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ARTICLE *in* CLINICAL & EXPERIMENTAL IMMUNOLOGY · SEPTEMBER 1992

Impact Factor: 3.04 · DOI: 10.1111/j.1365-2249.1992.tb06936.x · Source: PubMed

Clin. exp. Immunol. (1992) **89**, 223–229

IL-4 down-regulates the surface expression of CD5 on B cells and inhibits spontaneous immunoglobulin and IgM-rheumatoid factor production in patients with rheumatoid arthritis

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(Accepted for publication 1 April 1992)

SUMMARY

There is evidence to suggest that CD5$^+$ B cells may be associated with autoimmunity, e.g. they are increased in patients with rheumatoid arthritis (RA). In this study, we found that the expression of CD5 on RA B cells increased spontaneously, following culture for up to 4 days *in vitro* in the absence of T cells, supporting the idea that the CD5$^+$ B cell possesses distinctive features. The spontaneous increase of CD5 expression was down-regulated by recombinant IL-4 (rIL-4). Other cytokines studied (rIL-1α, rIL-2, rIL-5, rIL-6) did not alter CD5 expression. Studies of antibody production showed that rIL-4 could reduce spontaneous production of total IgG and IgM in non-stimulated RA T plus B cell cultures. Spontaneous production of IgM rheumatoid factor (IgM-RF), measured by a newly developed avidin-biotin complex ELISA, was also reduced by rIL-4. Furthermore, rIL-4 reduced the increase in IgM-RF production observed on stimulation with *Staphylococcus aureus* Cowan I (SAC) or pokeweed mitogen (PWM). Thus, IL-4 might act as a regulator of the development of abnormal B cell differentiation in patients with RA.

Keywords CD5$^+$ B cells IL-4 rheumatoid arthritis

INTRODUCTION

Recent studies have demonstrated that the pan-T cell antigen [1] CD5 is also expressed by subsets of B cells associated with autoantibody production [2,3], and that CD5$^+$ B cells are increased in patients with rheumatoid arthritis (RA) [2,4], hyperthyroid Graves' disease [5], and several other autoimmune diseases [6,7]. It has been reported that CD5 expression by activated B cells is specifically inhibited by IL-4; this has been demonstrated for normal splenic B cells [8], tonsillar B cells, and chronic lymphocytic leukaemia B cells (B-CLL) [9]. IL-4, originally described as a T cell-derived lymphokine, B cell stimulating factor 1 (BSF-1), has been found to induce proliferation of B cells and T cells [10,11], to stimulate IgE production [12,13], and to induce CD23 expression on B cells [14]. Human rIL-4 also induces highly purified B cells preactivated with *Staphylococcus aureus* Cowan I (SAC) to produce IgG and IgM [15]. In contrast, it was shown that addition of rIL-4 to SAC and rIL-2 co-stimulated B cells suppressed both proliferation and differentiation [16]. It was also reported that rIL-4 caused a marked suppression of IgM [13].

However, regulatory effects of IL-4 on CD5$^+$ B cells in autoimmune diseases are still unclear. In this study, we examined the effect of IL-4 on CD5 expression on B cells and on immunoglobulin production, including autoantibodies such as rheumatoid factor (RF) in patients with RA.

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PATIENTS AND METHODS

Patients

Subjects included 12 patients with definite and classical RA, diagnosed according to the criteria of the American Rheumatism Association (ARA) [17], and 10 sex- and age-matched normal subjects from the clinical and laboratory staff. Clinical characteristics of the 12 patients are listed in Table 1. All patients were being treated with a non-steroidal anti-inflammatory drug (NSAID) or with less than 5 mg/day prednisolone. The serum of all patients had more than 2560 titre by RA haemagglutination assay (RAHA).

Cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque density gradient. PBMC were separated into T and non-T enriched fractions with a sheep erythrocyte rosetting technique. Monocytes were depleted from the non-T cell fraction by adherence on plastic Petri dishes. The remaining non-T cells were further depleted of residual T cells, natural killer (NK) cells, and monocytes by cytokilling with OKT4,

OKT8, OKM1 (Ortho Diagnostic Systems, Raritan, NJ), Mo2 (Coulter Immunology, Hialeah, FL) MoAbs and rabbit complement. The resultant B cell-enriched population contained $<1\%$ $OKT3^+$ cells, $<1\%$ $OKT1^+$ cells, $<2\%$ $OKNK^+$ cells (Ortho), and $>90\%$ cells bearing surface immunoglobulin. T cell fractions were treated with mitomycin C (MMC) (Kyowa Hakko Ltd., Tokyo, Japan) at 25 μg/ml at 37°C for 30 min.

Recombinant cytokines

Recombinant human IL-1α (the specific activity was 2×10^7 U/mg protein) [18] was kindly provided by Otsuka Pharmaceutical Co. (Osaka, Japan). Recombinant human IL-2 (1 U = 7·5 ng/ml of purified IL-2) [19] was kindly provided by Takeda Chemical (Osaka, Japan).

Recombinant human IL-4 was produced by Chinese hamster ovary cells transfected with human IL-4 cDNA. Recombinant IL-4 was purified in three steps: Sepralyte C-1, Mono S and Superlose 12. Its purity was estimated as above 99%. These IL-4 preparations were kindly supplied by Ono Pharmaceutical Co. (Osaka, Japan). The specific activity was $1{\cdot}0 \times 10^6$ U/mg protein [20].

Recombinant human IL-5 expressed in Chinese hamster ovary cells purified by procedures reported by Tsujimoto [21,22] was kindly provided by Suntory Ltd. (Osaka, Japan). The preparation was shown to be biologically active in an *in vitro* assay for IgM secretion with a specific activity of $1{\cdot}7 \times 10^3$ U/mg [22].

Recombinant human IL-6 (specific activity $1{\cdot}7 \times 10^7$ U/mg protein) [23] was kindly provided from Ajinomoto Ltd. (Kanagawa, Japan).

Cell culture

For the kinetic analysis of CD5 expression, 1×10^6 enriched B cells were cultured with or without cytokines (rIL-1α, rIL-2, rIL-4, rIL-5, rIL-6) at the indicated concentrations in 1 ml of RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 10^3 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (FCS) (GIBCO, Grand Island, NY) for 2–6 days at 37°C in a humidified 5% CO_2 atmosphere. B cells were harvested and stained for flow cytometric analysis. In order to examine immunoglobulin and RF production, 5×10^5 enriched B cells and 5×10^5 MMC-treated T cells were cultured in 1 ml culture medium with or without 100 U/ml rIL-4 for 13 days. In some experiments, B cells and T cells without MMC treatment were cultured with the addition of formaldehyde-fixed SAC (final dilution 1:1000; GIBCO, Burlington, Ontario, Canada) or 1 μg/ml of pokeweed mitogen (PWM) (Sigma, St Louis, MO). All the cultures were duplicated. At the end of the culture period, the supernatant was harvested by centrifugation at 400 ***g*** for 5 min.

Immunofluorescence staining and analysis

Direct immunofluorescence staining was performed by using FITC-conjugated monoclonal anti-CD5 (Dakopatts, Denmark) and PE-conjugated B1 (CD20, Coulter Immunology). Two-colour immunofluorescence staining was analysed by Cytoron (Ortho Diagnostic Systems, Tokyo, Japan) equipped with an argon laser operating at 488 nm. Forward angle and 90° light scatter gates were established to exclude dead cells and cell debris from analysis. Fluorescence signals from the accumulated PE in cells were collected by the red photomultiplier and fluorescence signals from the accumulated FITC in cells were collected by green photomultiplier, converted to digital format and processed for storage and display in two parameters. Autofluorescence of unlabelled cells on flow cytometric analysis was used as negative control. The percentages of positive cells were expressed by counting the labelled cells which exceeds the upper limit of autofluorescence of control cells. Data analysed by flow cytometry were expressed as mean fluorescence intensity (MFI) and/or the percentages of positive cells.

Measurement of immunoglobulin and RF

Immunoglobulin secretion was determined by ELISA. Polystyrene microtitre wells (Nunc, Roskilde, Denmark) were incubated with anti-human IgG or IgM (Cappel Laboratories, Malvern, PA) at a concentration of 1 μg/ml, in carbonate buffer overnight at 4°C. After washing with PBS-Tween three times, culture supernatants and IgG or IgM standards (Cappel) were added to their respective wells and incubated at room temperature for 2 h. The wells were washed three times, and peroxidase-conjugated $F(ab')_2$ goat anti-human IgG or IgM (Cappel) was added to the wells. The plates were incubated for 2 h at 37°C and then washed three times. Finally, substrate solution (40 mg *o*-phenylenediamine dehydrochloride (Sigma), 40 μl H_2O_2 in 100 ml citrate phosphate buffer) was added and the colour was allowed to develop for 15 min. Absorption of light at 492 nm was determined using an EIA reader (Dynatic Instruments, Santa Monica, CA) and the concentration of immunoglobulin was determined by interpolation from the standard curve.

For the measurement of IgM-RF, rabbit IgG (5 μg/ml, Cappel) was coated on the wells. Non-specific binding was blocked by incubating the wells with 2% bovine serum albumin (BSA) for 2 h at room temperature. After culture supernatants and reference sera were applied, biotinylated goat anti-human IgM (EY Laboratories, San Mateo, CA) diluted with 1% goat serum containing PBS was added and incubated for 2 h. During this period, peroxidase conjugated-avidin biotin complex (Vector Laboratories, Elite, Burlingame, CA) was prepared, and applied to the wells. After washings, colour was developed and measured.

Statistical analysis

Results were analysed using the Wilcoxon rank sum test for paired and unpaired data Non-parametric tests were used, since the data were not normally distributed.

RESULTS

Percentage of $CD5^+$ B cells

The percentage of $CD5^+$ B cells in the peripheral blood was significantly higher in RA patients ($21{\cdot}2 \pm 11{\cdot}3\%$, mean ± s.d.) than in normal subjects ($9{\cdot}6 \pm 3{\cdot}1\%$) ($P < 0{\cdot}01$).

The effect of IL-4 on the expression of CD5 on RA B cells

B cell-enriched fractions (B cells) were cultured with or without IL-4 for up to 6 days. The expression of CD5 increased spontaneously without IL-4 for the first 4 days of culturing in four experiments. The percentages of B cells expressing CD5 increased following culture in the presence of IL-4 but not to the same levels as when they were cultured in the absence of IL-4 (Fig. 1). There was a significant difference in the surface expression of $CD5^+$ on RA B cells cultured for 2 days without

Table 1. Clinical features of 12 rheumatoid arthritis (RA) patients

Patient no.	Sex	Age (years)	ADL-class	Stage	Duration of disease	Morning stiffness (min)	Haemoglobin (g/dl)	ESR (mm/h)	CRP (mg/dl)	RF (U/ml)	CH50 (U/ml)	NSAIDs	Treatment, PSL doses (mg/day)
1	M	58	2	II	6 months	30	15·7	36	0·7	269	39·5	(+)	5
2	F	43	2	III	7 years	120	7·8	112	1·2	233	36·1	(+)	0
3	M	53	2	I	1 month	30	10·3	120	5·3	456	42·3	(+)	0
4	F	62	2	II	4 months	60	9·2	88	3·5	326	42·0	(+)	5
5	F	57	2	III	4 years	60	13·2	52	0·6	139	38·6	(+)	5
6	F	32	2	II	14 months	60	11·3	48	0·5	697	45·8	(+)	5
7	F	36	2	II	1 month	60	10·3	62	2·9	376	37·3	(+)	0
8	F	28	2	I	2 months	60	11·1	50	3·0	136	32·6	(+)	5
9	M	58	2	II	12 months	90	12·0	128	10·9	902	36·8	(+)	5
10	F	53	2	II	1 month	30	13·4	82	0·4	1331	40·9	(+)	0
11	F	50	2	II	13 months	90	11·8	44	0·7	494	36·3	(+)	5
12	M	66	2	II	8 months	90	12·4	56	1·4	263	47·0	(+)	5

NSAIDs, Non-steroidal anti-inflammatory drugs.

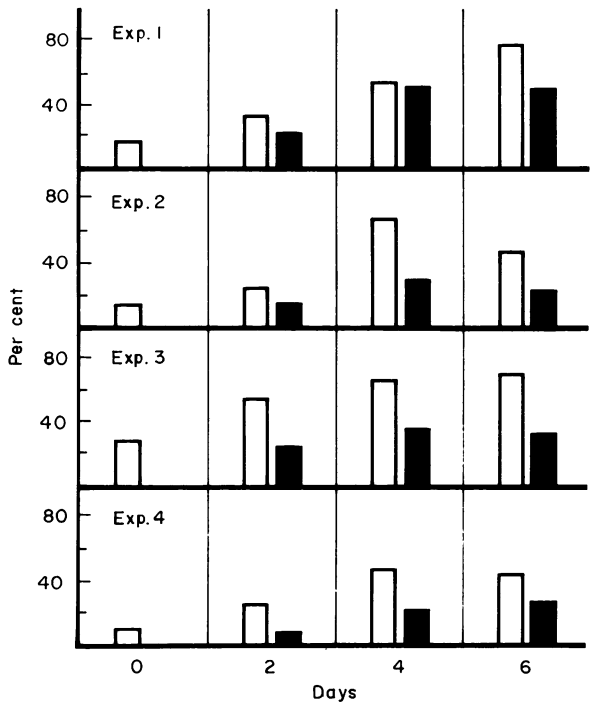

Fig. 1. Kinetic analysis of CD5 expression on rheumatoid arthritis (RA) B cells. B cell-enriched fractions were cultured with (■) or without (□) rIL-4 (100 U/ml) for up to 6 days and analysed by flow cytometry. Four replications are shown.

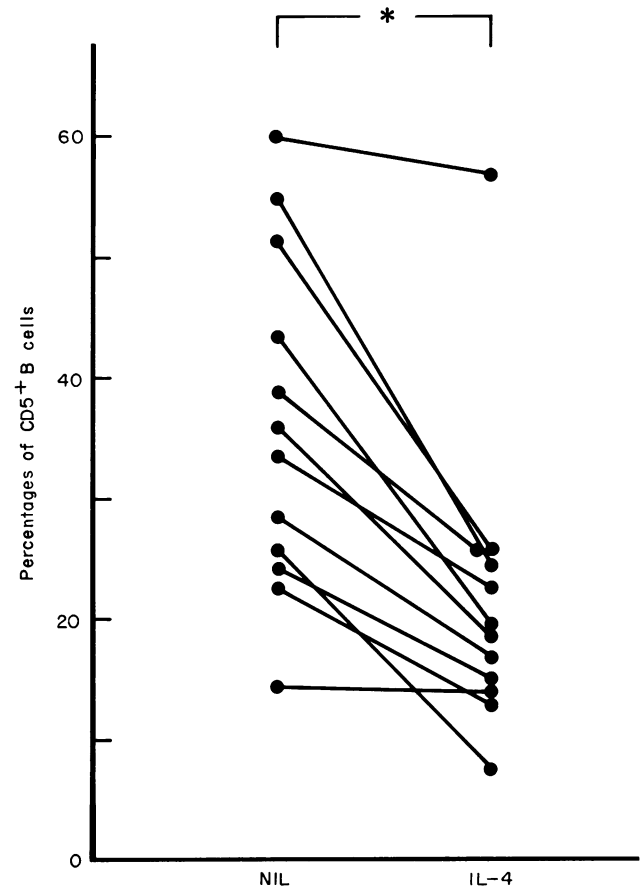

Fig. 2. The changes of percentages of $CD5^+$ B cells. B cell-enriched fractions were cultured with or without rIL-4 (100 U/ml) for 2 days. $*P < 0{\cdot}001$ by paired Wilcoxon rank sum test.

IL-4 and those cultured with IL-4 ($P < 0{\cdot}001$) (Fig. 2). IL-4 was shown to inhibit the up-regulation of CD5 expression. Representative flow cytometric profiles performed on 2-day, 4-day and 6-day cultures of B cell-enriched fractions with IL-4, IL-5 or without cytokines are presented in Fig. 3. In addition to the decrease of CD5 expression on B cells, CD20 expression was also down-regulated on B cells with rIL-4.

The effects of various cytokines on the expression of CD5 on RA B cells

B cell-enriched fractions were cultured for 2 days with rIL-1α, rIL-2, rIL-4, rIL-5 or rIL-6; cultures to which cytokines were not added served as controls. The experiments were repeated once. Only IL-4 displayed the capacity to decrease the surface expression of CD5 on RA B cells; the percentages of $CD5^+$ B cells in experiment 1 were 51%, 47%, 46%, 25%, 48% and 54%, and in experiment 2 were 44%, 38%, 37%, 19%, 42% and 48%

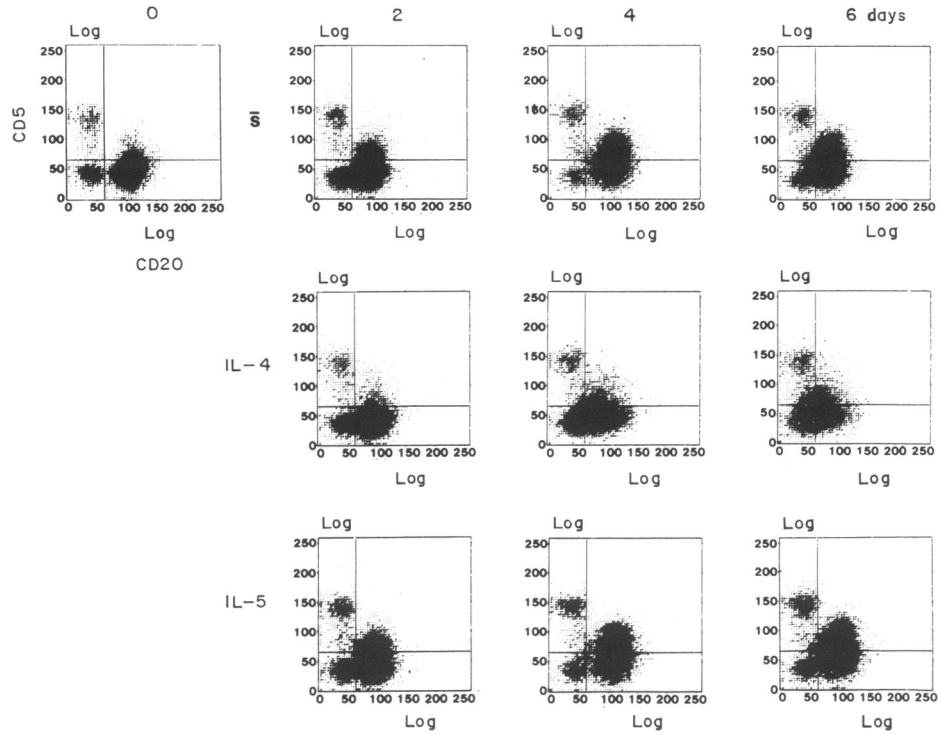

Fig. 3. Flow cytometric analysis of CD5 expression by 2-day, 4-day and 6-day cultures of rheumatoid arthritis (RA) with rIL-4 (100 U/ml), rIL-5 (10 μg/ml) or without (s̄) cytokines. Ordinate, green fluorescence of CD5; abscissa, red fluorescence of CD20.

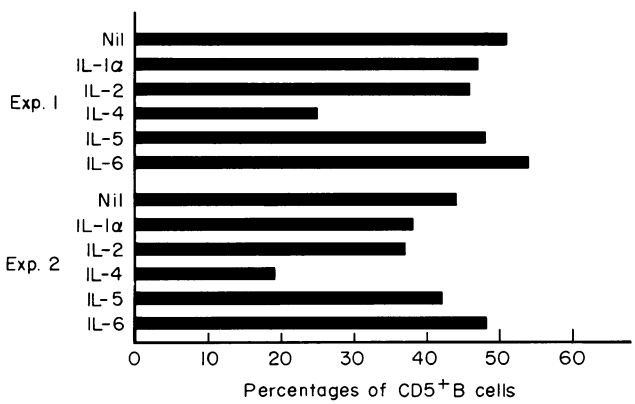

Fig. 4. Effect of various recombinant cytokines on the surface expression of CD5 on rheumatoid arthritis (RA) B cells. B cell-enriched fractions were cultured for 2 days with or without the following cytokines: 10 U/ml rIL-1α; 1 U/ml rIL-2; 100 U/ml rIL-4; 10 μg/ml rIL-5; 20 U/ml rIL-6.

for medium alone, rIL-1α, rIL-2, rIL-4, rIL-5 and rIL-6, respectively (Fig. 4).

Effect of IL-4 on spontaneous IgG, IgM and IgM-RF production

To investigate the effect of IL-4 on spontaneous IgG, IgM and IgM-RF production, purified T cells treated with MMC and B cells were cultured for 13 days with or without IL-4. IL-4 had a significantly negative effect on IgG, IgM, and IgM-RF production (Fig. 5). The amounts of IgG produced in the presence or absence of IL-4 were $3{\cdot}69 \pm 2{\cdot}39$ μg/ml and $8{\cdot}86 \pm 4{\cdot}93$ μg/ml, respectively ($P < 0{\cdot}01$); similarly, the amounts of IgM produced in the presence or absence of IL-4 were $0{\cdot}86 \pm 0{\cdot}86$ μg/ml and $3{\cdot}54 \pm 1{\cdot}91$ μg/ml, respectively ($P < 0{\cdot}01$). The amounts of IgM-RF expressed by the absorbance in ELISA were significantly suppressed by IL-4: $0{\cdot}251 \pm 0{\cdot}124$ and $0{\cdot}338 \pm 0{\cdot}135$ with or without IL-4, respectively ($P < 0{\cdot}001$). IL-5 (10 ng/ml to 30 μg/ml) had no significant effect on total IgG, IgM and IgM-RF production nor on the expression of CD5 (data not shown).

Effect of IL-4 on IgM-RF production by T plus B cells stimulated with SAC or PWM

RA T plus B cells were cultured for 13 days with or without SAC or PWM stimulators. In these experiments, adherent cells were not removed by adhesion to the Petri dishes, and T cells were not treated with MMC, in order to maintain the triggered effects of viable macrophage and T cells initiated by the stimulators. Spontaneous IgM-RF production, expressed as mean absorbance $\pm$ s.d. in ELISA, was suppressed by IL-4: $0{\cdot}284 \pm 0{\cdot}074$ *versus* $0{\cdot}131 \pm 0{\cdot}042$ for absence and presence of IL-4, respectively ($P < 0{\cdot}05$) (Fig. 6). In response to SAC, IgM-RF production was markedly increased without IL-4 ($P < 0{\cdot}05$), but the addition of IL-4 negated the effect of the SAC ($P < 0{\cdot}05$) (Fig. 6); the amounts of IgM-RF produced expressed as mean absorbance in ELISA with and without IL-4 were $0{\cdot}257 \pm 0{\cdot}052$ and $1{\cdot}268 \pm 0{\cdot}651$, respectively. In response to PWM, the amounts of IgM-RF produced with and without IL-4 were $0{\cdot}235 \pm 0{\cdot}043$ and $0{\cdot}153 \pm 0{\cdot}032$, respectively.

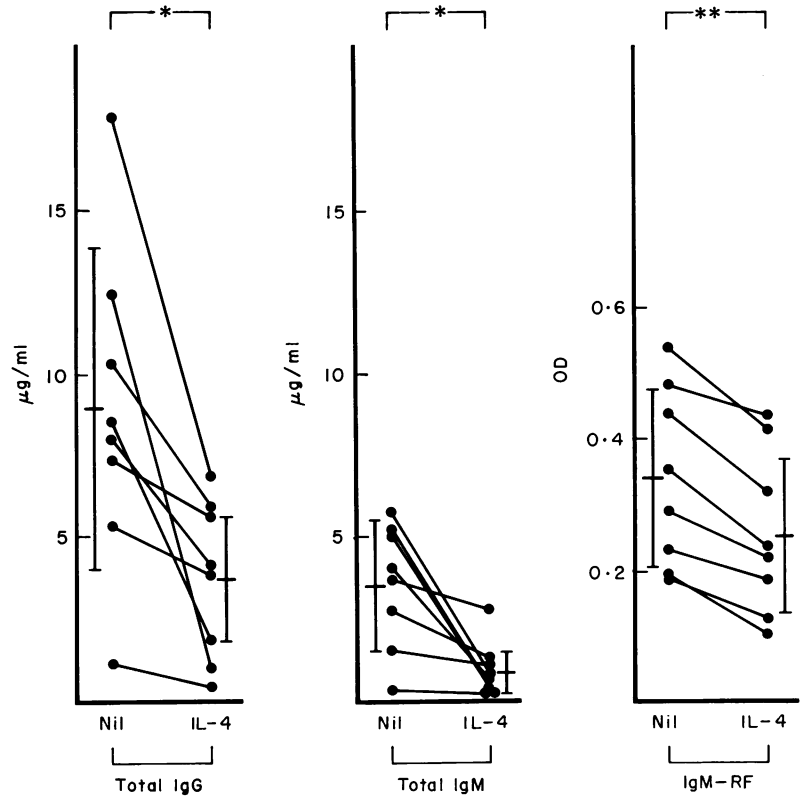

Fig. 5. Effect of IL-4 on spontaneous IgG, IgM and IgM-RF production. Purified T cells treated with mitomycin C (MMC) and B cells were cultured for 13 days with or without IL-4 (100 U/ml). $*P<0.01$; $**P<0.001$ by paired Wilcoxon rank sum test.

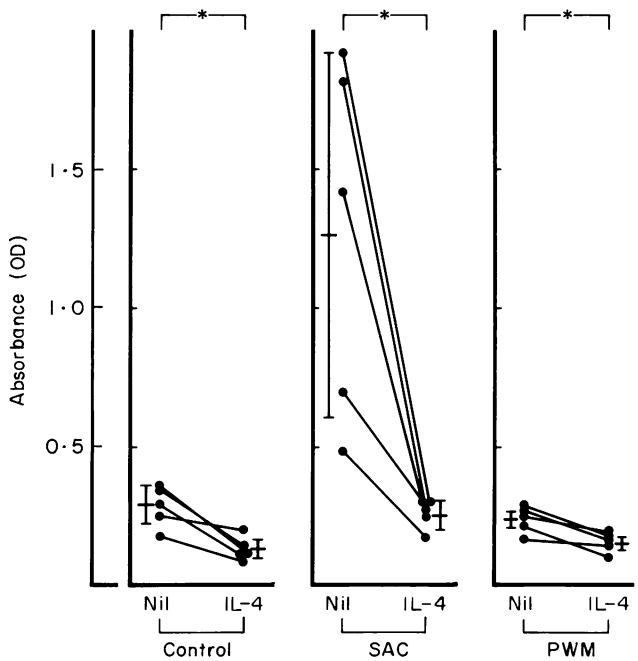

Fig. 6. Effect of IL-4 on IgM-RF production by T plus B cells stimulated with *Staphylococcus aureus* Cowan 1 (SAC) and pokeweed mitogen (PWM). Rheumatoid arthritis (RA) T plus B cells were cultured with or without stimulator (SAC or PWM) for 13 days. In these experiments, adherent cells were not removed by adhesion to Petri dishes, and T cells were not treated with mitomycin C (MMC) in order to maintain the triggered effects of viable macrophage and T cells initiated by the stimulators. $*P<0.05$ by paired Wilcoxon rank sum test.

DISCUSSION

$CD5^+$ B cells constitute a large proportion of B cells early in development [24,25]. It has been shown that $CD5^+$ B cells may be committed to production of IgM with RF-like activity [26], although recent studies have demonstrated that both $CD5^+$ and $CD5^-$ B cells produce anti-DNA antibodies [27] and that high-affinity IgG antibodies are produced mainly by $CD5^-$ B cells [28], and may constitute a lineage distinct from $CD5^-$ (Ly-1^-) B cells in the mouse [29]. We reported previously that anti-CD5 MoAbs augment B cell proliferation and IgM production in patients with RA [4]. However, regulation of their expression and the precise functional role of CD5 antigen on human B cells are still unclear. In this study, we found that the expression of CD5 on normal and RA B cells increases spontaneously on *in vitro* culture for up to 4 days, in the absence of T cells. It may also support the idea that the $CD5^+$ B cells possess distinctive features, although it has been reported that CD5 expression is a marker for activated human B cells [30].

It has been reported that the survival of $CD5^-$ cells could be increased by the addition of IL-4 [31], while $CD5^+$ B cell survival is independent of exogenous interleukins. In our study, for 4 days there was no significant change in absolute B cell numbers with or without IL-4. Cell viability was maintained at $>90\%$ during the culture period. Therefore, it could not be that the $CD5^-$ B cells were dying or the $CD5^+$ B cells proliferating, but suggests $CD5^-$ B cells are induced to express the CD5 antigen. It is unlikely that the decreased percentages of $CD5^+$ B cells observed following culture in the presence of IL-4 could be simply caused by the different survival rate. This question

should be addressed by culture of sorted $CD5^+$ and $CD5^-$ populations of B cells.

This study shows that IL-4 down-regulates the surface expression of CD5 on RA B cells, and that IL-4 also reduces immunoglobulin and RF production in SAC- or PWM-stimulated and in non-stimulated RA T plus B cell cultures. IL-4, T cell-derived B cell growth factor, was originally described as a co-factor for proliferation in response to soluble anti-immunoglobulin [32]. It was shown that rIL-4 enhanced IgE synthesis and preferentially induced IgG4 synthesis by normal peripheral blood lymphocytes [33] and IgG and IgM, but not IgE by highly purified B cells pre-activated with SAC [15]. IL-4 can also promote immunoglobulin secretion by lipopolysaccharide (LPS)-activated B cells and lymphoblastoid B cell lines [34]. On the contrary, IL-4 can inhibit the initial activation of human B cells in response to SAC and IL-2, leading to decreased immunoglobulin production [16,34]. These reports suggest that a single lymphokine may have multiple actions on the differentiation of B cells to immunoglobulin-secreting cells and that B cell activation of RA B cells is different from that of SAC or LPS pre-stimulated B cells, or of lymphoid B cell lines.

Recently it was revealed that IL-4 blocks the enhancement of mRNA for CD5 in murine 70Z/3 pre-B cell leukaemia cell line by LPS or NZB serum [35] and may be involved in the signal initiating immunoglobulin gene rearrangement, transcription, or translation [36]. Therefore it is possible that IL-4 regulates the immunoglobulin synthesis and autoantibody production directly or via CD5 expression.

Spontaneous and polyclonal activator-induced plaque-forming cell responses of mouse Ly-1 B cells were increased by IL-5. IL-5 also increased the frequency of peritoneal Ly-1 B cells induced to secrete certain autoantibodies [37]. It is reported that IL-5 is a potent stimulatory factor for mouse B cells but that seems to have very little, if any, significant effects on human B cells [38,39]. With respect to RA B cells, the presence or absence of IL-5 had no effect on the expression of CD5, immunoglobulin production or RF production in this study. It also showed that IL-5 might act differently on mouse and human B cells.

We conclude that IL-4 regulates the surface expression of CD5, which is associated with an early stage of B cell differentiation, and plays an important role in the regulation of immunoglobulin and autoantibody production.

ACKNOWLEDGMENTS

The authors thank Dr N. Yahagi (Ortho Diagnostic Systems, Japan) for his invaluable advice and expert technical help, and Miss Y. Miyazaki for her assistance in immunofluorescence analysis.

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