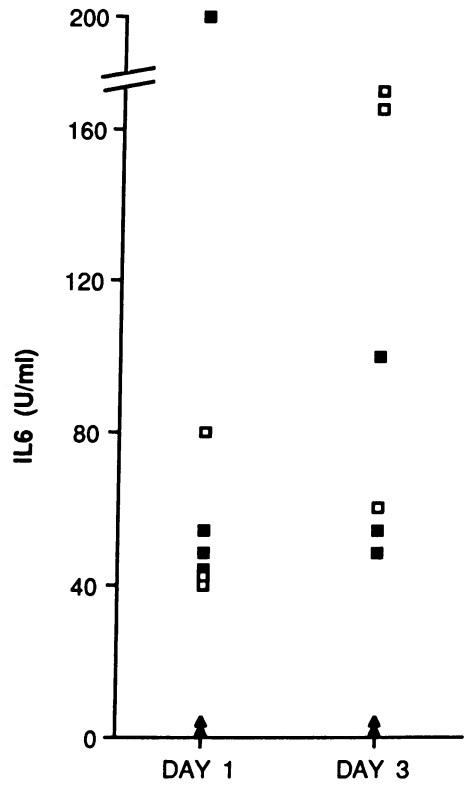

FIG. 2. IL-6 bioactivity [reference units (U)/ml] in supernatants collected at days 1 and 3 from cultures of purified B cells. Cells were from patients with (□) or without (■) overt lymphoid proliferation or from healthy control donors (▲).

DISCUSSION

Waldenström macroglobulinemia is characterized by the presence of a serum monoclonal IgM and a pleiomorphic monoclonal lymphoid infiltration of bone marrow and peripheral lymphoid organs by small lymphocytes, lymphoplasmacytic cells, and plasma cells (20). Hence, in this disease, the proliferating clonal B cells retain some capacity for terminal maturation into plasma cells, unlike B cells from most other lymphoproliferative diseases (such as chronic lymphocytic leukemias or B-cell lymphomas), which are believed to be frozen at a given step in the B-cell differentiation pathway. On the other hand, the presence of a monoclonal IgM in the serum is not invariably associated with an overt B-cell lymphoid proliferation. The latter may, however, become apparent after several years in some patients with a long follow-up, because the clinical consequences of the autoantibody activity of their serum monoclonal IgM led to

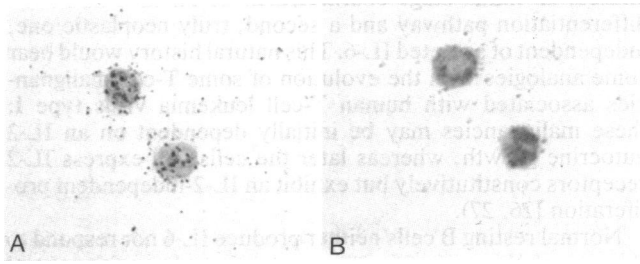

FIG. 3. Detection of IL-6 mRNA by *in situ* hybridization on uncultured purified B cells from a patient with monoclonal IgM (*A*) or from a healthy donor (*B*). (×93.)

its early discovery. This course has been observed, for instance, in patients with autoimmune hemolytic anemia and monoclonal IgM with cold agglutinin activity (21) and in patients with a peripheral neuropathy and serum monoclonal IgM directed to MAG (22). Whether these diverse clinicopathological patterns associated with monoclonal IgM correspond to two successive stages of the same lymphoproliferative process remains unsettled. In both instances, clonal B cells are often present in blood (20, 23) and may exhibit unusual phenotypic features such as the expression of the PCA1 antigenic marker (24).

We show here that T-cell-depleted or highly purified blood B cells from patients with serum monoclonal IgM with or without overt malignant lymphoid proliferation have the capacity to undergo spontaneous terminal plasma-cell differentiation *in vitro*. In six of eight cases, a dramatic generation of plasma cells (up to 45% of cells) occurred at the end of the culture period. That the differentiating B cells belong, at least partly, to the clonal process was shown by the finding of monotypic cIg and the presence of IgM with anti-MAG antibody activity in the supernatants of cells from patients with neuropathy. In our experimental conditions, similarly treated B cells from normal donors (or from patients with chronic lymphocytic leukemia) did not differentiate.

Since this spontaneous *in vitro* differentiation of B cells occurred without any cell proliferation, we investigated the possibility that IL-6 was involved in this phenomenon. Several lines of evidence indicated that this was indeed the case. IL-6 bioactivity was detected in all supernatants collected at days 1 and 3. That this IL-6 activity was constitutively produced by the B cells was demonstrated in the two cases studied by *in situ* hybridization experiments, which detected IL-6 mRNA in purified uncultured B cells, whereas no signal was obtained from control normal B cells. The detection of IL-6 mRNA (and bioactivity) in lymphoproliferative diseases is not without precedent, since rigorously purified malignant B cells from follicular or immunoblastic lymphomas were recently shown to contain IL-6 mRNA (25). However, the significance of the presence of IL-6 mRNA in these diseases is unknown.

In this study, we obtained evidence that the secreted IL-6 plays a major role in the process of terminal plasma-cell differentiation of the cultured B cells from those patients without overt underlying B-cell malignancy, since their differentiation was significantly inhibited in the presence of anti-IL-6 antibodies. Although we cannot formally rule out a synergism between IL-6 and other lymphokines in the differentiation process, it should be noted that the supernatants did not contain measurable IL-1, IL-2, IL-4, or IL-5 activities (data not shown) and that we used highly purified B cells. In sharp contrast with the above results, the addition of anti-IL-6 antibodies did not inhibit the differentiation of B cells from patients with overt lymphoid malignancy. These findings might have important implications for the pathogenesis of Waldenström macroglobulinemia, since they suggest that this lymphoproliferative disorder may evolve in two distinct phases, an initial stage characterized by an IL-6 autocrine differentiation pathway and a second, truly neoplastic one, independent of secreted IL-6. This natural history would bear some analogies with the evolution of some T-cell malignancies associated with human T-cell leukemia virus type I: these malignancies may be initially dependent on an IL-2 autocrine growth, whereas later the cells still express IL-2 receptors constitutively but exhibit an IL-2-independent proliferation (26, 27).

Normal resting B cells neither produce IL-6 nor respond to this lymphokine (6, 25, 28), as confirmed in our control experiments. Only the triggering of B-cell surface immunoglobulin by anti-immunoglobulin antibodies has been shown so far to induce the synthesis of IL-6 mRNA (25). A simple explanation of our results would therefore be that such a triggering by an autoantigen had occurred *in vivo*; this possibility would be most appealing for clonal B cells synthesizing anti-MAG IgM. In this context, it is of interest that the incidence of autoantibody activities is disproportionately high among monoclonal IgMs (29). On the other hand, *in vivo* activation of the clonal B cells may be secondary to lymphokine secretion by other cell types or idiotypic network regulation via antibodies sometimes found in such patients' sera (30) or via T cells. Finally, we cannot rule out the possibility that B cells constitutively produced IL-6 because of leukemia-related gene transcription.

Our data suggest that IL-6 may have paramount importance in the triggering and/or maintenance of some human B-cell lymphoproliferative disorders. Strikingly, the two known activities of IL-6 on B cells—namely, proliferation of plasma cells and terminal differentiation of B cells without proliferation—appear to be implicated in multiple myeloma and Waldenström macroglobulinemia, respectively. Further studies may give some insight into the mechanism(s) leading to this differential effect of IL-6 on B cells. Obvious hypotheses are that differentiating B cells express a variable density of surface IL-6 receptors or that different molecular forms of this receptor exist.

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