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Autostimulatory effects of IL-6 on excessive B cell differentiation in patients with systemic lupus erythematosus: analysis of IL-6 production and IL-6R expression

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SUMMARY

Introducing avidin–biotin complex ELISA for anti-DNA antibody, the mechanism of *in vitro* production of anti-ssDNA antibody as well as of polyclonal immunoglobulin mediated by an IL-6–IL-6R loop was studied in patients with systemic lupus erythematosus (SLE). Regardless of the presence or absence of T cells, B cells from SLE patients could produce IgG anti-ssDNA antibody as well as total IgG without any stimulation. Low density B cells obtained by Percoll gradient density centrifugation responded to rIL-6 to produce IgG and IgG anti-ssDNA antibody. rIL-2 and rIL-4 had lesser effects on the differentiation of low density B cells. In fact, IL-6R was preferentially expressed on low density B cells from active SLE patients, as detected by anti-IL-6R MoAb, MT18, which did not inhibit IL-6 binding. SLE B cells, especially high density B cells, produced greater amounts of IL-6 in culture supernatants than did T cells, regardless of whether disease was active or inactive. Normal T cells and B cells did not produce significant amounts of IL-6. Thus, endogenous IL-6 produced by high density B cells bound to the IL-6R preferentially expressed on the low density B cells, and drove them into terminal differentiation, especially in active SLE patients. Further, addition of polyclonal anti-IL-6 or anti-IL-6R MoAb (PMl), which inhibited IL-6 binding, both inhibited IgG anti-ssDNA antibody as well as total IgG production by SLE B cells in a dose-dependent manner. These results suggest that interruption of the autocrine IL-6 loop would be of therapeutic value in SLE.

Keywords systemic lupus erythematosus IL-6 IL-6 receptor anti-DNA antibodies

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease characterized by aberrant immune regulation and excessive production of immunoglobulin and a variety of autoantibodies [1–4]. Cultures of B cells from patients with SLE show spontaneous proliferation and excessive immunoglobulin production without any stimulation [5–7].

In order to analyse the hyperactivity of B cells from SLE patients, various B cell stimulatory factors (BSF) have been used to focus attention on the regulation of B cell growth and differentiation. In an earlier study [8], we reported abnormal BSF production by T cells and the response of B cells to BSF in SLE patients. Tanaka *et al.* [9] showed that SLE B cells spontaneously produce such BSF as IL-1α, IL-4, and IL-6, and that SLE B cells spontaneously proliferate and differentiate through an autocrine mechanism of BSF.

To analyse the activation stages of B cells in normal and diseased states, a technique for B cell fractionation on the basis of cell density has been developed using Percoll discontinuous density gradient [10,11]. We have demonstrated previously that peripheral lymphocytes from patients with active SLE contain B cells at various stages of activation and that low density B cells could differentiate into immunoglobulin secreting cells in response to rIL-6 without SAC preactivation [12].

The precise mechanism of spontaneous B cell differentiation and antibody synthesis, particularly excess autoantibody production (e.g. anti-DNA antibody), is unclear [13,14]. In the present study, we present evidence that high density B cells, not T cells, are significant producers of IL-6 and that IL-6R is expressed on large B cells in SLE. We also examine the effect of blocking the autocrine IL-6 loop on polyclonal IgG and on anti-DNA antibody production.

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MATERIALS AND METHODS

Subjects

Fifteen patients with SLE (six active, nine inactive) and nine healthy volunteers approximately matched for age and sex were used in this study. All patients met more than four of the American Rheumatism Association revised criteria for the diagnosis of SLE [15]. The activity of the disease was assessed on the basis of clinical and laboratory findings according to the Schur and Sandson activity index [16]. All the patients with active SLE had three abnormalities in their serological profiles: a high titre of anti-DNA antibodies, a severe grade of hypocomplementaemia, and a large amount of immune complex in the serum. Titres of anti-DNA antibodies were assessed by RIA commercial kit (Nippon DPC, Tokyo, Japan). Highly purified double-stranded plasmid DNA from *Escherichia coli* was used for the solid phase DNA. We also evaluated isotypic specific (IgG/IgM) ssDNA or dsDNA antibody using ELISA kit (MBL, Nagoya, Japan) according to the method described by Aotsuka *et al.* [17]. At the time of this study, patients with active SLE had not been receiving steroid therapy, and eight of the nine patients with inactive SLE were receiving low doses of prednisolone (2·5–12·5 mg/day) but none were receiving cytotoxic drugs.

Preparation of B cells

Mononuclear cells were isolated from heparinized peripheral blood by Ficoll–Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The recovered cells were separated into T cells and non-T cells by two cycles of rosetting with neuraminidase-treated SRBC. B cells were obtained by depletion of remaining monocytes and T cells in the non-T cell fraction by adhesion to Petri dishes and by complement-mediated cell lysis with OKT4, OKT8, OKM1 (Ortho, Raritan, NJ) and Mo2 (Coulter, Hialeah, FL). B cell-rich fractions were further treated with 5 mM L-leucin methyl ester (LME, Sigma Chemical Co., St Louis, MO) for 40 min at room temperature (RT), utilizing the technique developed by Thiele and Lipsky [18]. After washing, B cells prepared in this fashion contained more than 95% surface immunoglobulin$^+$ cells, and less than 1% OKM1$^+$, OKT3$^+$, and OKNK$^+$ cells (as detected by fluoresceinated MoAb). In some experiments, B cells were obtained directly by magnetic separation procedures using anti-CD19 MoAb (AB1) coated magnetic beads (Dynabeads M450, Dynal A.S., Oslo, Norway) as developed by Lea *et al.* [19]. The resultant B cell population contained more than 95% CD20$^+$ (B1, Coulter) cells and less than 0·5% OKT3, OKM1, or OKNK cells.

Density gradient separation of B cells

Purified B cells were further fractionated by Percoll (Pharmacia) density gradient centrifugation, as previously described [12]. Cell recovery from the 30/40, 40/50, 50/60, 60/70% interface and from the bottom was 7 ± 6, 36 ± 12, 50 ± 16, 6 ± 5, and 3 ± 2%, respectively, in patients with SLE. High density B cells were taken from the 50–60% interface, and low density, larger B cells were from the 40–50% interface.

Culture conditions for B cell differentiation

Purified B cells (1×10^5/well) with or without equal numbers of T cells were cultured in 0·2 ml RPMI medium containing 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Gibco, Grand Isalnd, NY) in round-bottomed microtitre culture plates (Linbro, Flow Laboratories, McLean, VA) at 37°C in 5% CO_2. Total IgG production was assessed after 7 days of culturing and IgG anti-ssDNA antibody production was assessed after 12 days of culturing. All cultures were triplicated, and at the end of culturing the culture supernatant was collected and stored at −70°C until used. In some experiments, the following cytokines and/or antibodies were added to the cultures at the indicated concentrations: rIL-2 (Takeda Chemical Ind., Osaka, Japan); rIL-4 (Ono Pharmaceutical Co., Osaka, Japan); rIL-6 (Ajinomoto, Tokyo, Japan); rabbit anti-IL-2 antibody (Collaborative Research Inc., Bedford, MA); rabbit anti-IL-6 antibody (kindly provided by Dr Y. Akiyama, Ajinomoto); and as a control, non-immunized rabbit IgG (Cappel, Cochraneville, PA). Anti-IL-6R MoAb (PM1, kindly donated by Dr T. Taga, Institute for Molecular and Cellular Biology, Osaka Univ., Osaka, Japan [20]) and control mouse myeloma IgG1 (Sigma) were also added to the cultures for the inhibition study of B cell differentiation.

Assay for IL-6 production

Purified T cells, unfractionated B cells, or fractionated B cells by Percoll density gradient were cultured in RPMI medium with 10% FCS in microtitre culture plates for up to 5 days. The culture supernatant was collected and the amount of IL-6 in the supernatant was determined by two methods, namely, bioassay and immunoassay. For the bioassay, IL-6-dependent hybridoma cells, MH-60. BSF-2 (1×10^4) (kindly donated by Dr T. Matsuda, Osaka Univ., Osaka [21]) were cultured in 0·2 ml RPMI-10% FCS with serially diluted supernatant or rIL-6 as standard for 48 h in triplicate cultures and pulsed with [^{3}H]TdR for the last 16 h. The cells were harvested and incorporated radioactivity was measured. Immunoassay was performed by a commercially available ELISA kit (Genzyme Co., Boston, MA). Results were expressed as units calculated from the data of standard rIL-6.

Assay of total IgG production

For the evaluation of polyclonal B cell differentiation, total IgG in culture supernatants was determined by sandwich ELISA, as described previously [12]. The absorbance at 492 nm was measured by easy reader EAR400 (SLT Instrument, Grödig, Austria).

Assay for anti-ssDNA production

IgG anti-ssDNA antibody was measured by ELISA using an avidin–biotin complex system [22] by which sensitivity was improved over the original avidin–biotin system. Microtitre plates were coated with 100 μl of poly L-lsain (mol. wt 30 000, 10 μg/ml, Sigma) in 1·5 M Tris buffer (pH 8·5) overnight at 4°C. Then, 100 μl of ssDNA (10 μg/ml) in Tris buffer was added to the plates. ssDNA was prepared by heat denaturation of calf thymus DNA (high mol. wt, Boehringer-Mannheim GmbH, Germany) for 5 min at 90°C. PBS with 1% BSA was added to the wells to inhibit non-specific binding to the unsaturated areas. After washing, undiluted culture supernatants and serially diluted anti-ssDNA positive sera were added to the plates for 2 h at RT. The plates were washed and incubated with biotinylated goat anti-human IgG (EY laboratories, San

Mateo, CA) at a dilution of 1000 × in PBS with 1% goat sera for 2 h at RT. After washing, 100 μl of avidin–biotin–peroxidase complex (ABC Elite kit, Vector Laboratories, Burlingame, CA), which had been prepared by mixing each drop of avidin and biotin-peroxidase reagent diluted to 10 ml PBS for 2 h, was added, and the colour was developed. This ABC-ELISA was 5-fold more sensitive than the original avidin–biotin ELISA.

Flowcytometric analysis of IL-6R expression

Fractionated lymphocytes were treated with anti-IL-6R MoAb (MT18, [20]; kindly donated by Dr T. Hirano, Osaka Univ., Osaka) or control myeloma IgG2b (Sigma) at the appropriate dilution in PBS with 0·02% NaN_3 and were stained with fluorescein-conjugated $F(ab')_2$ goat anti-mouse IgG (Tago). Stained cells were analysed by Cytoron (Ortho Diagnostic Systems, Tokyo, Japan) on a linear scale.

Statistical analysis

Two-tailed paired Student's and Wilcoxon's *t*-tests were used where appropriate.

RESULTS

Production of total (polyclonal) IgG and IgG anti-ssDNA antibodies by B cells from SLE patients

Purified B cells (1×10^5) with or without T cells (1×10^5) from normal controls or SLE patients were cultured and spontaneous production of total IgG and anti-ssDNA antibodies in the culture supernatants were assayed by ELISA. The maximum rate of total IgG production by SLE B cells was observed on day 7 and the amounts of total IgG and IgG anti-ssDNA antibodies reached a plateau around the 10th to the 14th day. We also examined the production rates of low and high density B cells, respectively. We found that the low density B cells produced major amounts of total IgG and IgG anti-ssDNA, and the production rate of low density B cells is similar to that of total B cells. In contrast, high density B cells had little effect on rates and amounts of total B cell production. Moreover, SLE B cells could be differentiated as usual throughout 7 and 12 day culturing periods without T cell help. Considering these results, production of total IgG was thereafter determined on day 7 and of IgG anti-ssDNA antibody on day 12. Unstimulated normal B cells without or with T cells produced polyclonal IgG (92 ± 53 ng/ml without and 191 ± 111 ng/ml with T cells) (Fig. 1a). In contrast, unstimulated B cells from SLE patients markedly produced polyclonal IgG. The addition of T cells did not materially enhance production of total IgG and actually decreased production in some active patients (699 ± 618 ng/ml without and 849 ± 628 ng/ml with T cells, respectively) (Fig. 1a). Similar results were observed in the production of total IgM (data not shown).

As for the production of IgG anti-ssDNA antibody, normal B cells without or with T cells produced only marginal amounts (0·066 ± 0·008 without and 0·073 ± 0·012 with T cells), as expressed by absorbance at 492 nm (Fig. 1b). In contrast, B cells from SLE patients spontaneously produced large amounts of IgG anti-ssDNA antibody (Fig. 1b). B cells from active patients seem to produce greater amounts of IgG anti-ssDNA antibody (0·415 ± 0·097 for active SLE patients vs 0·160 ± 0·059 for inactive SLE patients). The addition of T cells had no significant effect on the production of IgG anti-ssDNA antibody (0·262 ± 0·148 without vs 0·302 ± 0·169 with T cells) (Fig. 1b). We also assessed production of IgM anti-ssDNA antibody and anti-dsDNA antibodies, but the amounts *in vivo* and *in vitro* were so low as to be undetectable in seven of the 15 patients. In the other eight patients, the amounts produced *in vitro* were much lower than that of IgG anti-ssDNA antibody, even as assessed by ABC enhanced ELISA (data not shown). The patient's serum levels of IgG anti-ssDNA (U/ml) were correlated with levels produced by unstimulated B cells from both active and inactive SLE patients in culture ($y = 2{\cdot}232e^{-2} + 0{\cdot}220\log(x)$; $r = 0{\cdot}767$; $P < 0{\cdot}05$).

Effects of various cytokines and their antibodies on total IgG and anti-ssDNA antibody production by SLE B cells

SLE B cells were cultured with or without rIL-2, rIL-4, rIL-6, rabbit control IgG, anti-IL-2, and/or anti-IL-6, in order to determine the effect of these cytokines and antibodies on total IgG and IgG anti-ssDNA antibody production. High density B cells responded marginally to these cytokines, whereas low density B cells produced considerable amounts of total IgG and IgG anti-ssDNA antibody without cytokines, and these productions were enhanced by rIL-6 (Table 1). rIL-2 and rIL-4 did not always show effective enhancement of total IgG and anti-ssDNA antibody production, and rIL-4 rather decreased anti-ssDNA antibody. Furthermore, addition of anti-IL-6 antibody resulted in a notable decrease in the spontaneous production of both total IgG and IgG anti-ssDNA antibody by SLE B cells (Table 1). This decrease by anti-IL-6 antibody was observed even in the presence of rIL-2. The substitution of anti-IL-2 antibody for anti-IL-6 antibody resulted in less inhibition. These results suggest that the differentiation of SLE B cells is driven preferentially by IL-6, especially by endogenous IL-6. Therefore in the following experiments, we analysed the production of endogenous IL-6.

IL-6 production by T cell or B cell populations from SLE patients

Results from a patient with active SLE showed that T cells produced marginal amounts of IL-6 during 5 days of culturing, whereas B cells produced significant amounts of IL-6 on the 1st day and augumented its production during the next 3 days (data not shown). Therefore, the amounts of IL-6 in culture supernatants of T cell or B cells during 3 day culture periods were measured by proliferation of MH60. BSF-2 cells. It has been reported that none of the other cytokines tested, such as recombinant human IL-1α, IL-1β, IL-2, IL-3, IL-4, G-CSF, IFN-β, IFN-γ, murine IL-3, IL-4, IL-5, or NGF could induce [^{3}H]TdR uptake in MH60.BSF2 [21]. The lack of effect of these cytokines on [^{3}H]TdR uptake was confirmed on an MH60. BSF2 culture maintained in our laboratory. Again, B cells were separated by anti-CD19 coated magnetic beads. T cells from normal controls produced marginal amounts of IL-6, but B cells produced significant amounts of IL-6 in some cases (2·4 ± 1·1 U/ml, 6·0 ± 4·6 U/ml, respectively) (Fig. 2). In patients with SLE, a slight production of IL-6 by T cells was observed, whereas B cells produced large amounts of IL-6 (4·7 ± 2·4 U/ml, 30·2 ± 21·1 U/Ml, respectively, $P < 0{\cdot}001$) (Fig. 2). Disease activity was not related to the amounts of IL-6 production (active patients, 32·4 ± 16·8 U/ml, vs inactive patients, 28·7 ± 25·5 U/ml, respectively).

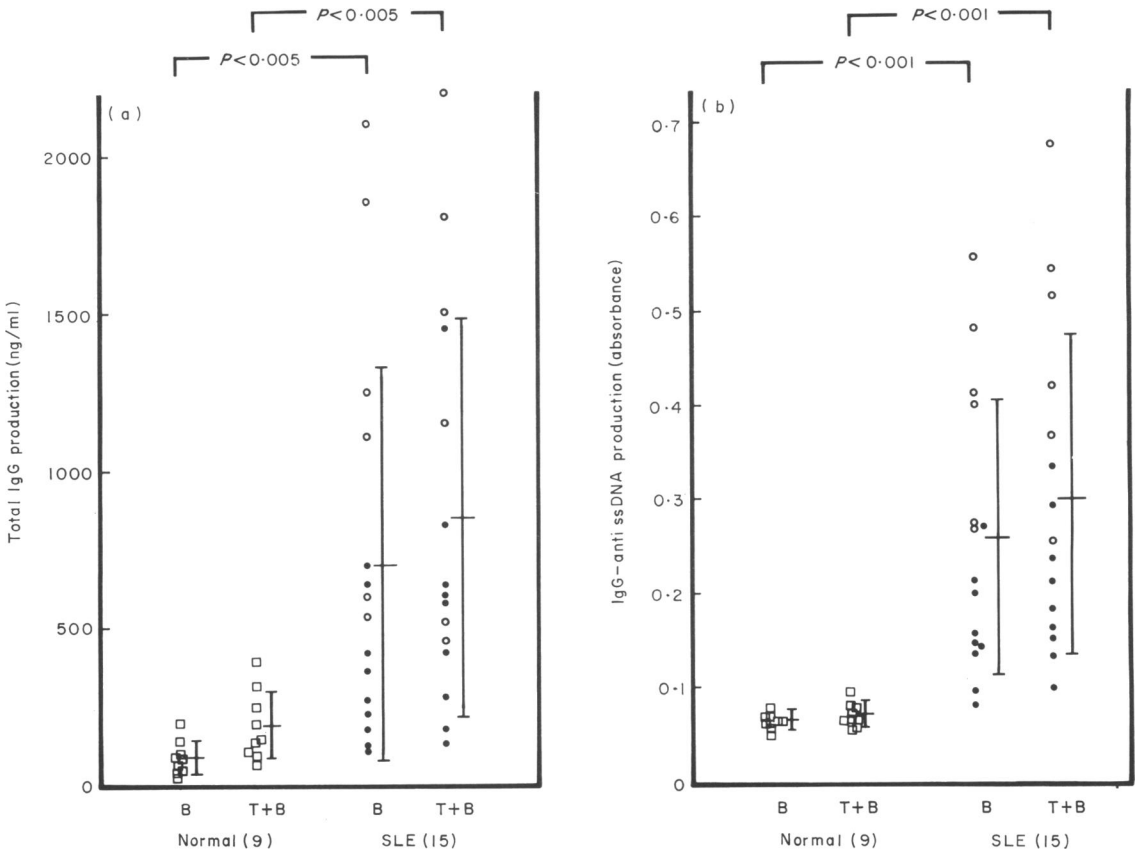

Fig. 1. Spontaneous total IgG and IgG anti-ssDNA antibody production in culture supernatants by B cells (1×10^5) with or without T cells (1×10^5). (a) Total IgG production is expressed as ng/ml determined by ELISA on day 7. (b) IgG anti-ssDNA production is expressed as absorbance at 492 nm, as determined by ABS-ELISA on day 12. Each point (normal control (□), active SLE (○) and inactive SLE (●)) represents the mean of amounts produced in triplicate cultures from individual persons. The bars indicate means and SD in each group.

IL-6 production by Percoll-fractionated SLE B cells

The absolute amounts of IL-6 produced by T cells, B cells and fractionated B cells from SLE patients were measured by ELISA. High density B cells produced greater amounts of IL-6 than did low density B cells (Table 2).

Expression of IL-6 receptor on SLE lymphocytes

To determine whether or not IL-6R was expressed on SLE B cells, we used anti-IL-6R MoAb (MT 18) which did not inhibit the binding of IL-6 and could detect IL-6R expression even in the presence of an autocrine mechanism [20]. A slight expression of IL-6R was observed on T cells from both normal controls and active SLE patients (Fig. 3a, b). A slight expression of IL-6R was also observed on whole B cells from active SLE patients but not on whole B cells from normal controls (Fig. 3c, d) nor from patients with inactive SLE. According to the Percoll density fractionation, low density B cells from active SLE patients preferentially expressed IL-6R. High density B cells from neither normal controls nor active patients expressed IL-6R (Fig. 3e, f) nor did those from inactive SLE patients. Low density B cells from normal controls did not express IL-6R (Fig. 3g). Low density B cells from all four recently diagnosed active patients significantly expressed IL-6R (Fig. 3h), but those from only two out of seven inactive patients showed any such expression.

Effect of anti-IL-6 antibody on polyclonal B cell differentiation and anti-ssDNA antibody production in active SLE

Rabbit polyclonal anti-IL-6 antibody (or normal rabbit IgG, used as a control) was added in graded concentrations to the initial cultures of B cells (with or without T cells) from five active SLE patients. Although the possibility of communication between T and B cells sustained by any cytokines produced by T cells except for IL-6 can not be excluded, IgG production was inhibited by the addition of anti-IL-6 antibody. At concentrations of 25 μg/ml, anti-IL-6 reduced total IgG production in 7-day cultures by 76·1–96·1% in T plus B cell cultures (Fig. 4a), and by 82·4–94·2% in B cell cultures (Fig. 4b).

At concentrations of 25 μg/ml, polyclonal anti-IL-6 antibody reduced IgG anti-ssDNA antibody in culture supernatants from 12-day cultures by 51·2–86·3% in T plus B cell cultures (Fig. 4c) and by 62·4–84·7% in B cell cultures (Fig. 4d). Normal rabbit IgG (1–25 μg/ml) was added to the cultures in a similar fashion but no detectable effect was observed in ELISA for both total IgG and ssDNA antibody.

Table 1. Effects of various cytokines and their antibodies on total IgG and IgG anti-ssDNA antibody production by SLE B cells

Percoll density fractionated cells	Cultured with		Production of	
	Cytokine	Antibody	Total IgG (ng/ml)	IgG anti-ssDNA (OD)
Case 1				
High-density B cells	—	—	20	ND
(50–60%)	rIL-2	—	30	ND
	rIL-4	—	25	ND
	rIL-6	—	35	ND
Low-density B cells	—	—	380	0·549
(40–50%)	rIL-2	—	175	0·336
	rIL-4	—	740	0·231
	rIL-6	—	1300	0·669
Case 2				
High-density B cells	—	Control IgG	110	0·207
(50–60%)	rIL-2	Control IgG	90	0·181
	rIL-4	Control IgG	170	0·065
	rIL-6	Control IgG	200	0·217
Low-density B cells	—	Control IgG	840	0·337
(40–50%)	rIL-2	Control IgG	1070	0·284
	rIL-4	Control IgG	1030	0·126
	rIL-6	Control IgG	1860	0·380
	—	Anti IL-2	780	0·212
	—	Anti IL-6	120	0·079
	rIL-2	Anti IL-6	165	0·113

Fractionated B cells (1×10^5/well) from active SLE patients were cultured with or without the following cytokines or antibodies: rIL-2 (1 U/ml), rIL-4 (100 U/ml), rIL-6 (20 U/ml), rabbit control IgG (10 μg/ml), anti IL-2 (10 μg/ml), and/or anti IL-6 (10 μg/ml) in triplicated microcultures. The unit activity designations of cytokines were determined by the providers. SDs of triplicate measures were < 15% in total IgG production, < 10% in IgG-ssDNA antibody production, respectively. The data are representative of five experiments.

Effect of anti-IL-6R MoAb on SLE B cell differentiation

Anti-IL-6R MoAb PM1, which blocked the binding of IL-6, was also added to the B cell cultures from two active SLE patients, in addition to anti-IL-6. Total IgG (Fig. 5A) and IgG anti-ssDNA antibody (Fig. 5B) in culture supernatants was reduced by the addition of anti-IL-6R MoAb or anti-IL-6 antibody in a dose-dependent manner.

DISCUSSION

In our previous paper [12], we showed that low density B cells from active SLE patients could directly differentiate into immunoglobulin secreting cells in response to rIL-6. In the present study, we further analysed the mechanism of excessive SLE B cell differentiation as mediated by autocrine production of IL-6. A sensitive ABC-ELISA was used to determine anti-ssDNA antibody production, and enabled us to analyse the involvement of IL-6 in the multistage differentiation of polyclonal B cells and anti-DNA antibody secreting B cells.

We first determined which cytokines affected the differentiation of B cells from SLE patients. Since rIL-6 was more effective than rIL-2 or rIL-4 in driving low density B cells to differentiate, and since the spontaneous differentiation was inhibited by anti-IL-6 antibody but not by anti-IL-2 antibody regardless of the presence of rIL-2, we concluded that IL-6 plays a major role in the later stages of SLE B cell differentiation, especially in active SLE patients, even though it had been reported that IL-2 was one of the essential differentiation factors of B cells [23,24] and that IL-4 could induce differentiation of high density B cells [25]. Modulatory (both inhibiting and promoting) roles of IL-4 on the secretion of immunoglobulin was reported to depend upon the differentiation states of B cells [26]. A suppressive effect of IL-2 on immunoglobulin secretion is generally not observed in the stimulation of normal B cell cultures. IL-2 induces the IL-6 responsiveness of SAC-activated normal B cells to differentiate in later stages of the culture, and that the addition of anti-IL-6 antibody reduced IgG secretion by 50–70%, indicating an important role of autocrine IL-6 in the IL-2 driven B cell differentiation [27]. These results raise the possibility that IL-2 and IL-4 have reciprocal effects on each other's receptors and thereby affect the expression of IL-6R and its associated molecule.

In earlier studies [9], culture supernatants of B cells from patients with SLE had various B cell stimulating activities. They found that the supernatants possessed IL-1, IL-4 and IL-6 activities, but did not possess IL-2 and IFN-γ activities, and that those BSF-producing cells were enriched in the higher density fraction by Percoll density gradient centrifugation. They also reported that the cells responding to the BSF produced by SLE B cells were higher density but not lower density B cells.

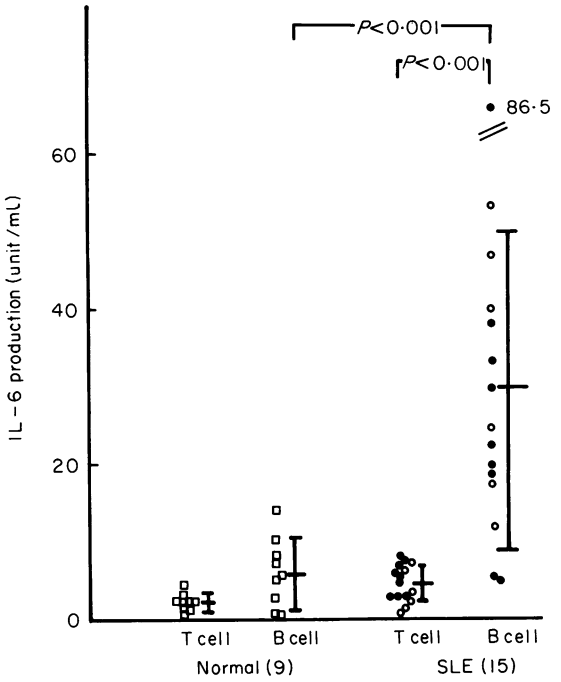

Fig. 2. IL-6 production by T cells or B cells from nine normal controls (□), nine inactive (●) and six active (○) patients with SLE. E-rosetted T cells or B cells isolated by anti-CD19 coated magnetic beads were cultured for 3 days and the amounts of IL-6 in culture supernatants were assessed by the proliferation of IL-6 dependent MH60. BSF-2 cells. Results were expressed as U/ml calculated from the standard curve.

Table 2. Spontaneous IL-6 production by T cells, whole B cells, and fractionated B cells according to Percoll density

		B cells		
Case	T cells	High density (50 to 60%)	Low density (40 to 50%)	Whole
1	1·0±0·1	26·2±1·6	12·4±0·5	17·5±1·5
2	4·8±0·3	24·3±1·5	14·5±0·8	18·4±1·6
3	1·2±0·1	38·2±2·4	25·5±1·5	29·3±2·4

Amounts of IL-6 produced in 3 day-culture supernatant were determined by ELISA. B cells were purified from non-T cells by cytokilling with anti-CD4, CD8, CD11b, and CD14 MoAb and C, followed by LME treatment. Data are expressed as mean±s.d. (U/ml) of three patients with active SLE.

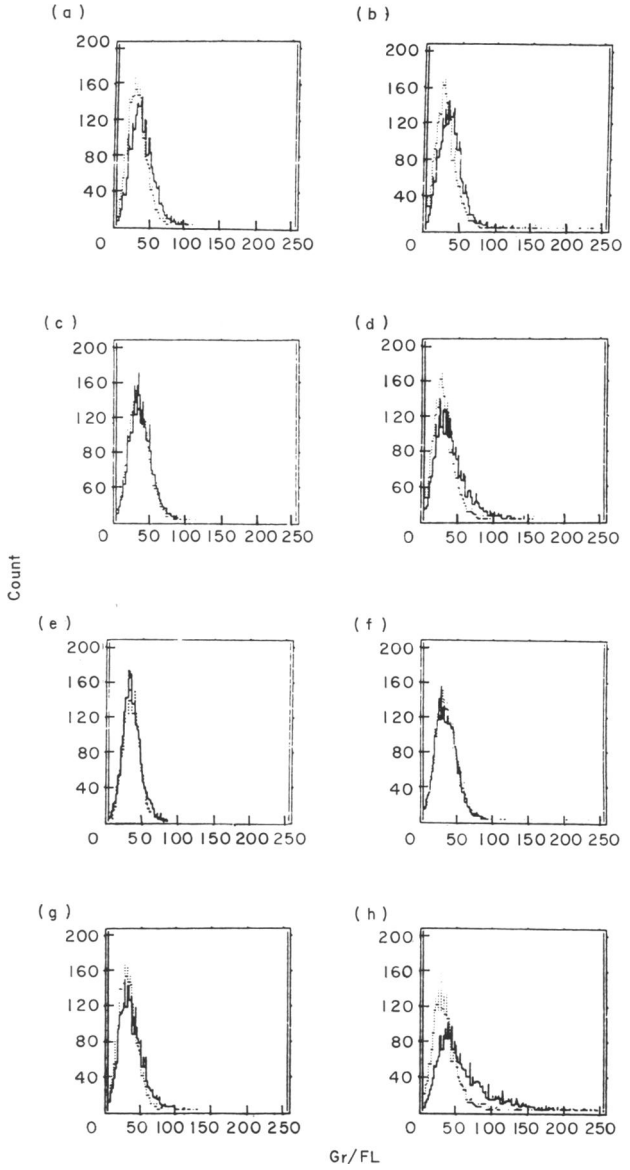

Fig. 3. Flow-cytometric analysis of IL-6R expression on T cells and B cells. Anti-IL-6R MoAb (MT18) or control IgG2b was added to the cells and indirect fluoro-staining with FITC-F(ab′)$_2$ anti-mouse IgG was performed. Five thousand stained cells were analysed by Cytoron on a linear scale. Representative results from a normal control (a, c, e, g) and from a patient with active SLE (b, d, f, h) are shown as follows: T cells (a, b); whole B cells (c, d); high density B cells (e, f); and low density B cells (g, h).

However, contrary to the results of Tanaka *et al.* [9], we found that the responder SLE B cells for both exogenous rIL-6 and endogenous IL-6 from SLE B cells seem to belong to the low density B cells. The difference between our results and those reported by Tanaka *et al.* [9] might stem from several causes. First, the density fractionations used by Tanaka *et al.* were 45, 55 and 65%, while ours were 40, 50 and 60%. Nakagawa *et al.* reported that the higher density fractions contained resting and early activated B cells and the terminal differentiating immunoglobulin secreting cells, whose density is concentrated again following the low density B cell stage [10]. Secondly, Tanaka *et al.* [9] evaluated the BSF response by the number of plaque-forming cells while we used ELISA to measure the absolute amounts of secreted antibodies in culture supernatants. Culture periods also might affect the results of BSF response. For the SAC-activated large, low density B cells from normal donors, it was reported that more than 7 days were required for the IL-6 response [10,12]. Furthermore, it was also reported that the PFC

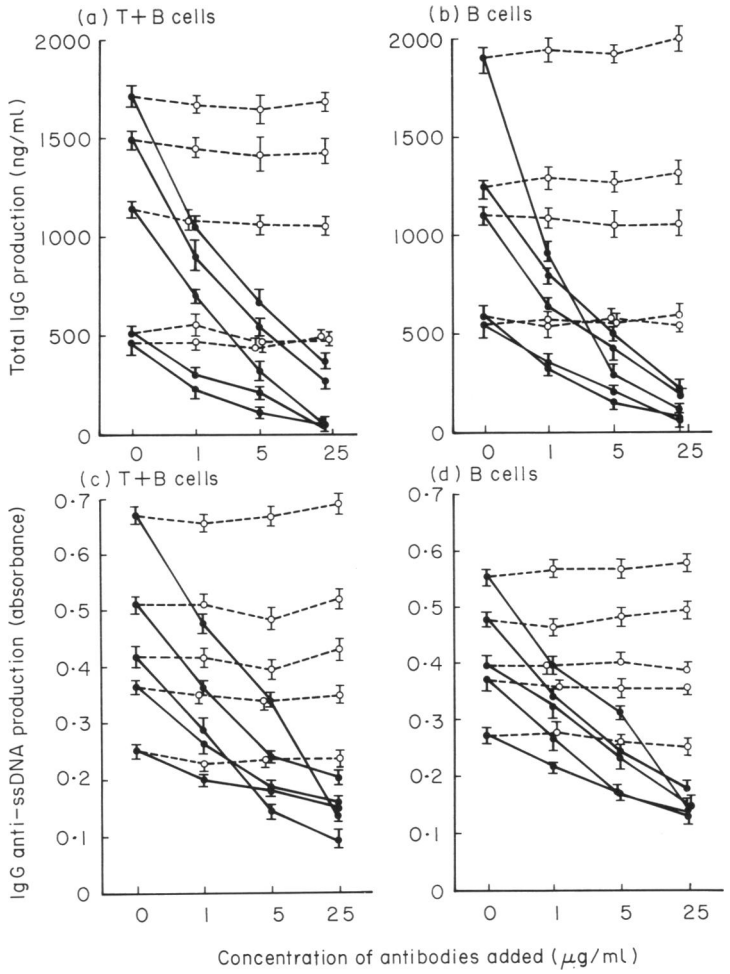

Fig. 4. Effect of rabbit anti-IL-6 antibody on total IgG and IgG anti-ssDNA antibody production with or without T cells from active patients with SLE ($n = 5$). At the initiation of the cultures, anti-IL-6 antibody (——) or normal rabbit IgG (- - -), as a control was added at the indicated concentrations. Amounts of total IgG in 7 day-culture supernatants of B cells plus T cells (a) and B cells alone (b) were determined by ELISA. Curves represent means ± s.d. of triplicated cultures. The amounts of IgG anti-ssDNA antibody in 12 day-culture supernatants of B cells plus T cells (c), and B cells alone (d) were determined by ABC-ELISA. Curves represent means ± s.d. of triplicated cultures.

responses to T cell soluble factors by the SAC-activated low density B cells was significantly higher in patients with SLE as compared with normal controls [28]. In the present study, we also observed that the low density B cells expressed IL-6R preferentially. Thus, endogenous IL-6 produced by high density B cells bound to IL-6R preferentially expressed on low density B cells and drove them into terminal differentiation, especially in active SLE patients.

In order to analyse the expression and function of IL-6R molecules on spontaneously activated SLE B cells, we used two MoAbs, MT18 and PM1, which recognize different epitopes on human IL-6R [20]. PM1 blocked the binding of ^{125}I-IL-6 to myeloma cell line U266, whereas MT 18 does not compete with IL-6 for the binding to IL-6R. Addition of anti-IL-6 and anti-IL-6R (PM1) into SLE B cell cultures inhibited spontaneous B cell differentiation in a dose-dependent manner, suggesting that an IL-6 autocrine mechanism plays a critical role in SLE B cell hyperactivity. In addition, the excessive production of IL-6 observed in high density B cells, but not in T cells, supports the suggestion of an autostimulatory mechanism of B cell lineage. It was also reported that culture supernatants of T depleted PBL had elevated levels of IL-6, and anti-IL-6 antibody inhibited polyclonal immunoglobulin synthesis *in vitro* [29].

IL-1 plays an essential role in B cell activation by directly delivering an early requisite signal to the B cells [30,31]. Since it has been shown that IL-6 production is enhanced by IL-1 in various types of cells [32,33], there is a possibility that IL-1 and IL-1R induction in precursor B cells would promote the development of later differentiation mediated by IL-6-IL-6R in patients with SLE.

One critical point for increased IL-6 in SLE B cells may be the preparation of the B cell population. For some measures of IL-6 production, CD19$^+$ B cells were positively isolated by anti-CD19 MoAb coated magnetic beads, yielding more than 95% CD20 positive and less than 0·5% CD3, CD14, and CD56 positive cells. It was reported that the cells isolated by magnetic beads are still in a resting state after isolation, as judged by cell cycle analysis and by various parameters for cell activation, and that the isolation procedure does not interfere with either

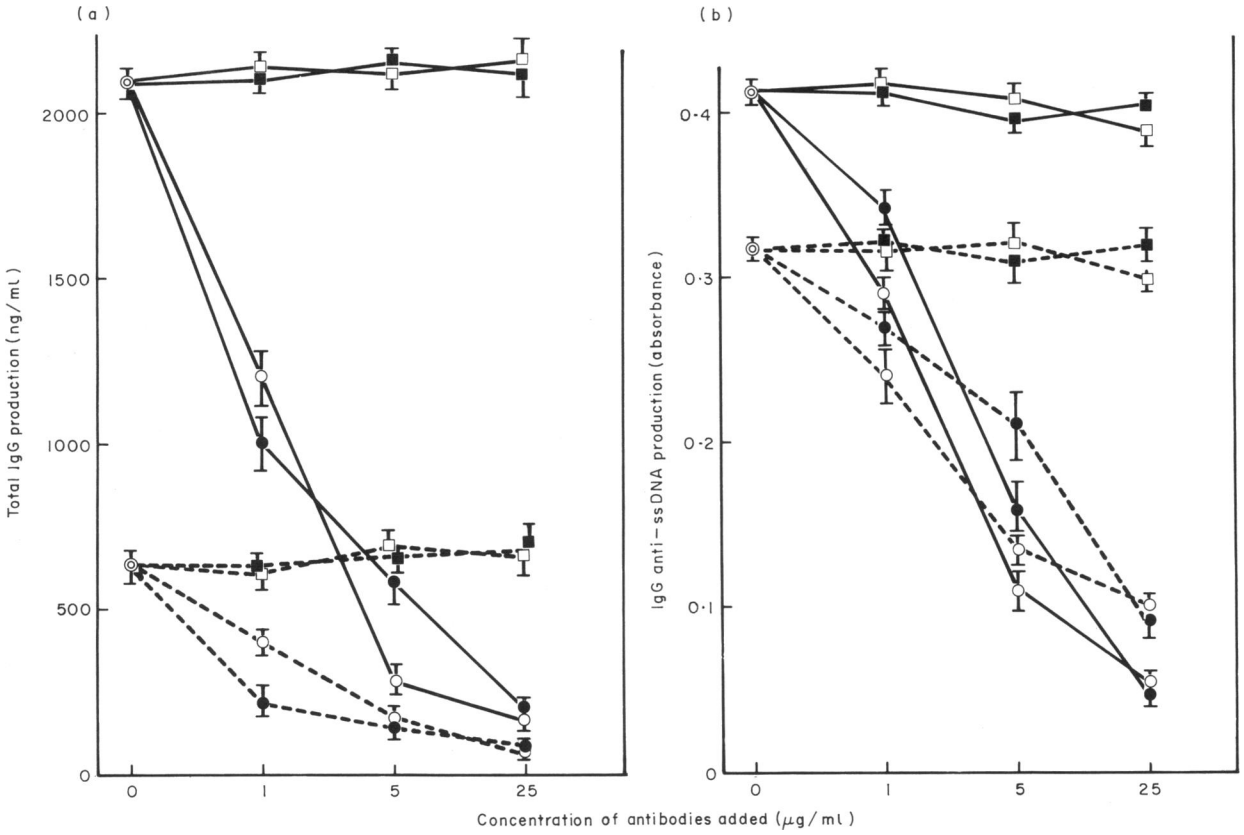

Fig. 5. Effect of anti-IL-6 antibody and anti-IL-6R MoAb on the production of total IgG (A) or IgG anti-ssDNA antibodies (B) by purified SLE B cells. At the initiation of the cultures, anti-IL-6 (○), normal rabbit IgG (□), anti-IL-6R, PM1 (●), and mouse IgG1 (■) were added at the indicated concentrations. The results (mean ± s.d.) of triplicated cultures from two representative cases (——, - - -) of active SLE patients are shown.

activation or proliferation/differentiation of CD19 selected cells compared with negatively isolated cells in different functional assays [34]. In fact, a significant difference was not observed in the amounts of IL-6 production in B cells isolated positively by anti-CD19 MoAb coated magnetic beads as compared to negatively isolated B cells. Further, culture supernatant derived from the same numbers of T cells, monocytes and NK cells as found contaminating the SLE B cell population did not produce detectable levels of IL-6. Considering these facts, we conclude that participation by T cells and monocytes is not essential for IL-6 mediated spontaneous B cell differentiation in SLE.

There are two major models explaining autoantibody production in SLE, abnormal polyclonal activation of B cells, and clonal selection by self-antigens [35,36]. Considering the inhibition of overproduction of anti-DNA antibody, as well as of polyclonal immunoglobulin by the addition of both anti-IL-6 and anti-IL-6R antibodies, we suggest that polyclonal activation of B cells is driven by autocrine IL-6 and contributes to the generation of anti-DNA antibody as well as hypergammaglobulinaemia in SLE patients. Therefore, interruption of the autocrine IL-6 loop could lead to reduced immunoglobulin production, and thus be of potential therapeutic value in SLE. Analysis of the intracellular IL-6 transduction system through gp 130 molecule [37] will further elucidate our understanding of the mechanism of abnormal B cell differentiation in SLE.

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