

Immunodeficiency and other clinical immunology

Enhancement of IgE production by anti-CD40 antibody in atopic dermatitis

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It has been recently recognized that the obligate requirement for T cells in the development of IgE responses can be substituted for by anti-CD40 antibody. In this study of patients with atopic dermatitis and high IgE levels, we have analyzed the role of the CD40 molecule in IgE production. Costimulation of peripheral blood mononuclear cells (PBMCs) from normal donors with interleukin-4 (IL-4) and anti-CD40 monoclonal antibody resulted in a selective increase in IgE production; either reagent alone, however, was ineffective. In contrast, addition of anti-CD40 monoclonal antibody alone to PBMCs or B cells from patients with atopic dermatitis markedly increased IgE production, even in the absence of exogenous IL-4. With the use of an ELISA spot assay, this increase in IgE production was attributed to an expansion of IgE-secreting B cells. In anti-IgM-stimulated lymphocyte cultures from patients with atopic dermatitis the costimulation with anti-CD40 induced strong lymphocyte proliferation. Similar results were observed with anti-IgM plus IL-4. The augmentation induced by anti-CD40 was inhibited by addition of anti-IL4 to anti-CD40-treated atopic dermatitis cells. In normal subjects the effects of anti-CD40 alone on IgE production could be observed after pretreatment of normal PBMCs with IL-4 for 3 days. The effects of anti-CD40 in atopic dermatitis may be explained in part by differences in CD40 expression. In freshly isolated PBMCs from patients with atopic dermatitis, the mean fluorescence intensity of CD40 expression on B cells was increased when compared with PBMCs from nonatopic donors, and stimulation of normal or atopic dermatitis PBMCs with IL-4 increased the intensity of CD40 staining of cells. (J ALLERGY CLIN IMMUNOL 1994;93:658-68.)

Key words: CD40, B cells, IgE, atopic dermatitis, interleukin-4

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is characterized by elevated serum IgE levels.¹⁻³ IgE production is under the regulatory control of T cells and requires at least two distinct signals. The first signal can be delivered through cognate interaction between acti-

Abbreviations used

AD:	Atopic dermatitis
EBV:	Epstein-Barr virus
IFN- γ :	Interferon- γ
IL-4:	Interleukin-4
mAb:	Monoclonal antibody
MFI:	Mean fluorescence index
PBMCs:	Peripheral blood mononuclear cells

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Supported in part by grants AI-26490, AI-29704, and PO1-36577 from the National Institutes of Health; USPHS grants HL 36577, AR 41256, and HL 37260; and a post-doctoral fellowship grant from the Deutsche Forschungsgemeinschaft (Re737/1-1). Dr. Gelfand is a Scholar of the Raymond and Beverly Sackler Foundation.

Received for publication Aug. 25, 1992; revised Feb. 9, 1993; accepted for publication Aug. 20, 1993.

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0091-6749/94 \$3.00 + 0 1/1/50954

vated CD4⁺ T lymphocytes and B cells.^{4, 5} The second signal is provided by interleukin-4 (IL-4), which is secreted by activated T cells. IL-4 is a potent stimulator of B-cell proliferation^{6, 7} and acts as an isotype switch factor for IgE.⁸⁻¹⁰

In a number of different culture systems, the requirement for cognate interaction between T and B cells can be bypassed. Infection of B cells with Epstein-Barr virus (EBV) induces responsiveness to IL-4 for IgE production.¹¹ Also, in a T-cell-independent culture system of purified B

cells from normal donors, cross-linking of the B-cell surface receptor, CD40, with different anti-CD40 antibodies can substitute for the T-cell-dependent signal and results in IgE production in the presence of IL-4.¹² Similar results have been reported in unfractionated peripheral blood mononuclear cells (PBMCs) from normal donors.¹³ CD40 is a 48 kd phosphorylated membrane glycoprotein, which is found on normal and malignant B cells.^{14, 15} The CD40 receptor has been shown to be related to the nerve growth factor receptor and the 55 and 80 kd tumor necrosis factor receptors.¹⁶⁻¹⁸ This homology suggests that CD40 is a member of a new receptor superfamily.¹⁹ Anti-CD40 antibodies augment proliferation of B cells treated with anti-immunoglobulin, anti-CD20, and phorbol myristate acetate; and cotreatment with anti-CD40 and IL-4 results in an increase in cell proliferation.^{20, 21} The present study was undertaken to examine the function of CD40 in AD, a disease in which IL-4 production is enhanced and IgE synthesis is elevated. This disorder can provide an excellent model for delineating the functional interactions between IL-4 and CD40 in IgE production.

METHODS

Reagents

Recombinant IL-4 was kindly provided by Paul Trotta (Shering Plough Corp., Bloomfield, N.J.) and used at a concentration of 400 U/ml. Anti-CD40 monoclonal antibody (mAb), G28-5 (used at a concentration of 500 ng/ml), was a kind gift of Dr. Edward Clark (Seattle, Wash.). Monoclonal anti-IL-4 antibody (IL-4 mAb) was a gift from Dr. M. P. Beckmann (Immunex Corp., Seattle, Wash.) and was used at a concentration of 1.5 µg/ml. Cycloheximide was used in a concentration of 50 µg/ml, which did not affect cell viability. The concentrations of the various reagents used in this study were previously shown to produce maximal effects in the culture systems used here.²²

Study population

Seven patients with severe AD were studied. All patients or guardians gave written informed consent before participation in the study. Patients were between the ages of 7 and 53 years and had severe AD diagnosed according to the criteria of Hanifin and Rajka.²³ Ten nonatopic healthy age-matched volunteers served as control subjects.

Blood sampling and cell preparations

PBMCs were prepared by Ficoll-Hypaque (Pharmacia, Uppsala Sweden) gradient centrifugation for 15 minutes at room temperature. The interface was collected and washed three times in phosphate-buffered saline. B cells were purified by E-rosette depletion and

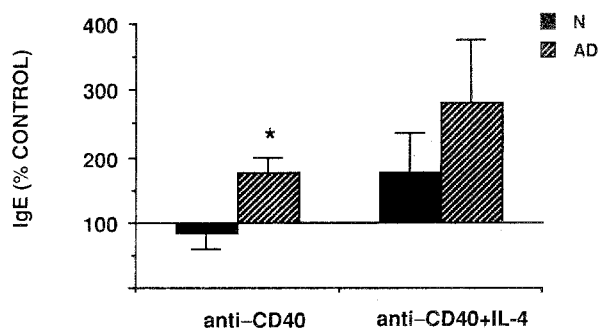


FIG. 1. Induction of IgE production after incubation with anti-CD40 or the combination of anti-CD40 and IL-4. PBMCs from five atopic and five nonatopic donors were cultured with anti-CD40 (0.5 µg/ml), IL-4 (400 U/ml), or tissue culture medium alone for 14 days. IgE production was then analyzed in culture supernatants by ELISA. In each individual the effect of anti-CD40 stimulation was compared with control levels (culture with medium alone) and calculated as percent control (means: atopic donors, 4536 pg/ml; normal donors, 508 pg/ml). Similarly, in IL-4-stimulated cultures the effect of anti-CD40 was compared with cultures treated with IL-4 alone (control) and expressed as percent control (means: atopic donors, 11,144 pg/ml; normal donors, 4046 pg/ml). For each individual, cultures were performed in duplicate. Shown are means $\pm$ SD for each experimental group. The asterisk indicates significant ($p < 0.05$) differences between the groups.

plastic adherence as described previously.²² After this procedure, more than 95% of cells expressed the surface markers CD19/CD20. In addition, this population of cells failed to proliferate in response to addition of the T-cell mitogen phytohemagglutinin. Cells were counted and resuspended in RPMI 1640 tissue culture medium (Gibco, Grand Island, N.Y.), supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 2-mercaptoethanol (5 mmol/L), L-glutamine (2 mmol/L), and 10% heat-inactivated fetal calf serum. For immunoglobulin production, 2×10^6 PBMCs or 1×10^6 B cells were incubated in 24-well flat-bottom tissue culture plates (2 ml) for 14 days. Cell-free culture supernatants were then harvested and stored at -20°C until analysis.

Determination of immunoglobulin concentrations in culture supernatants

The amounts of IgG, IgM, IgA, and IgE in culture supernatants were determined by ELISA as described previously.²² Immulon II round-bottom plates (Dynatech Laboratories, Burlingame, Calif.) were coated with goat anti-human IgG, IgM, IgA, or IgE (Tago, Inc., Burlingame, Calif.) diluted in 0.1 mol/L NaHCO₃ at a concentration of 3 µg/ml. After overnight incubation at 4°C , the wells were blocked with 1% (wt/vol) bovine serum albumin-Tris buffer for 2 hours at 37°C . Supernatants were incubated in triplicate overnight at 4°C , with parallel standards for human Ig isotypes. After

TABLE 1. Anti-CD40 alone enhances IgE production from purified B cells of atopic donors

	IgE (pg/ml)			
	Patient 1 with AD		Patient 2 with AD	
	PBMCs	B cells	PBMCs	B cells
Medium	3,200	1,600	8,255	7,265
IL-4	12,335	1,335	15,365	8,455
α CD40	13,920	7,940	17,005	12,780
IL-4 + α CD40	18,625	16,465	18,710	14,095

PBMCs (2×10^6) or B cells (2×10^6) from two patients with AD were purified and cultured for 14 days in the presence or absence of IL-4 (200 U/ml), anti-CD40 antibody (500 ng/ml), or a combination of the two. Cell-free supernatants were harvested and analyzed for IgE. Results (means of duplicate cultures) of two of five similar experiments are shown.

washing the plates, alkaline phosphatase-conjugated goat anti-human IgG, IgM, IgA or IgE (Tago) were added and the plates were incubated for an additional 2 hours at 37° C. After reaction with phosphatase substrate (Sigma Chemical Co., St. Louis, Mo.); plates were read in a microplate ELISA reader (Bio-Rad, Pleasanton, Calif.) at 410 nm, and the amounts of Ig were calculated according to the standard curve (Microplate Manager, Bio-Rad). The limits of detection in our ELISA were 1 ng/ml IgG, 1 ng/ml IgM, 1 ng/ml IgA, and 300 pg/ml IgE. Control incubations showed no cross-reactivity between Ig isotypes.

Quantitation of immunoglobulin-secreting B cells

The frequency of IgE-secreting cells was determined by ELISA spot assay.²⁴ Flat-bottom 96-well plates (Corning Glass Works, Corning, N.Y.) were coated overnight at 4° C with purified goat anti-human IgE (Tago) at a concentration of 3 mg/ml diluted in 0.1 mol/L NaHCO₃. After washing, wells were blocked with 0.1 mol/L Tris and 1% (wt/vol) bovine serum albumin for 2 hours at 37° C. At the end of the 14-day incubation, cells were harvested, washed three times with medium, and resuspended in medium containing 10% fetal calf serum. These cells were transferred immediately onto the anti-human IgE-coated ELISA plates at a concentration ranging from 1×10^5 to 1×10^3 cells per well for an additional 18 hours at 37° C in a humidified atmosphere of 95% air and 5% CO₂. Thereafter, plates were washed and goat anti-human alkaline phosphatase-conjugated IgE (Tago) was added for an additional 2 hours. The plates were developed by adding 5-bromo-4-chloro-3-indoyl phosphate substrate diluted in 1.2% agarose (Sigma). After 2-hour incubation at 37° C, spots were counted with an inverted microscope. To determine the sensitivity and the specificity of this assay, control incubations were carried out as described previously.²⁴ No cross-reactivity with other classes of Ig was found. The lowest amount of detectable IgE was 10 to 50 pg IgE per spot as determined by microinjection of known amounts of IgE into agarose substrate-treated plates.²⁴

Lymphocyte proliferation

Triplicate cultures of PBMCs were incubated at a concentration of 0.5×10^6 cells per well for 72 hours. Cells were stimulated with anti-IgM (0.5 μ g/ml) (Tago), anti-CD40 (500 ng/ml), and IL-4 (400 U/ml). During the last 6 hours of the culture period, cells were pulsed with 1 μ Ci of tritiated thymidine (ICN Biomedicals, Inc., Costa Mesa, Calif.), and cells were harvested with a Cambridge cell harvester (Cambridge Technology, Cambridge, Mass.) Tritiated thymidine incorporation was measured in a scintillation counter.

Analysis of cell surface markers by flow cytometry

Expression of CD40 and CD20 was monitored by indirect immunofluorescence and flow cytometry with mAbs directed against human CD40 and CD20. For staining, 5×10^5 cells were suspended in 50 μ l of antibody solution for 45 minutes at 4° C. The cells were then washed with staining buffer (phosphate-buffered saline + 2% bovine serum albumin + 0.1% sodium azide) and subsequently incubated with goat anti-mouse IgG fluorescein isothiocyanate for 30 minutes. IgE+, and CD40+ cells were identified by two-color flow cytometry. In this assay cells were stained first with anti-CD40 mAb followed by a second incubation with phycoerythrin-labeled rat anti-mouse IgG₁ (Sigma), which was simultaneously added with fluorescein isothiocyanate-conjugated goat anti-human IgE antibody (Tago). Cells were analyzed with an EPICS Profile (Coulter Electronics, Hialeah, Fla.).

Statistical analysis

Groups were compared by means of two-tailed Student's *t* test.

RESULTS

Stimulation of atopic PBMCs with anti-CD40 alone selectively enhances IgE production

The effect of anti-CD40 (mAb G28-5) on IgE production in PBMCs from patients with AD and normal control subjects was studied in the pres-

ence and absence of IL-4. As observed by other investigators,^{12, 13} when PBMCs were stimulated with IL-4, coincubation with anti-CD40 resulted in a marked increase in IgE synthesis (Fig. 1). As demonstrated in Fig. 1, this effect was found in PBMCs from patients with AD or nonatopic donors. IgE production in PBMCs from nonatopic donors was not enhanced if cultured in the presence of anti-CD40 alone. In contrast to normal PBMCs, culture of AD PBMCs with anti-CD40 alone significantly augmented IgE production.

The effect of anti-CD40 alone on IgE production in AD did not appear to be dependent on the presence of T cells or monocytes, because similar effects were observed when anti-CD40 was added to purified AD B cells. As shown in Table I, when anti-CD40 was added to purified B cells from two representative patients with AD, an increase in IgE secretion was found, which was similar to the results with PBMCs. However, in contrast to PBMCs, IL-4 alone had no effect on the purified B cells. Overall, in five patients with AD, IL-4 alone was without effect, whereas anti-CD40 or anti-CD40 plus IL-4 resulted in increases of $284\% \pm 123\%$ and $353\% \pm 158\%$, respectively.

The increases in IgE production were due to net new synthesis of IgE and not related to the release to cytoplasmic preformed IgE, since pretreatment of the cells with the protein synthesis inhibitor cycloheximide reduced the amount of IgE to less than 5% of the amount measured in culture supernatants from untreated cells (data not shown). The effect of anti-CD40 stimulation was specific for the IgE isotype because spontaneous (Fig. 2, A) and IL-4-modulated (Fig. 2, B) IgG, IgM, and IgA production were not found to be enhanced by anti-CD40 in this culture system.

Anti-CD40 enhances the number of IgE-secreting B cells

To examine the possible mechanisms of anti-CD40-induced IgE synthesis in AD, we used the ELISA spot assay for enumeration of IgE-producing B cells. In this assay system, PBMCs were transferred to the spot assay plates at the end of the 14-day culture period. Table II shows that the stimulation of AD PBMCs but not of normal PBMCs with anti-CD40 markedly enhanced the number of IgE-secreting B cells. A comparison of the amount of total IgE secreted into the culture supernatants with the increases in the number of IgE-secreting B cells indicated that anti-CD40 most likely acts by increasing the number of

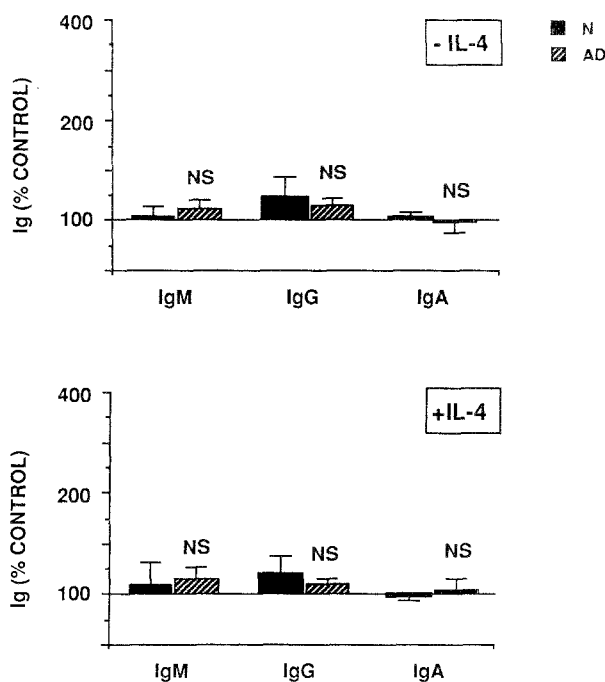


FIG. 2. Effect of anti-CD40 treatment on IgG, IgM, and IgA production. PBMCs from five atopic and five nonatopic donors were cultured with anti-CD40 (0.5 μ g/ml) $\pm$ IL-4 (400 U/ml) for 14 days, and Ig isotype production was measured in culture supernatants by ELISA. The effect of anti-CD40 was expressed as percent control (cultures with tissue culture medium alone). The baseline levels were: for atopic donors, IgM 868 ng/ml, IgG 926 ng/ml, IgA 95 ng/ml; for normal donors, IgM 716 ng/ml, IgG 672 ng/ml, IgA 104 ng/ml. Shown are means $\pm$ SD for each experimental group. NS, Not significant.

IgE-secreting B cells rather than by stimulating individual cells to secrete more IgE.

IL-4 alone stimulated IgE synthesis in both normal and AD PBMCs. This increase was also attributed to an expansion of IgE-secreting B cells (Table II). The addition of anti-CD40 to IL-4-stimulated cells further enhanced the number of IgE-secreting cells. Under these conditions, the number of IgE-producing cells demonstrated parallel increases compared with the amounts of IgE in culture supernatants. This correlation between numbers of IgE-secreting cells and amount of supernatant IgE also indicated that CD40 and IL-4 together also act by enhancing the number of IgE-producing B cells rather than by increasing the amount of IgE produced by a single B cell.

Anti-CD40-induced IgE production was reduced to baseline by anti-IL4 antibody

We recently demonstrated that PBMCs from patients with AD produce higher amounts of IL-4 than those of normal donors.²⁵ One explanation

TABLE II. Enumeration of IgE-secreting cells

Subjects	- IL-4				+ IL-4			
	Medium		Anti-CD40		Medium		Anti-CD40	
	Spots/2 × 10 ⁶ cells	SN-IgE (pg/ml)	Spots/2 × 10 ⁶ cells	SN-IgE (pg/ml)	Spots/2 × 10 ⁶ cells	SN-IgE (pg/ml)	Spots/2 × 10 ⁶ cells	SN-IgE (pg/ml)
N1	0	<350	4 ± 4	890 ± 630	12 ± 4	2320 ± 1430	30 ± 8	5660 ± 1025
N2	0	<350	6 ± 2	480 ± 120	8 ± 2	1280 ± 630	18 ± 6	3860 ± 460
AD1	6 ± 6	3950 ± 415	30 ± 10	7260 ± 865	18 ± 8	11380 ± 2650	46 ± 12	23690 ± 2380
AD2	18 ± 6	4650 ± 1160	42 ± 10	12860 ± 1260	52 ± 14	12750 ± 2350	76 ± 18	18390 ± 2160

After culture of cells (2×10^6 PBMCs) as described for 14 days, culture supernatants (SN) were harvested and analyzed for total IgE production by ELISA. In parallel, cells were collected, washed, and plated in ELISA spot assay plates in complete culture medium overnight. After development, IgE-secreting cells were counted, and the numbers of secreting cells adjusted to 2×10^6 cells plated. Values are expressed as means $\pm$ SD from triplicate cultures.

SN, Supernatant; N, normal.

TABLE III. Anti-IL-4 inhibits anti-CD40-stimulated IgE production by AD PBMCs

Donor No.	Cells	Stimulation	IgE (pg/ml)		
			Treatment		
			Medium	Anti-IL-4	Control antibody
1	PBMCs	Medium	2860 $\pm$ 465	1650 $\pm$ 850	2750 $\pm$ 358
	PBMCs	Anti-CD40	9860 $\pm$ 1260	2680 $\pm$ 260	9760 $\pm$ 950
2	PBMCs	Medium	4150 $\pm$ 1055	4618 $\pm$ 960	3960 $\pm$ 1250
	PBMCs	Anti-CD40	8760 $\pm$ 1765	4655 $\pm$ 1250	8990 $\pm$ 870
1	B cells	Medium	1600 $\pm$ 380	1560 $\pm$ 260	1750 $\pm$ 200
	B cells	Anti-CD40	4750 $\pm$ 960	4960 $\pm$ 850	3950 $\pm$ 1200
2	B cells	Medium	2580 $\pm$ 490	1680 $\pm$ 540	2430 $\pm$ 620
	B cells	Anti-CD40	9860 $\pm$ 2540	11280 $\pm$ 1950	12200 $\pm$ 3100

PBMCs and purified B cells from two donors with AD were stimulated with or without anti-CD40 (500 ng/ml) in the presence or absence of anti-IL-4 antibody (1.5 μ g/ml). Addition of an isotype and species-matched control antibody had no effect. Cells were cultured for 14 days, and supernatants were analyzed for IgE. B cells were purified as described in the Methods section. Values are expressed as means $\pm$ SEM from one of two experiments.

TABLE IV. Anti-CD40 induces IgE in IL-4-primed cells

First treatment	Second treatment	IgE (pg/ml)	
		Subject No. 1	Subject No. 2
—	—	1150 $\pm$ 250	<300
IL-4	—	2246 $\pm$ 596	350 $\pm$ 150
—	CD40	940 $\pm$ 180	<300
IL-4	CD40	8990 $\pm$ 1350	4650 $\pm$ 1260

PBMCs from two normal donors were incubated with or without IL-4 (400 U/ml) for 3 days, then washed three times and recultured with or without anti-CD40 (500 μ g/ml) for an additional 14 days. Culture supernatants were harvested and analyzed for IgE. Shown are two of four similar experiments. Values are expressed as means $\pm$ SEM from triplicate cultures.

for the present observations is that anti-CD40 alone is capable of enhancing IgE production in AD because the second signal (IL-4) is being spontaneously provided by the AD PBMCs. To

examine this possibility, PBMCs of two patients with AD were stimulated with anti-CD40 in the presence and absence of anti-IL4 antibodies. As demonstrated in Table III, when anti-IL4 was

added to anti-CD40-treated PBMC cultures, the stimulatory effect on IgE production was reduced to baseline levels. These data indicate that anti-CD40-mediated increases in IgE production by PBMCs can be inhibited by blocking the effects of IL-4 in culture. In contrast, addition of anti-IL-4 antibody to cultures of anti-CD40-stimulated purified B cells did not inhibit IgE production (Table III), indicating an apparent IL-4-independent effect of anti-CD40-stimulated IgE production on B cells. This could reflect that B cells from AD were already activated in vivo by IL-4.

IL-4 primes normal PBMCs to synthesize IgE in response to anti-CD40

On the basis of these data, the major difference between lymphocytes obtained from patients with AD and control subjects may reside in the enhanced production of IL-4 by AD PBMCs. To determine whether preexposure of normal lymphocytes to IL-4 may render the cells competent to respond to subsequent anti-CD40 stimulation with IgE synthesis, PBMCs from control subjects were treated sequentially with IL-4 and anti-CD40. In these experiments, normal PBMCs were pretreated with optimal concentrations of IL-4 (400 U/ml) for 3 days. After extensive washing, the cells were then stimulated with anti-CD40 in the absence of IL-4 (second culture phase). Table IV shows that only in cultures pretreated with IL-4 was anti-CD40 effective in inducing IgE production.

Anti-CD40 and IL-4 stimulate lymphocyte proliferation

To analyze the effects of anti-CD40 and IL-4 on lymphocyte proliferation, PBMCs from normal and atopic donors were incubated with anti-IgM, anti-CD40, or IL-4. When each of these reagents was added alone, only minimal or no proliferative responses were detected when compared to medium controls (Fig. 3). These effects were observed in PBMCs from both normal and atopic donors. In contrast, the combination of anti-IgM antibody plus anti-CD40-stimulated lymphocyte proliferation in atopic donors (mean increase, 700%) and to a much lower degree in normal donors (mean increase, 182%). A similar effect was found with IL-4. When lymphocytes were stimulated by IL-4 and anti-IgM, cell proliferation was increased compared with the response induced by each of the reagents alone. Again, PBMCs from atopic donors showed a higher degree of lymphocyte proliferation than

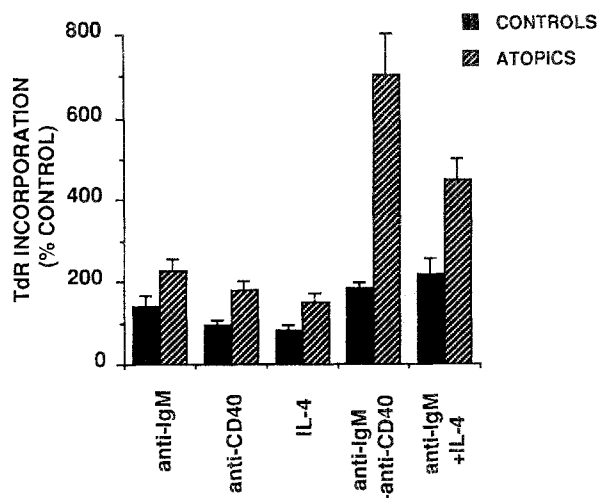


FIG. 3. Lymphocyte proliferation induced by anti-IgM, anti-CD40, and IL-4. PBMCs from five atopic patients and five control donors were incubated with anti-IgM (0.5 μ g/ml), anti-CD40 (500 ng/ml), and IL-4 (400 U/ml) in different combinations as shown in the figure. The cell cultures were carried out for 72 hours. During the last 6 hours of the culture period tritiated thymidine (TdR) was added (1 μ Ci/well). In each donor the proliferative response to the reagents was compared with the medium control, and the effect was expressed as percent of the medium control (100%). Expressed are means $\pm$ SD from all donors in each group. Mean counts per minute of the medium control were 1345 cpm in the control group and 1947 cpm in the group of atopic patients.

that found in normal donors. These data demonstrate that atopic PBMCs respond to a higher degree to the costimulatory effects of anti-CD40 and IL-4.

Mean fluorescence intensity of CD40 expression is higher in AD

Because the activity of anti-CD40 is mediated through the CD40 receptor, which is expressed on B cells, we examined the expression of this receptor in patients with AD. The proportion of CD40-positive lymphocytes was only slightly higher in fresh PBMCs from AD ($12.7\% \pm 4.7\%$) than in normal PBMCs ($9.82\% \pm 2.4\%$). In both populations the number of CD40-positive cells correlated with the number of CD20-positive B cells (Table V). Furthermore, as shown in Fig. 4, CD40-positive lymphocytes were demonstrated only in the CD3-negative cell population. Although the number of CD40-positive cells was not significantly different in patients with AD and control subjects, there were differences in the levels of CD40 expression, monitored by mean fluorescence intensity (MFI). As summarized in Table V and illustrated in Fig. 3, AD PBMCs

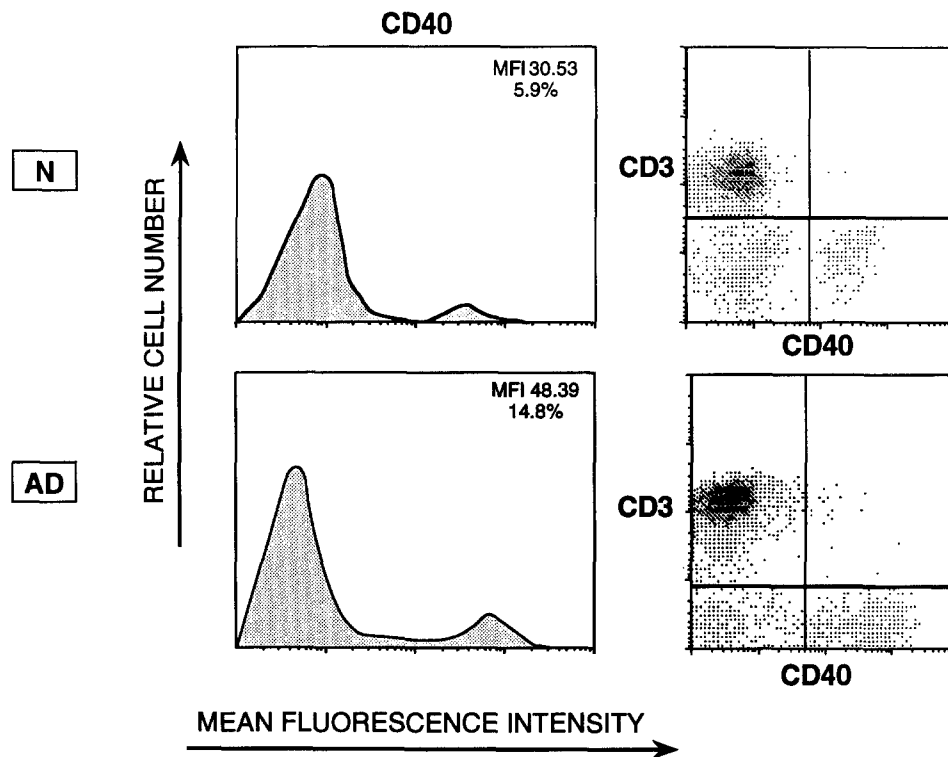


FIG. 4. Analysis of CD40 and CD3 expression on lymphocytes from one normal nonatopic donor (N) and one patient with atopic dermatitis (AD) by double staining. Indicated are MFI and the percentage of CD40-positive cells. Cells were analyzed with a gate for lymphocytes.

TABLE V. The MFI of CD40 expression is higher in lymphocytes from patients with AD than in those from normal donors

Subjects	CD40		CD20	
	%	MFI	%	MFI
N	9.82 ± 2.4	5.7 ± 0.9	7.98 ± 1.85	18.4 ± 10
AD	12.7 ± 4.7	12.5 ± 1.4*	10.78 ± 1.3	20.5 ± 11

Fresh PBMCs from seven normal donors (N) and seven patients with AD were stained with anti-CD40 and anti-CD20 mAbs and CD40, and CD20 expression was analyzed by flow cytometry with a gate for lymphocytes as described in the Methods section.

The analysis of surface markers on normal donors and patients with AD was performed during the same experiments. Expressed are the percent positive cells and the MFI for each group. Values are expressed as means ± SD.

N, Normal.

*Significant difference ($p < 0.01$).

exhibited a significantly higher ($p < 0.05$) MFI than PBMCs from normal donors.

IL-4 enhances MFI of CD40 expression on B cells

Fig. 5 demonstrates that the level of B-cell CD40 expression can also be upregulated by IL-4. At the same time that culture supernatants were obtained for the analysis of total IgE production, expression of CD40 was determined in parallel on normal B cells. In these studies lymphocytes were

harvested after culture for 14 days in the presence or absence of IL-4, and the expression of CD40 was determined by flow cytometry. Although the number of CD40-positive cells was similar under the two culture conditions, the MFI for CD40 was significantly increased when the cells were cultured together with IL-4. This increase in CD40 expression was found in PBMCs from both patients with AD and normal donors. These data indicate that a 14-day exposure of AD and normal PBMCs to IL-4 resulted in an upregulation of

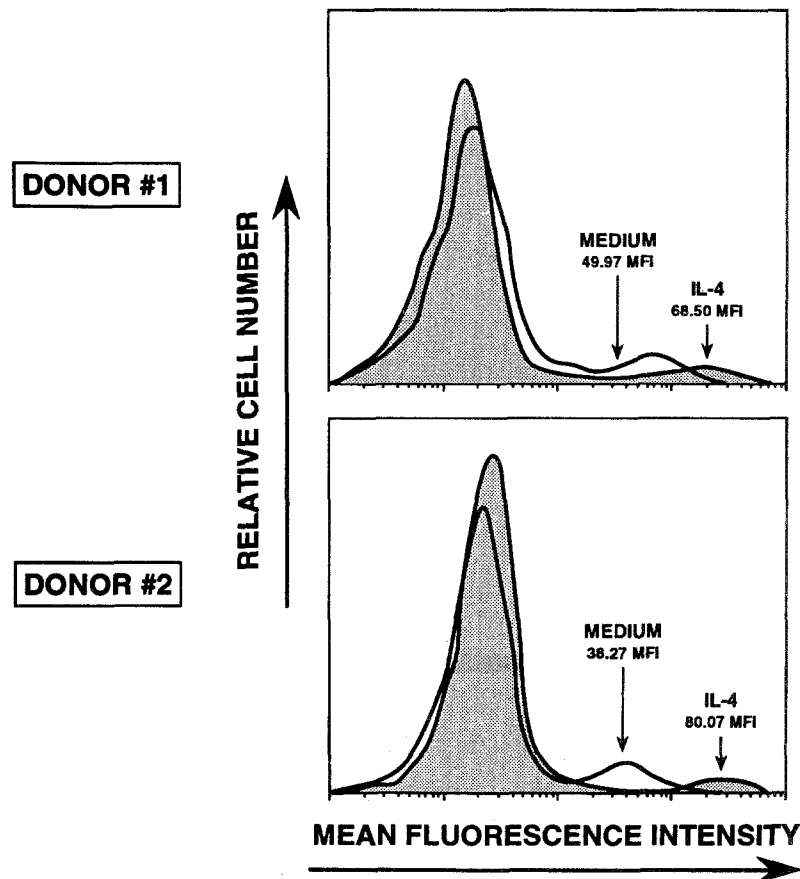


FIG. 5. Regulation of CD40 expression by IL-4. PBMCs from two normal donors were incubated with either IL-4 or medium alone for 14 days. At the end of the culture period, lymphocytes were recovered and stained with anti-CD40 as described in the **Methods** section. Shown are the percentage of CD-40-positive cells and the MFI. The percent of CD-40-positive cells was identical to the percent of CD20-expressing cells (data not shown).

TABLE VI. Simultaneous presence of surface IgE and CD40

	% IgE+	% CD40+	% CD40+ of IgE+
Experiment 1	3.5	20.1	95.8
Experiment 2	5.4	21.4	96.4

PBMCs from two patients with AD were cultured with IL-4 (400 U/ml) for 72 hours. Frequencies of surface IgE+ B cells and CD40-expressing lymphocytes were characterized by flow cytometry. The percentages of IgE+ B cells that simultaneously expressed CD40 were calculated from two-color analysis, which was performed as described in the **Methods** section. Expressed are the values from two representative experiments. The analysis of cell surface markers by flow cytometry was performed on populations of lymphocytes.

CD40 surface expression. A similar upregulation of CD40 was observed after 3 days of incubation with IL-4 (data not shown). Two-color flow cytometry indicated that all B cells expressed the CD40 receptor, including surface IgE-positive B cells (Table VI).

DISCUSSION

This study demonstrates that cross-linking of the B-cell surface receptor CD40 with antibody

exerts a potent enhancing effect on IgE production in patients with AD. Earlier studies on B cells from normal donors indicated that the increase in IgE production induced by anti-CD40 was dependent on the simultaneous presence of IL-4.^{12, 13} It is now well established that the induction of IgE requires the presence of two distinct signals. This first signal can be provided by T cells, which stimulate B cells after cognate interaction.⁵ The second signal is provided by IL-4. IL-4 exerts

its activity on B cells in several ways. It not only induces proliferation of activated B cells^{6, 7} but is an isotype switching factor for IgE.⁸⁻¹⁰ Although the first signal can be substituted by activation of human B cells with EBV¹¹ and by stimulation of mouse B cells with lipopolysaccharide,²⁶ IL-4 is the only known factor to induce ϵ -switching. Because activation of B cells through CD40 also bypasses the need for T cells in IgE production,^{12, 13} the role of this receptor in conditions associated with elevated circulating levels of IgE is of interest.

The data presented here revealed that in PBMCs or B-cell preparations from patients with AD, anti-CD40 alone was capable of enhancing IgE production. That is, the addition of exogenous IL-4 to AD cultures was not required to induce IgE synthesis. This finding confirms and extends the previous observations by Zhang et al.¹³ who described a similar effect in two patients with hyperimmunoglobulinemia E syndrome. From our data it appears that the major reason for this response in AD but not normal cells is the ongoing endogenous production of IL-4 in these patients. In a recent study we found that spontaneous, as well as phytohemagglutinin-induced, IL-4 production was enhanced in PBMCs from patients with AD when compared with cells from normal donors.²⁵ In contrast, results of interferon- γ (IFN- γ) production indicated lower responses in AD. Stimulation of normal PBMCs with lectins resulted in the production of high levels of IFN- γ , whereas PBMCs from atopic donors produced significantly lower levels of IFN- γ .²⁵ These data, together with results from other studies,²⁷⁻²⁹ suggest that AD is a disease characterized by an endogenous cytokine profile, which shows a trend toward higher levels of IL-4 production and lower levels of IFN- γ .

These earlier findings are further supported by the data presented here. When AD PBMCs that were stimulated with anti-CD40 alone were treated with anti-IL-4 antibody, the anti-CD40-induced augmentation of IgE synthesis was markedly reduced. A conclusion from this experiment is that endogenous IL-4 production is still ongoing in cultured AD PBMCs, and second, it is this endogenous production of IL-4 that plays an important role in anti-CD40 enhancement of IgE production by PBMCs. The inhibitor effect of anti-IL-4 antibody in cultures of AD PBMCs was not observed in cultures of B cells from these patients. At present it is unclear whether this indicates that a proportion of B cells from patients with AD have already re-

ceived an IL-4 signal. In cultures of PBMCs, the IL-4-independent effect on the B cells may be minimized, given the lower numbers of B cells in these cultures compared with the cultures of purified B cells. It is also possible that in cultures of PBMCs, IL-4 is necessary to potentially overcome some inhibitory influences mediated by T cells (or monocytes). The experiments involving sequential treatment of normal PBMCs first with IL-4, and then with anti-CD40 stimulation, revealed that these two signals do not have to be present simultaneously. IL-4 somehow triggers the cells to become susceptible to anti-CD40, resulting in the selective increases in IgE production. Whether this is a direct effect of IL-4 on B cells or whether IL-4 acts by stimulating the production of other interleukins requires further evaluation.

The data from the cell proliferation experiments indirectly support the hypothesis that anti-CD40 and IL-4 trigger the expansion and proliferation of B cells. Anti-IgM antibodies specifically stimulate surface IgM⁺ B cells. This stimulus by itself had only minimal effects on the proliferative response of PBMCs from both normal and atopic subjects. The combination of anti-IgM plus anti-CD40 or IL-4 strongly enhanced lymphocyte proliferation. This effect was especially observed in cultures of atopic cells. Furthermore, stimulation of PBMCs from patients with AD with anti-CD40 antibodies was found to increase the number of IgE-secreting B cells as shown in the ELISA spot assay. This finding can be explained by either an expansion of IgE-committed B cells, which are present in patients with AD or induction of ϵ -switching by anti-CD40 stimulation. When naive IgD-positive B cells were exposed to IL-4 plus anti-CD40, IgE production was induced, indicating that the combination of both stimuli leads to ϵ -switching.¹² Whether the switching was directly induced by stimulation of the CD40 receptor or whether signaling through CD40 acts at a different level in the B-cell maturation pathway has not been elucidated.

The CD40 receptor, a 48 to 50 kd surface glycoprotein, has been described virtually exclusively on B cells and is not expressed on T cells. The numbers of CD40-positive cells correlate with numbers of CD20-positive cells. Antibodies to CD40, such as G28-5 or S2C6, have been shown to provide a progression signal for proliferation of preactivated B cells.^{14, 15} These effects of anti-CD40 mAbs and their relatedness to other growth factor receptors (nerve growth factor and tumor necrosis factor receptors) suggest that

CD40 may have a role in the regulation of B-cell activation and differentiation. In human beings T-cell-independent synthesis of IgE can be induced by costimulation of the cells with either EBV and IL-4¹¹ or with anti-CD40 and IL-4.^{12, 13} These two stimuli are quite distinct because EBV is a polyclonal activator for B cells inducing synthesis of other Ig isotypes as well, whereas anti-CD40 and IL-4 provide a specific signal or signals for IgE production. It was shown that anti-CD40 increased the levels of IL-4-induced germline ϵ transcripts and induced the production of mature messenger RNA. One explanation for these effects of anti-CD40 is that B cells underwent deletional switch recombination when stimulated with anti-CD40 and IL-4.³⁰

Although anti-CD40 and IL-4 clearly deliver distinct signals, they do appear to act through interrelated mechanisms. Both are synergistic for IgE production and maintenance of DNA synthesis in preactivated B cells. IL-4 also appears to regulate CD40 expression. In patients with AD the MFI of CD40 expression was increased in B cells compared with that of nonatopic donors, who were analyzed at the same time. This implies an increase in numbers of CD40 receptors in patients with AD. It has been reported that IL-4 can upregulate the intensity of CD40,^{20, 21} and because we found in *in vitro* experiments that exposure of atopic PBMCs to IL-4 indeed increases the MFI of CD40-positive cells, it was possible that the enhanced intensity of CD40 on fresh atopic cells was due to the continuous endogenous overproduction of IL-4.

These activities of CD40 were restricted to the induction of IgE. Other isotypes (IgG, IgM, and IgA) were not enhanced by stimulation with anti-CD40. This is in agreement with results of another study by Zhang et al.¹³ who reported that normal PBMCs stimulated with the combination of anti-CD40 and IL-4 exclusively resulted in an increase of IgE production, but not of other isotypes. Zhang et al.¹³ used the same anti-CD40 antibody (G28-5) that was used in our experiments. However, in another study in which a different CD40 antibody was used for the stimulation of purified B cells instead of unseparated PBMCs, an increase in IgG and IgM was reported.¹² Further experiments are necessary to determine whether these differences are due to the activities of different antibodies reacting with the CD40 molecule or whether the presence of T cells in the culture system may lead to different effects on various isotypes.

In conclusion, this study demonstrates that stimulation of the B-cell surface receptor CD40 and IL-4 provide two important signals to induce IgE production in normal and atopic PBMCs. PBMCs from patients with AD are characterized by enhanced endogenous IL-4 production, and as a result *in vitro* stimulation of lymphocytes with anti-CD40 mAb alone appears to be sufficient to increase IgE production. The interaction between IL-4 and CD40 has been further linked by our demonstration that IL-4 induced an upregulation of the CD40 receptor on AD and normal lymphocytes. These data provide further evidence for a unique imbalance of interleukin production and the expression of certain receptors in AD. These observations may provide new insight into the immunopathogenesis of this disease and may account to some extent for the high IgE levels in these patients. Lymphocytes from patients with AD provide a valuable means for additional evaluation of the role of CD40 in IgE synthesis.

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