Role of Interleukin 10 in the B Lymphocyte Hyperactivity and Autoantibody Production of Human Systemic Lupus Erythematosus

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Summary

Interleukin-10 (IL-10) is produced at a high level by B lymphocytes and monocytes of patients with systemic lupus erythematosus (SLE). In the present work, we analyzed whether this increased production of IL-10 contributed to the abnormal production of immunoglobulins (Ig) and of autoantibodies in SLE. The role of IL-10 was compared with that of IL-6, another cytokine suspected to play a role in these abnormalities. The spontaneous in vitro production of IgM, IgG, and IgA by peripheral blood mononuclear cells from SLE patients was weakly increased by recombinant IL (rIL)-6, but strongly by rIL-10. This production was not significantly affected by an anti-IL-6 mAb but was decreased by an anti-IL-10 mAb. We then tested the in vivo effect of these antibodies in severe combined immunodeficiency mice injected with PBMC from SLE patients. The anti-IL-6 mAb did not significantly affect the serum concentration of total human IgG and of anti-double-stranded DNA IgG in the mice. In contrast, the anti-IL-10 mAb strongly inhibited the production of autoantibodies, and, to a lesser extent, that of total human IgG. These results indicate that the Ig production by SLE B lymphocytes is largely IL-10 dependent, and that the increased production of IL-10 by SLE B lymphocytes and monocytes may represent a critical mechanism in the emergence of the autoimmune manifestations of the disease.

The immunological imbalance of SLE is characterized by an increased B lymphocyte hyperactivity, mainly reflected by the production of autoantibodies (1) and decreased in vitro and in vivo cellular immune responses (2, 3). The latter defect is related to a dysfunction of both T helper lymphocytes (4) and antigen-presenting cells (5). IL-10 is a potent in vitro inducer of B lymphocyte differentiation (6) as well as an inhibitor of T helper lymphocyte (7) and antigen-presenting cell function (8). Thus, the immunological imbalance of SLE may be related to an abnormally high production of IL-10, or to a hypersensitivity of immune cells to this cytokine.

Consistent with the putative implication of IL-10 in the pathophysiology of SLE, we recently showed that SLE is characterized by an increased production of IL-10 by PBMC. In untreated SLE patients, PBMC spontaneously express the IL-10 gene at a significantly higher level than that in healthy

individuals. Moreover, PBMC from SLE patients spontaneously release large amounts of IL-10. T lymphocytes are not involved in the hyperproduction of IL-10, which arises from both B lymphocytes and monocytes (9, 10). These results suggest that B lymphocyte hyperactivity in human SLE results from the conjunction of IL-10-mediated autocrine and paracrine stimulations.

Murine studies suggest a role for IL-10 in the pathophysiology of SLE. Treatment of NZB/W F1 mice with an anti-IL-10 mAb delays the onset of autoimmune manifestations and the production of autoantibodies (11). However, the role of IL-10 as an inducer of autoantibody production in human SLE has not yet been documented.

In addition to IL-10, IL-6 has been suggested to be involved in the induction of autoimmune manifestations of SLE. Indeed, serum concentrations of IL-6 are high in SLE pa-

tients, and the in vitro polyclonal B lymphocyte hyperactivity and production of autoantibodies in SLE are at least partly IL-6 dependent (12).

We investigated the respective effects of rIL-6, rIL-10, as well as neutralizing anti-IL-6 and anti-IL-10 mAbs, on the production of total Ig and autoantibodies in SLE patients. These questions were addressed by experiments both in vitro and in vivo using the SCID mouse model.

Materials and Methods

Patients. 17 patients were included in the study. All were women and they fulfilled at least four of the American College of Rheumatology criteria for SLE (13). Their ages ranged from 21 to 46 yr (mean 33.8 yr). They had received no immunosuppressive or corticosteroid treatment during the previous month, and no nonsteroid antiinflammatory drugs for at least 3 d. Clinical disease activity was scored on a 0–16 point scale according to the Mex-SLEDAI index (14). 2 patients had inactive disease; 3 patients, with indices of 1–2, were grouped as having mild active disease; 12 patients, with indices of 7–16, had severe multisystem disease. 19 healthy female volunteer blood donors served as control subjects. All patients and controls were informed about the objectives and methods of the study and gave their consent.

Reagents. Human recombinant IL-6 was kindly provided by N. Vita (Sanofi Bio Recherches, Labège, France). Human recombinant IL-10 was provided by K. Moore (DNAX, Palo Alto, CA). The neutralizing anti-IL-6 mAb (BE-8) has already been described (15). The neutralizing anti-IL-10 mAb (B-N10, IgG1) was obtained by fusing P3X63-Ag8.653 mouse myeloma cells to spleen cells from Balb/c mice immunized with human recombinant IL-10. The screening for specific antibodies was performed by ELISA: Hybridoma supernatants were incubated in microtiter plates coated with a goat anti-mouse antibody followed by incubation with biotinylated IL-10. The assay was then terminated with streptavidin peroxydase and substrate. Several antibodies were selected in this assay, and B-N10 was chosen for its ability to block IL-10 activity in a bioassay. In the latter, PBMC (4.106 cells/ml) were activated for 24 h with LPS (1 μ g/ml) in the absence or presence of IL-10 (1 ng/ml) and of graded concentrations of anti-IL-10 mAb. The inhibition of IL-6 production by IL-10 was determined in the supernatants of these cultures by an IL-6-specific ELISA. 500 ng B-N10 was needed to neutralize the activity of 1 ng IL-10.

As control antibodies, we used, for in vitro studies, the anti-KLH mAb (Becton Dickinson and Co., Mountain View, CA) and, for SCID mice studies, the F3.14.1 mAb (recognizing the hepatitis B virus [HBV]¹ envelope protein). This antibody was obtained by fusing P3X63Ag-8.653 myeloma cells with spleen cells from Balb/c mice immunized with 42-nm viral particles purified from the serum from an HBV-infected patient. The screening of the clones was performed using a radioimmunoassay in which HBV particles were coated, the clone supernatants were added to the wells, and the fixation of the mAb was revealed using anti-mouse Ig antibodies. The F3.14.1 is an IgG1 mAb which does not recognize any serum protein from healthy individuals but binds in Western blot analysis to two HBV envelope antigens of 55 kD and 66 kD, respectively. Antibodies for in vitro experiments were used at the final concentration of 10 µg/ml.

Cell Separations. PBMC were isolated on Histopaque (Sigma

Chemical Co., St. Louis, MO) by gradient centrifugation. For in vitro studies, PBMC were rosetted twice with 2-aminoethylisothiouronium bromide (Sigma)-treated sheep red blood cells. Non-T cells were resuspended in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 100 U/ml penicillin, 2 mM glutamine, and 10% gammaglobulin-free fetal calf serum (complete medium, CM), (GIBCO). For in vivo studies in SCID mice and for bcl-2 expression analysis, PBMC were kept frozen in liquid nitrogen until tested.

Cell Cultures. One million non-T cells were cultured in CM for 24 h, washed, and cultured for an additional 5 d. Either rIL-6, rIL-10, anti-IL-6 mAb, or anti-IL-10 mAb were added during each step of the cultures. The concentrations of IgM, IgG, and IgA were measured in the supernatant using an ELISA assay. Briefly, 96-well flat-bottom microtitration plates (Linbro Chemical Co., Hamden, CT) were coated with goat anti-human polyvalent immunoglobulin (10 µg/ml) overnight at 4°C. After washing and saturating the plastic plates with 1% bovine serum albumin in PBS, culture supernatants or standard human IgM, IgG, and IgA (Sigma) were added to the coated wells in duplicate and incubated for 4 h at room temperature. After washing out unbound antibodies, alkaline phosphatase-conjugated goat IgG, specific for human IgM, IgG, or IgA (Sigma), were added to correspondent wells and incubated overnight at 4°C. The enzyme reaction was measured by its absorbance at 405 nm with a Titertek Multiscan® microtiter plate spectrophotometer (Flow Laboratories, Inc., McLean, VA). A paired Student's t test was used for statistical analysis.

Flow Cytometric Analysis. Two-color flow cytometric analyses were performed using a FACScan® (Becton Dickinson). Non-T cells were washed twice in PBS containing 2% fetal calf serum and stained for 20 min at 4°C with anti-CD19 PE-conjugated mAb (Dako Corp., Glostrup, Denmark). Washed cells were fixed in 1 ml 0.25% paraformaldehyde for 15 min at room temperature. Then, cells were permeabilized in 1 ml 70% methanol for 1 h. After two washes, cells were incubated with mAb anti-bcl-2 FITC (Dako), which had been conjugated for 20 min. After three washes, cells were analyzed. As controls, mAbs conjugated with either PE or FITC and of the same isotype as the anti-CD19 or anti-bcl-2 mAbs were used.

SCID Mice Experiments. 6-10-wk-old SCID mice from Iffa-Credo (L'Arbresle, France), maintained in a sterile isolator, were used. Of the 32 SCID mice tested for murine IgG (Biosys, Compiègne, France), two had serum levels of above 10 ng/ml and were excluded from the study. Frozen PBMC from SLE patients were thawed, washed twice in RPMI 1640, and resuspended in PBS. 15 million cells were injected intraperitoneally to each mouse in a final vol of 200 µl. The mAbs were injected intraperitoneally twice a week (100 µg each time, from day 3 to day 21). Thus, a total of 600 µg mAb was administered to each mouse. On day 30, the mice were killed, and the serum was collected and stored frozen in aliquots until tested. Total human IgG were measured using an ELISA assay (Biosys, Compiègne, France), and human anti-dsDNA IgG were measured using a Farr assay (Kodak Diagnostic SA, Les Ulis, France). A paired Student's t test was used for statistical analysis.

Results

In Vitro Effect of rIL-6 and rIL-10 on Ig Synthesis of SLE Patients. Non-T cells from five healthy individuals and seven SLE patients were cultured for 24 h in CM, washed, and cultured for an additional 5 d, either alone, or in the presence of rIL-6 or rIL-10. At the end of the culture, the con-

¹ Abbreviations used in this paper: CM, complete medium; ds, double stranded; HBV, hepatitis B virus.

centrations of IgM, IgG, and IgA were measured in each supernatant.

In healthy individuals, the spontaneous production of Ig was low. It was not affected by the addition of either rIL-6 or rIL-10, regardless of the isotype considered (Fig. 1 A). The spontaneous production of the three isotypes by SLE non-T cells was much higher than that of healthy individuals' non-T cells. The addition of rIL-6 only weakly stimulated this production. The increase was, however, constant for both IgG and IgA (P = 0.034 and P = 0.043, respectively). The addition of rIL-10 dramatically increased the production of Ig by SLE non-T cells (P = 0.02, P < 0.001, and P = 0.002 for IgM, IgG, and IgA, respectively). This stimulating effect of IL-10 was particularly strong for IgG production (the increase of which was on average [± SEM] 435% [± 76%] [Fig. 1 B]).

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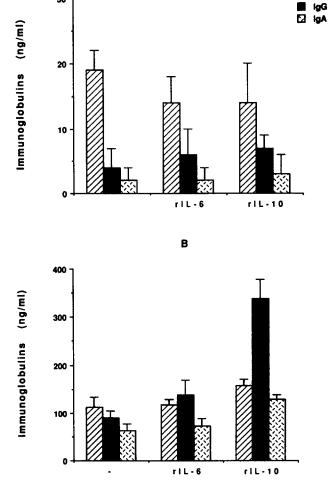


Figure 1. Effect of rIL-6 and rIL-10 on the in vitro production of Ig by normal and SLE PBMC. Non-T cells from either healthy individuals (n = 5) (A), or SLE patients (n = 7) (B) were cultured alone for 24 h, then washed and cultured for an additional 5 d either alone, in the presence of rIL-6 (4 ng/ml), or in the presence of rIL-10 (10 ng/ml). The concentrations of IgM, IgG, and IgA in the supernatant were then determined. Results are expressed as means ± SEM.

In Vitro Effect of an Anti-IL-6 mAb or an Anti-IL-10 mAb on Ig Synthesis of SLE Patients. We then asked whether the endogenous production of IL-6 or IL-10 contributed to the spontaneous hyperactivity of B lymphocytes from SLE patients. Anti-IL-6 mAb did not significantly inhibit the production of IgM and IgG by non-T cells from SLE patients, and weakly inhibited that of IgA (P = 0.05). Production of the three isotypes was more sensitive to the anti-IL-10 mAb. which on average inhibited the production of IgM, IgG, and IgA by 54.2 \pm 9.5%, 44.6 \pm 7.7%, and 60.5 \pm 7.1%, respectively (P = 0.015, P = 0.005, and P = 0.004, respectively)(Fig. 2 B). Neither the anti-IL-6 nor the anti-IL-10 mAb

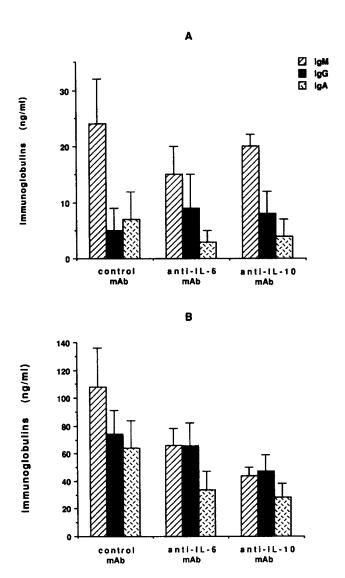


Figure 2. Effect of an anti-IL-6 mAb and of an anti-IL-10 mAb on the in vitro production of Ig by normal and SLE PBMC. Non-T cells from either healthy individuals (n = 5) (A), or SLE patients (n = 7) (B) were cultured for 24 h in the presence of a control mAb, an anti-IL-6 mAb, or an anti-IL-10 mAb. They were then washed and cultured with the same mAb for an additional 5 d. The concentrations of IgM, IgG, and IgA were then determined in the supernatant. Results are expressed as means ± SEM.

altered the spontaneous production of Ig by non-T cells from healthy individuals (Fig. 2 A).

These in vitro experiments showed that the spontaneous production of Ig by SLE B lymphocytes is particularly sensitive to IL-10, and that the increased synthesis of this cytokine by non-T cells contributes to the spontaneous B lymphocyte hyperactivity of SLE patients.

Expression of bcl-2 by SLE B Lymphocytes in the Presence of an Anti-IL-10 mAb. The findings described above may reflect an effect of IL-10 on B lymphocyte differentiation of SLE patients. Alternatively, they may result from an increased bcl-2 expression and B lymphocyte survival (16, 17). We thus examined, by double labeling flow cytometry analysis, the effect of an anti-IL-10 mAb on the level of bcl-2 expression by B lymphocytes.

In the absence of anti-IL-10 mAb, >90% of SLE B lymphocytes cultured for 3 d contained bcl-2. Of the two healthy individuals tested, one had 94% of the B lymphocytes which expressed bcl-2, whereas the other had 51% bcl-2-positive B lymphocytes. Presence of the anti-IL-10 mAb during the culture did not affect bcl-2 expression by B lymphocytes from either healthy individuals, or SLE patients (Fig. 3). Consistent with this finding, cell viability and recovery at the end of the culture were unaffected by the presence of the anti-IL-10 mAb (data not shown).

Effect of the Anti-IL-6 and Anti-IL-10 mAbs on IgG and Autoantibody Production by SLE Cells in the SCID Mouse Model. We then asked whether the in vitro effect of the anti-IL-6 and anti-IL-10 mAbs on the B lymphocyte hyperactivity of SLE patients could be extended to an in vivo model. PBMC from individual SLE patients were injected into two or three SCID mice, which were subsequently treated with either a control mAb, an anti-IL-6 mAb, or an anti-IL-10 mAb.

On day 30, human IgG were detected in the serum of all mice, although at a variable level. As compared to control mice, total human IgG concentration was decreased in mice treated with the anti-IL-6 mAb or with the anti-IL-10 mAb. However, the decreases were moderate (P > 0.1 and P = 0.047, respectively) (Fig. 4 A).

Anti-double-stranded (ds)DNA IgG was detected in the serum of all mice treated with the control mAb. Treatment

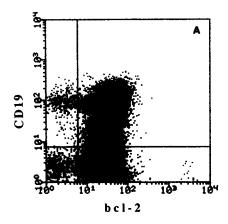
of SCID mice with anti-IL-6 mAb inconsistently decreased the serum concentration of anti-dsDNA IgG. In contrast, administration of anti-IL-10 mAb consistently decreased autoantibody concentration, which dropped below the threshold level of detection of 10 out of 12 cases (P < 0.001) (Fig. 4 B). This indicates that in vivo, in the SCID mouse model, the production of autoantibodies by SLE PBMC is largely dependent on the endogenous production of IL-10, whereas endogenous production of IL-6 does not make a major contribution. Moreover, anti-IL-10 mAb affects the production of autoantibodies more than that of total IgG.

Discussion

We compared the roles of IL-6 and IL-10, two major B lymphocyte-differentiating cytokines, on the polyclonal Ig secretion and on the autoantibody production of SLE patients. These experiments underlined the prominent role of IL-10 rather than that of IL-6 on these phenomena. In vitro experiments analyzing the spontaneous production of Ig by SLE PBMC showed that IL-6 or an anti-IL-6 mAb only minimally affected Ig production. Consistent with these in vitro findings, the in vivo production of human IgG and of anti-dsDNA antibodies in SCID mice injected with SLE PBMC was not significantly affected by anti-IL-6 mAb administration.

In contrast, the IL-10 dependency of SLE B lymphocyte hyperactivity was clearcut by all experimental approaches used. The addition of rIL-10 to SLE PBMC increased the spontaneous production of all isotypes tested, particularly that of IgG. This enhancement of Ig production by SLE PBMC contrasted with the absence of effects of IL-10 on the spontaneous Ig production by PBMC from healthy individuals. The differentiating effect of IL-10 on normal B lymphocytes requires preactivation of the cells with another signal, such as triggering through CD40 or surface Ig (6). This suggests that some sort of triggering stimuli had already been given in vivo in SLE patients and that IL-10 is a limiting factor for production of Ig.

Consistent with this hypothesis, neutralization of endogenously produced IL-10 during cultures of SLE PBMC significantly decreased the production of IgM, IgG, and IgA. We recently showed that IL-10 is produced at a high level



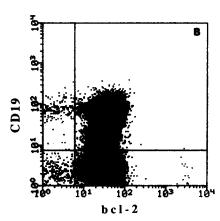
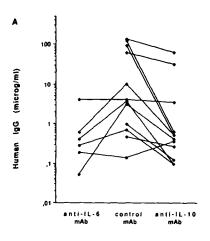


Figure 3. Effect of an anti-IL-10 mAb on bcl-2 expression by SLE B lymphocytes. Non-T cells from SLE patients were cultured for 3 d without (A) or with (B) an anti-IL-10 mAb. bcl-2 expression by CD19+ lymphocytes was then determined by flow cytometry analysis. The fraction of bcl-2+CD19+ and bcl-2-CD19+ cells was 48.6 and 2.4%, respectively, in A, and 45.7 and 1.6%, respectively, in B. Results shown are from one typical case of five SLE patients.



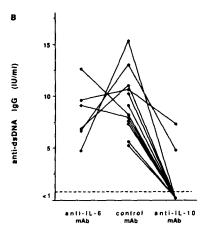


Figure 4. Effect of an anti-IL-6 mAb and an anti-IL-10 mAb on the production of human IgG and anti-dsDNA autoantibody production in the SCID mice model. PBMC from individual SLE patients (n=12) were injected into 2–3 SCID mice, which were subsequently treated with a control mAb, an anti-IL-6 mAb, or an anti-IL-10 mAb. On day 30, the serum concentrations of total human IgG (A) and of anti-dsDNA IgG (B) were determined.

by monocytes and B lymphocytes from SLE patients (9, 10). The present results are the first evidence that this combined autocrine and paracrine production is directly involved in the spontaneous B lymphocyte hyperactivity of SLE patients. This conclusion is also supported by our results in vivo with the SCID mouse model (in which treatment of the mice with an anti–IL-10 mAb significantly decreased the production of human IgG). Interestingly, anti–IL-10 mAb had a larger effect on the production of anti-dsDNA IgG than on that of total human IgG: Anti-DNA autoantibody production was completely inhibited in most mice. This indicates that the requirement for IL-10 may be larger for the production of autoantibodies than for that of other Ig.

We previously showed that IL-10 increases bcl-2 expression by germinal center B lymphocytes and that it prevents their death (17). Thus, the enhancing effect of IL-10 on Ig production may have been partly due to increased B lymphocyte survival. However, we found no modification of bcl-2 expression when culturing peripheral B lymphocytes from SLE patients in the presence of an anti-IL-10 mAb. Thus, the in vitro role of IL-10 on peripheral SLE B lymphocytes primarily reflects its differentiating potency on such cells. This, however, does not rule out an in vivo role of IL-10 in SLE involving an abnormal regulation of B lymphocyte bcl-2 expression. It is possible that the abnormal production of IL-10 by B lymphocytes and monocytes in SLE alters the Ig repertoire selection by preventing B lymphocyte cell death in germinal centers, for example. Considering the importance of this step in the constitution of a normal Ig repertoire, such a phenomenon may lead to the emergence of autoantibodies. This hypothesis is consistent with the observation that, in mice, peripheral deletion of autoreactive B1 lymphocytes is abrogated when the bcl-2 gene is overexpressed by the B lymphocyte compartment (16). Further experiments analyzing the role in SLE of IL-10 on bcl-2 expression and apoptosis of B lymphocytes from lymphoid organs are needed to address this question.

In humans, an increased in vivo production of IL-10 by monocytes has been reported in a number of clinical conditions. Malignant B lymphocytes are potent producers of IL-10 in vivo (18). However, in vivo production of IL-10 by nonmalignant B cells has been observed only in clinical conditions associated with B lymphocyte-mediated autoimmunity (as in SLE, rheumatoid arthritis, and Sjögren's syndrome [9, 10]). In HIV infection, another condition characterized by polyclonal B lymphocyte hyperactivity, peripheral B lymphocytes do not significantly contribute to IL-10 production (19) suggesting that increased production of IL-10 could be restricted to B lymphocytes with autoreactive properties. This is consistent with murine studies which have shown that B lymphocyte-derived IL-10 primarily arises from the B1 (Ly1+) subpopulation (20). In the murine model, the autocrine production of IL-10 may be involved in the expansion of autoreactive B lymphocytes (21). Our present results suggest that, in SLE, a similar phenomenon may occur and that the abnormal production of IL-10 may contribute to the expansion of B lymphocytes with autoreactive properties. Our in vitro results, performed on non-T cells, indicate that T lymphocytes are not required for the differentiating effect of IL-10 on SLE B lymphocytes. Whether an additional effect of IL-10 on T lymphocytes also contributes to our results in the SCID mice model cannot be formally ruled out. In addition to stimulating autoreactive B lymphocytes, the increased production of IL-10 may account for the inhibition of cell-mediated immune responses observed in SLE patients (2).

In addition to monocytes and B lymphocytes, it is possible that extraimmunological sources of IL-10 influence the immunological imbalance of SLE. In mice, the placenta (22) and UV-irradiated keratinocytes (23) produce large amounts of IL-10. If this is also true in humans, it may contribute to the exacerbation of SLE by pregnancy and sun exposure (24).

These hypotheses, as well as the strong inhibition of autoantibody production in the SCID mice treated with an anti-IL-10 mAb, indicate that neutralizing the spontaneous production of IL-10 in SLE patients may represent a new way to interfere with the development of the autoimmune process. Considering the poor long-term tolerance of the immunomodulating agents currently used in this condition (24), this potential new therapeutic approach warrants investigation, particularly in those patients who require high doses of corticosteroids to maintain control of the disease.

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