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Inhibition of LFA-1-dependent human B-cell aggregation induced by CD40 antibodies and interleukin-4 leads to decreased IgE synthesis

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SUMMARY

Antibodies to CD40 have been shown to induce homotypic aggregation of human resting B cells and B-cell lines via an LFA-1-dependent mechanism. We show here that interleukin-4 (IL-4) is a strong potentiator of this process and stimulation of tonsillar B cells for 4 days with IL-4 and CD40 antibodies resulted in the formation of large, dense aggregates. Also in this case, aggregation appeared to be chiefly dependent on the activation of LFA-1, although the small clusters of cells remaining after blocking with LFA-1 antibodies suggest the involvement of another adhesion system(s). When testing the relationship between aggregation and IgE synthesis, a known consequence of IL-4/CD40 stimulation, IgE levels were found to be significantly decreased in the presence of LFA-1 antibodies. In contrast to these observations, proliferation occurring in response to the IL-4/CD40 stimulation was not inhibitable by LFA-1 antibodies. Rather, in most cases, this was slightly enhanced, suggesting that aggregation may have a limiting effect on cell growth. Isolated aggregates, each of which could comprise more than 10^5 cells, were also examined by electron microscopy. This revealed a tissue-like structure of the aggregates with large contact areas and with minimal intercellular space between the adjacent cells. As the apparent inhibitory effect of aggregation on proliferation may reflect a negative autocrine signalling, which is enhanced by the close cell contact, we also tested the effect of neutralizing antibodies to IL-6, one of the factors known to be produced in the system. Such treatment did not affect aggregation but in most experiments enhanced proliferation. The results suggest that a possible effect of aggregation may be to enhance differentiation of cells and that this may also be associated with the difficulties in growing B cells *in vitro*.

INTRODUCTION

Cellular contact between the different members of the immune system is of critical importance to obtain an efficient and specific immune response. For B cells this includes the interaction with antigen-specific T helper cells but also with, for example, dendritic cells within particular sites of the follicular lymph nodes.[1,2] As a result of these interactions and the exposure to lymphokines, B cells will proliferate and differentiate into Ig-producing cells. Important components which enable efficient cell contact are the different adhesion molecules. Among those relevant to B cells is LFA-1, a member of the integrin family, which binds to either of three cell-surface ligands: ICAM-1 (CD54), ICAM-2 or ICAM-3.[3-5] The LFA-1 molecule is a heterodimer, composed of an α-chain (CD11a) and a β-chain (CD18), whereas the ICAM-1 and ICAM-2 molecules are single-chain structures belonging to the immunoglobulin superfamily.[3,6] Stimulation of this adhesion system in B cells with the phorbol ester, PMA[7] and, as more recently shown, with antibodies to several B-cell antigens including CD19, CD20, CD22 and CD40[8-11] leads to rapid aggregation of freshly isolated B cells and/or B-cell lines. Recently we have also found that IL-4 and, to some extent, IL-2, which by themselves do not induce adhesion in B cells, are able to synergize with anti-IgM to induce LFA-1-dependent aggregation.[12] In contrast to the rapid but relatively modest aggregation seen with most other agents, the IL-4/anti-IgM-induced aggregation was characterized by slower kinetics and the formation of very large cell clusters.

In the present report we show that IL-4 is a strong potentiator of aggregation induced by CD40 antibodies as well as by antibodies to CD19 and CD20. As IL-4 and CD40 antibodies are known to induce proliferation and IgE synthesis in B cells we also investigated the relationship between aggregation and these events. Our results show that aggregation is necessary for IgE production whereas proliferation occurs

Abbreviations: BSA, bovine serum albumin; FCS, foetal calf serum; GH, growth hormone; ICAM, intercellular adhesion molecule; IL, interleukin; LFA-1, leucocyte function-associated antigen 1; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; TSH, thyroid-stimulating hormone.

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independently of aggregation. The possible *in vitro* and *in vivo* consequences of these findings are discussed.

MATERIALS AND METHODS

Reagents

Recombinant IL-1β, IL-2, IL-3, IL-4 and IL-6, IFN-γ and anti-IL-6 antibodies (IgG1) were from Genzyme, Boston, MA. IL-1α was a kind gift from Dr P. T. Lomedico, Hoffmann-La Roche Inc., Nutley, NJ. Growth hormone (GH) was kindly provided by Dr H.-P. Ekre, Kabi-Pharmacia, Sweden. Thyroid-stimulating hormone (TSH) was a kind gift from Dr J. Pierre, UCLA, CA and prolactin was from Dr P. Roos, Dept. of Natural Sciences, BMC, Uppsala, Sweden. The biological activity of the various cytokines was confirmed by IL-1-induced production of IL-2 by the cell line LBRM 33 1A5 (ATCC CRL 8079; IL-1α and IL-1β), proliferation of bone marrow precursor cells (IL-3), by co-stimulation with anti-IgM beads (IL-2, IL-4 and IFN-γ) and by IL-6-dependent growth of the mouse hybridoma cell line B45. Antibodies against CD18 (MHM23, IgG1), CD19 (HD37, IgG1) and CD20 (L26, IgG2a) were obtained from Dakopatts a/s, Copenhagen, Denmark. The CD21 antibody THB5 (IgG2a) was from the American Type Culture Collection (ATCC), Rochester, MD. CD40 antibodies (S2C6, IgG1), a monospecific rabbit antibody against CD40[13] and the CD43 antibody B1B6 (IgG1)[14] were produced in our laboratory. Anti-IL-4 antibodies (IgG1) were from Mabtech, Stockholm, Sweden. FITC-conjugated antibodies to CD18 (MHM23) and CD54 were from Dakopatts a/s and Immunotech, Marseilles, France, respectively.

Cells

B cells were prepared from human tonsils as described earlier.[15] Briefly, tonsils obtained from patients undergoing routine tonsillectomy (Danderyds Hospital, Stockholm, Sweden) were cut into fragments and dispersed into single-cell suspensions. T cells were removed by E rosetting followed by separation on Ficoll–Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). The remaining cells were suspended in RPMI-1640 supplemented with 30% foetal calf serum (FCS; Gibco, Paisley, U.K.) and monocytes were depleted by adherence to plastic tissue-culture flasks for 1 hr at 37°. The non-adherent cells were thereafter layered on a step gradient of 65%, 52·5% and 42·5% Percoll (Pharmacia) and centrifuged for 10 min at 800 ***g***. The resting B cells were collected from the 65–52·5% interphase and the *in vivo*-activated B cells from the 52·5–42·5% interphase. Both B-cell populations contained 96–98% CD20-positive cells (B1; Coulter Electronics, Hialeah, FL) and $\leqslant$1% CD3- or CD11b-positive cells (OKT3 and OKM1, respectively, ATCC).

Aggregation and proliferation

All cultures were carried out in HEPES-buffered RPMI-1640 supplemented with 10% FCS or 0·3% bovine serum albumin (BSA), penicillin (100 IU/ml), streptomycin (100 μg/ml), glutamine (2 mM) and 2-mercaptoethanol (5×10^{-5} M Serva Fein Biochimica, Heidelberg, Germany). B cells were cultured at 1×1^6 cells/ml or $1{\cdot}5 \times 10^6$ cell/ml (Table 3) in flat-bottomed multi-well tissue culture plates (Linbro Chemical Co., New Haven, CT) in a final volume of 0·2 ml/well.

Cells were cultured with or without IL-1α or IL-1β, IL-2, IL-3, IL-4, IL-6, IFN-γ, GH, TSH, prolactin or antibodies to CD19, CD20, CD21 or CD43. To prevent Fc interactions, cells were incubated with 20 μg/ml of human gammaglobulin for 1 hr at 37° before being added to the cultures.

Cellular aggregation was estimated in an inverted microscope (Nikon, Yokohama, Japan) and scored according to a scale ranging from negative (−) to very strong (+++), reflecting the density as well as the size of the aggregates (Fig. 1).

For determination of proliferation, cells were cultured for 84 hr and pulsed with [^{3}H]thymidine (1 μCi/well, 18·0 Ci/mmol; Amersham International, Amersham, U.K.) during the last 18 hr, and thereafter harvested onto glass fibre filter paper (Skatron, Lier, Norway). [^{3}H]thymidine incorporation was determined as triplicates in a liquid scintillation counter (LKB/Pharmacia).

Flow cytometry

For determination of antigen expression by flow cytometry, cells were cultured at a cell density of 1×10^6 cells/ml in 24-well plates (Linbro) in the presence or absence of CD40 antibodies and/or IL-4. Cells were stained with appropriately diluted direct FITC-conjugated CD18 or CD54 antibodies for 30 min and washed twice in Tris-buffered Hanks' balanced salt solution containing 0·02% sodium azide. An isotype-matched unrelated antibody (P7A5-FITC directed against a bladder carcinoma antigen) was used as a control. The cells were analysed with a FACScan (Beckton-Dickinson, Mountain View, CA).

Electron microscopy

Aggregates from cultures stimulated for 4 days with CD40 antibodies and IL-4 were prepared for examination by electron microscopy. Aggregates were fixed at room temperature with 2·5% glutaraldehyde in 0·1 M Na-cacodylate buffer for 1 hr. After washing with buffer for 5–10 min, the cultures were post-fixed in osmium tetroxide for 1 hr at 4° before dehydration in graded ethanol, transfer to a dipenten as an intermediate and embedding in epoxide.[16] The preparations were examined in a Jeol 100-S electron microscope (Tokyo, Japan).

IgE ELISA

Resting tonsillar B cells were cultured for 10 days in round-bottomed multi-well tissue culture plates (Linbro) at a cell density of $0{\cdot}5 \times 10^6$ cells/ml and supernatants were assayed for IgE content by ELISA as described earlier.[17] Briefly, 96-well ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 10 μg/ml of rabbit anti-human IgE over night at 4° in 0·05 M sodium carbonate buffer (pH 9·6) and saturated with phosphate-buffered saline (PBS) and 0·5% bovine serum albumin (BSA) for 1·5 hr at 37°. After four washes in PBS containing 0·05% Tween 20, duplicates of the samples, 100 μl/well, were added and incubated over night at room temperature. For detection, biotinylated anti-IgE antibodies were added, incubated for 1·5 hr at 37°, washed and streptavidin-ALP (Mabtech) was added. After further incubation for 1·5 hr at 37°, p-nitrophenyl substrate was added and OD_{405} was read in an ELISA reader. The sensitivity of the assay was determined with a calibrated standard and found to be 30 pg/ml.

IL-6 assay

For detection of IL-6, resting B cells were cultured with different stimuli at a cell density of 1×10^6 cells/ml in flat-bottomed 24-well plates (Linbro) and supernatants were harvested and filtered (0·22 μm) after 4 days of culture. Supernatants were

thereafter added in serial dilutions in Dulbecco's modified medium supplemented with antibiotics, glutamine and 10% FCS. The IL-6-sensitive mouse hybridoma cell line B45 was added at a density of $2{\cdot}5 \times 10^4$ cells/ml and rIL-6 (Genzyme, specific activity 10^7 U/mg) was used as a standard. For determination of proliferation, cell were cultured for 48 hr and pulsed with [^{3}H]thymidine (1 μCi/well, 18·0 Ci/mmol; Amersham) for the last 6 hr of culture and thereafter harvested onto glass fibre filter papers (Skatron). [^{3}H]thymidine incorporation was determined as triplicates in a liquid scintillation counter (LKB/Pharmacia).

RESULTS

IL-4 enhances aggregation of CD40-stimulated human B cells

Highly purified, resting tonsillar B cells were cultured together with anti-CD40 antibodies, with IL-4 or a combination of these reagents. As reported previously, CD40 stimulation by itself induced some aggregation as well as a low but significant proliferation. IL-4, on the other hand, displayed neither of these effects but induced a motile phenotype. However, when combining the two stimuli a striking enhancement of both aggregation and proliferation was observed (Table 1), resulting in large dense aggregates which involved most cells in the cultures (Fig. 1). Apart from the dramatic difference in size of these aggregates and those induced by only CD40 antibodies, there was also a clear difference in the kinetics of aggregate formation. Thus, whereas the latter occurred rapidly and was almost fully developed within the first 24 hr, the IL-4/CD40-induced aggregates reached their maximal size around day 4, at which time they were usually macroscopically visible. This effect was seen with the monoclonal CD40 antibody, S2C6, and was even more pronounced with a monospecific rabbit antibody to this molecule. In contrast, and as shown earlier for proliferation, Fab fragments were incapable of inducing aggregation but could be made to do so after crosslinking with secondary antibody (Table 1). Furthermore, the doses of antibodies as well as the concentration of IL-4 required to obtain optimal co-stimulation were about the same for both proliferation and aggregation.

To rule out that the observed aggregation merely reflected an agglutination of the cells, tests were also performed at 4° or in the presence of 0·02% sodium azide. In both cases aggregation was completely inhibited, indicating that it is an energy-dependent process which is likely to involve the activation of specific adhesion molecules. To further assure that the observed effects were not due to contaminants in the antibody or IL-4 preparations, soluble CD40 fragments containing the external part of the molecule linked to the heavy chain of mouse IgG or neutralizing antibodies to IL-4 were added to IL-4/CD40-stimulated cultures. Both these agents were shown to efficiently block both proliferation and aggregation (data not shown).

Antibodies to LFA-1 inhibit aggregation and IgE synthesis but not proliferation

To test whether aggregation was due to an activation of LFA-1, blocking antibodies to the β-chain of this molecule (CD18) were included in the IL-4/CD40-stimulated cultures. As shown in Fig. 1e, aggregation was almost completely inhibited by this treatment. Although this indicates that LFA-1 may be principally responsible for the increased adhesiveness, the small clusters of cells which remained and which were roughly equivalent to those seen after stimulation with CD40 antibodies alone, suggest the involvement also of another adhesion mechanism(s). However, with aggregation being clearly dependent on the activation of LFA-1, we further investigated whether stimulation affected the surface expression of this molecule as well as of its ligand ICAM-1. As seen in Fig. 2, both molecules were significantly up-regulated in response to IL-4/CD40 stimulation, with ICAM-1 showing more rapid kinetics than LFA-1. Some up-regulation of ICAM-1, but not of LFA-1, was also observed in cells treated only with CD40 antibodies, whereas IL-4 alone had no effect on the expression (data not shown).

Table 1. Effect of aggregation and proliferation on resting tonsillar B cells cultured for 4 days with CD40 antibodies and/or IL-4

	Aggregation	Proliferation
Medium	−	630 ± 109
IL-4 (100 U/ml)	−	782 ± 65
S2C6 (25 μg/ml)	+	2987 ± 436
S2C6 + IL-4 (10,000 U/ml)	+ + +	23,155 ± 1177
S2C6 + IL-4 (5000 U/ml)	+ + +	22,428 ± 697
S2C6 + IL-4 (1000 U/ml)	+ + +	22,518 ± 2635
S2C6 + IL-4 (100 U/ml)	+ + +	6654 ± 335
S2C6 + IL-4 (10 U/ml)	+ +	3577 ± 514
S2C6 + IL-4 (1 U/ml)	+	3315 ± 104
S2C6 + IL-4 (0·1 U/ml)	+	2760 ± 233
S2C6 + IL-4 (0·01 U/ml)	+	2667 ± 82
Rabbit CD40 Ab (25 μg/ml)	+	4922 ± 383
Rabbit CD40 Ab + IL-4 (100 U/ml)	+ + +	13,380 ± 611
Rabbit anti-CD40 Fab fragments (1 μg/ml)	−	641 ± 66
Rabbit anti-CD40 (Fab) + IL-4	−	857 ± 41
Sheep anti-rabbit IgG (Fab) (0·5 μg/ml) + IL-4	−	228 ± 66
Rabbit CD40 Fab + anti-rabbit IgG-Fab + IL-4	+ +	1232 ± 63

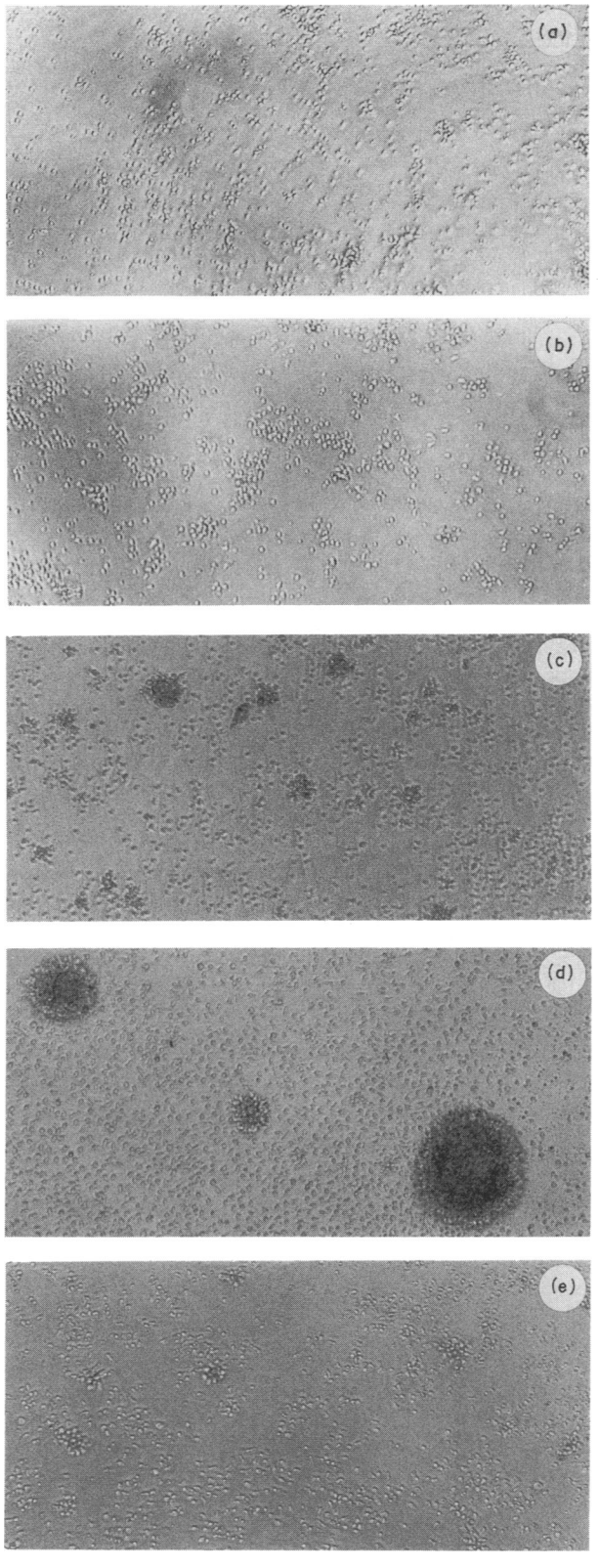

Figure 1. Effects of CD40 antibodies and IL-4 on homotypic B-cell aggregation. Resting tonsillar B cells (1×10^6/ml) were cultured for 4 days in (a) medium alone, (b) IL-4 (100 U/ml), (c) CD40 antibodies (25 μg/ml), (d) IL-4 and CD40 antibodies; and (e) IL-4, CD40 antibodies and CD18 antibodies (10 μg/ml of MHM23).

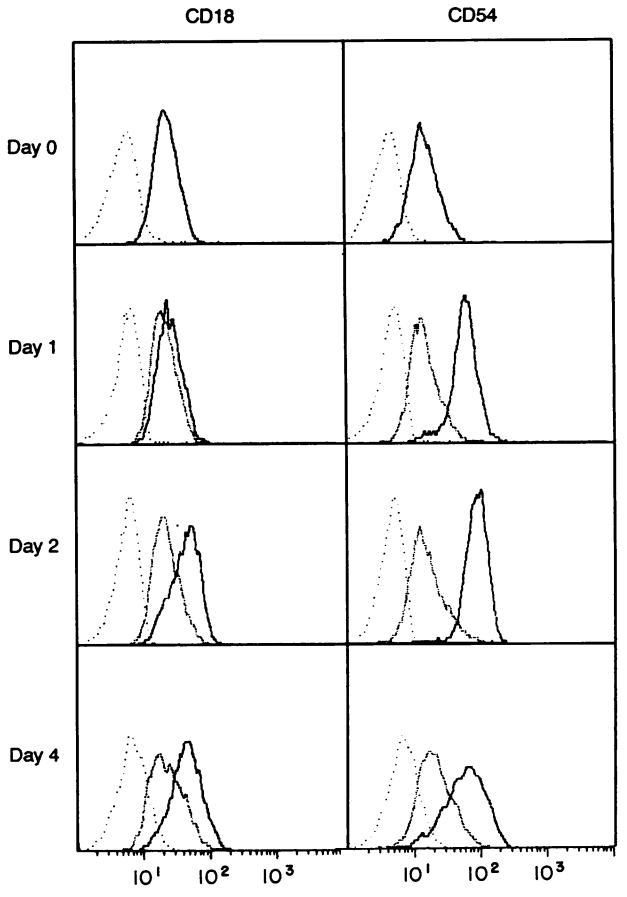

Figure 2. Kinetic analysis of CD18 and CD54 expression. FACS analysis of resting tonsillar B cells (1×10^6/ml) cultured for 0, 1, 2 or 4 days in the presence or absence of IL-4 (100 U/ml) and CD40 antibodies (25 μg/ml). Stimulated (solid line) or unstimulated (hatched line) cells were stained with CD18- or CD54-FITC conjugated antibodies. Dotted line represents stimulated cells stained with an isotype-matched control antibody (P7A5-FITC, IgG1, directed against a bladder carcinoma antigen).

It has previously been shown that IL-4/CD40 stimulation leads to induction of IgE synthesis. To test whether aggregation was required for IgE production, blocking LFA-1 antibodies were added to stimulated cultures and the IgE content of 10-day supernatants was determined by ELISA. As seen in Table 2, addition of antibodies either to the α- or the β-chain of LFA-1 (CD11a and CD18, respectively) caused a significant decrease in the IgE production. This inhibition, which was even more pronounced if both antibodies were added together, suggests that cell contact is intimately associated with their differentiation into IgE-secreting cells. As expected, a similar suppression in IgE secretion was seen when a neutralizing antibody to IL-4 was added. In contrast to the inhibitory effect of CD18 mAb on aggregation and IgE synthesis, proliferation was not affected or was slightly enhanced by the addition of antibodies (Table 3). This indicates that proliferation occurs independently of aggregation and may even be counteracted by it. Interestingly, a similar, positive effect on proliferation was seen when neutralizing antibodies to IL-6 were added (Table 3).

Table 2. LFA-1 antibodies inhibit IgE synthesis in IL-4/CD40-stimulated human B cells

	Experiment (pg/ml)				
Stimuli	1	2	3	4	5
Medium	<30	<30	<30	<30	<30
IL-4 (200 U/ml)	<30	<30	<30	<30	<30
S2C6 (25 μg/ml)	<30	<30	36	<30	<30
IL-4+S2C6	450	1450	420	850	775
IL-4+S2C6+CD18 mAb (10 μg/ml)	60	370	42	460	<30
IL-4+S2C6+CD11a mAb (10 μg/ml)	75	300	46	<30	<30
IL-4+S2C6+CD18 mAb+CD11a mAb	40	160	<30	<30	<30
IL-4+S2C6+IL-4 mAb	NT	NT	<30	<30	<30

NT, not tested.

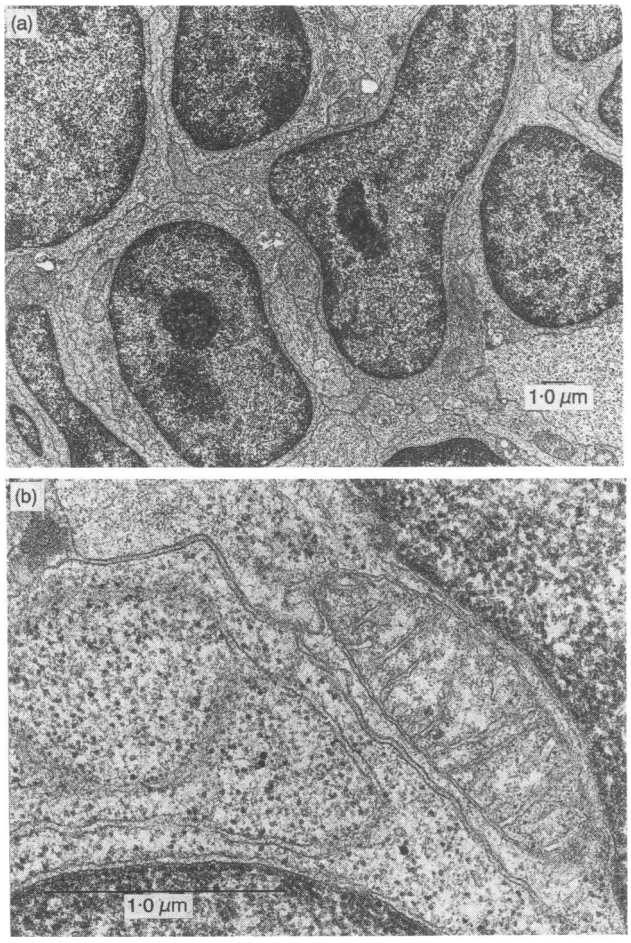

Figure 3. Aggregate morphology. Isolated aggregates from B-cell cultures stimulated for 4 days with IL-4 (100 U/ml) and CD40 antibodies (25 μg/ml) were examined by electron microscopy at (a) 10,000 × or (b) 70,000 × magnification.

Close cell contact may facilitate uptake of endogenous factors

Observations of the aggregated cells in a light microscope showed that they were assembled in very tight spherical clusters that each could contain more than 10^5 cells. These aggregates were very stable and could be separated only by trypsin treatment or by harsh pipetting in the presence of EDTA. Isolated aggregates were also investigated by electron microscopy and, as seen in Fig. 3a, the cells within aggregates displayed an almost tissue-like conformation with a compressed cytoplasm and with little intercellular space (< 20 nm) between the cells (Fig. 3b). Although there was no evidence of any type of junction, the large contact areas are likely to facilitate not only direct cell contacts such as those represented by LFA-1/ICAM-1 interactions but also indirect contacts via soluble factors. One such factor known to be produced by B cells in an autocrine manner is IL-6. It has, for example, been shown that IL-6 is produced in response to IL-4/CD40 stimulation and it may contribute to the development of IgE-producing cells. By tests on the IL-6-dependent mouse hybridoma cell line B45, we could confirm this induction of IL-6 and could furthermore demonstrate significantly increased levels of this interleukin in cultures where aggregation had been blocked by LFA-1 antibodies (Table 4).

Effects of other B-cell signals on aggregation and proliferation

Since antibodies to other B-cell antigens, including CD19 and CD22, have recently been reported to induce homotypic B-cell aggregation, we also examined how these compared to the CD40-induced aggregation and to what extent they were also synergistic together with IL-4. Out of four antibodies tested (CD19, CD20, CD21 and CD43), antibodies to CD19 and CD20 by themselves induced a rapid aggregation (< 24 hr) that was somewhat more extensive than the one seen with CD40 antibodies (Table 5). As for CD40, this aggregation could be further enhanced by the addition of IL-4. However, there appeared to be no co-stimulatory effects on aggregation when combinations of CD19 and CD20 antibodies were used or when these were used together with antibodies to CD40. Unlike CD40, stimulation via CD19 and CD20 did not induce any proliferation either alone or in combination with IL-4. Instead, both CD19 and CD20 antibodies efficiently inhibited the CD40-induced proliferation.

In addition to the agonistic antibodies and IL-4, we also tested the effect on aggregation and proliferation of several other cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-6, IFN-γ) as well as some hormones (GH, TSH, prolactin) that have been shown to be involved in B-cell stimulation. None of these displayed any stimulatory activity when tested alone. However, when tested in

Table 3. Effect of aggregation and proliferation on resting tonsillar B cells stimulated with CD40 antibodies, IL-4 and with LFA-1-antibodies (CD18) or IL-6 antibodies

Stimuli	Experiment 1		2		3		4	
	Proliferation	Aggregation	Proliferation	Aggregation	Proliferation	Aggregation	Proliferation	Aggregation
Medium	199 ± 42	−	99 ± 36	−	297 ± 44	−	748 ± 101	−
IL-4 (100 U/ml)	1930 ± 423	−	802 ± 56	−	785 ± 177	−	2811 ± 153	−
Rabbit anti-CD40 (25 μg/ml)	12,675 ± 680	+	4258 ± 299	+	6920 ± 343	+	4024 ± 605	+
IL-4 + rabbit anti-CD40	34,458 ± 3878	+ + +	64,630 ± 4147	+ + +	13,920 ± 1557	+ + +	44,751 ± 2856	+ + +
IL-4 + rabbit anti-CD40 + CD18 mAb	39,623 ± 663	+	73,186 ± 3744	+	17,852 ± 2732	+	37,526 ± 1258	+
IL-4 + rabbit anti-CD40 + IL-6 mAb (20 μg/ml)	44,286 ± 10,587	+ + +	66,869 ± 14,498	+ + +	17,728 ± 2581	+ + +	NT	NT

NT, not tested.

Table 4. Determination of IL-6 production by CD40/IL-4-stimulated resting tonsillar B cells

Supernatant from cells stimulated with	Experiment (pg/ml) 1	2
Medium	80	73
IL-4 (200 U/ml)	507	159
S2C6 (25 μg/ml)	1408	104
IL-4 + S2C6	10,812	2535
IL-4 + S2C6 + CD18 mAb (10 μg/ml)	40,766	21,856

Table 5. Effects of other antibodies/cytokines on aggregation (A) and proliferation (P)

	IL-2				IL-4	
	A	P	A	P	A	P
Medium	−	−	−	−	−	−
CD19Ab	+ +	−	+ +	−	+ + +	−
CD20Ab	+ +	−	+ +	−	+ + +	−
CD21Ab	−	−	−	±	−	−
CD40Ab	+	+	+ +	+ +	+ + +	+ + +
CD43Ab	−	−	−	−	±	+

a criss-cross manner, IL-2 was shown to be co-stimulatory with CD40 antibodies both with regard to aggregation and proliferation (Table 5). Furthermore, while antibodies to CD21 and CD43 did not by themselves have any effect on the cells they were slightly co-stimulatory with IL-2 (CD21) or IL-4 (CD43). All other combinations of cytokines, hormones and/or antibodies failed to induce aggregation or proliferation (data not shown).

DISCUSSION

This study extends and confirms the previous observations of homotypic adhesion of B cells in response to stimulation with CD40 antibodies.[11,18] It further shows that IL-4 is a strong potentiator of this process and, under the influence of these two signals, B cells formed large spherical aggregates with a dense configuration, as revealed by electron microscopy. Both the CD40- and CD40/IL-4-induced adhesion was shown to be dependent on the activation of LFA-1/ICAM-1 and the inclusion of blocking antibodies to this molecule resulted in a reduced and delayed aggregation. However, the fact that aggregation was never completely blocked by this treatment also suggests the involvement of another adhesion mechanism(s). In addition to the stimulation with CD40 antibodies and IL-4, a number of other B-cell antibodies and lymphokines were examined for their ability to induce aggregation. Out of these only antibodies to CD19 and CD20 were active and induced a rapid aggregation that was somewhat more pronounced than the corresponding aggregation with CD40 antibodies. Together with the previous observation that IL-4 enhances aggregation of anti-IgM-stimu-

lated B cells[12] it seems reasonable to assume that IL-4 plays a similar role in all these cases. As shown here for CD40 and previously for anti-IgM[12] co-stimulation with IL-4 led to an up-regulation of both LFA-1 and its ligand ICAM-1. Although this may reflect a common mechanism by which IL-4 can stimulate adhesion, others have reported that such up-regulation of LFA-1/ICAM-1 is not required to promote aggregation via this adhesion system.[9] Furthermore, as up-regulation of LFA-1 was late and relatively modest, one can not exclude that it may to a large extent be caused by an increase in cell size. Another function of IL-4 that may be relevant to the situation is its activity as an inducer of locomotion. Thus, IL-4 has been shown to stimulate cell motility both in human and mouse B cells[19,20] and it is likely that such activation of cell movement would increase the chances for cell contact and aggregation provided that the proper adhesion molecules are present in an activated form.

As previously reported, stimulation of B cells with IL-4 and CD40 antibodies also led to considerable proliferation.[21] However, as opposed to aggregation, this was not inhibitable by LFA-1 antibodies. Rather, in most experiments proliferation was enhanced by this treatment, suggesting that proliferation occurs independently of aggregation and may even be counteracted by it. Analogously, proliferation was not required for cell adhesion since extensive aggregation was also observed in serum-free cultures where there was little or no proliferation (data not shown). Similarly, aggregation induced by IL-4 in combination with antibodies to CD19 or CD20 occurred in the absence of any proliferation.

In spite of several reports on B-cell aggregation[4,9] there appears so far to be little information as to the function of this phenomenon. Apart from the general notion that adhesion is likely to be important in the cellular contacts required for the collaboration between, for example, B and T cells[1] we also believe that homotypic adhesion may play a role in B-cell activation. The intimate cell contact found in the aggregates may serve here to facilitate the exchange of direct signalling through LFA-1[22,23] or indirect signals between cells and it can be easily envisaged that, for example, lymphokines released from the cells are retained at high local concentration in the intercellular space. That such indirect communication via autocrine factors may indeed take place in this type of culture has been shown by the fact that IL-4/CD40-induced IgE synthesis could be inhibited by neutralizing endogenous IL-6 with antibodies.[24] Interestingly, we found that IgE production was similarly inhibited in cultures where aggregation had been prevented by antibodies of LFA-1. This decrease in IgE synthesis could not be explained by a lowered IL-6 production since more of this factor was regularly found in the supernatants of cultures where aggregation had been blocked compared to control cultures (without LFA-1 antibodies). However, in agreement with the suggestion above, aggregation may be essential in creating high local concentrations of IL-6 which in turn are likely to improve the accessability and responsiveness to this factor.

The results obtained here support previous observations that autocrine signalling may be an important feature during certain stages of B-cell activation[25,26] and suggests further that the IL-6, and possibly other lymphokines produced in response to IL-4/CD40 stimulation, acts primarily as a maturation factor and has a limiting effect on proliferation. In apparent contradiction to this notion of B-cell growth as being self-limiting, it has recently been shown that IL-4/CD40 stimulation can sustain long-term growth of B cells, provided that the CD40 antibodies are presented on Fc-receptor-bearing cells.[27,28] Also under these conditions aggregates are formed, but these tend to be smaller and are more dispersed in the cultures, probably as a consequence of physical association to the Fc-receptor-positive cells (P. Björck *et al.*, unpublished observation). As a result large aggregates take longer to develop and the cells are retained in a proliferative phase for longer periods of time.

The observations made here may have several implications for studies of B cells *in vitro*. Thus, for example, the realisation that aggregation can represent a major obstacle to obtain long-term growth of normal B cells may prove helpful in defining new strategies for growing B cells. Furthermore, determinations of the individual factors that affect proliferation, maturation and Ig-switching may be facilitated by using culture conditions in which the influence of endogenous factors has been minimized by inhibiting aggregation. *In vivo*, the primary function of adhesion may be to promote contact with T cells during the early phases of B-cell activation. However, it seems likely that the homotypic aggregation of B cells also has a role in the later stages of activation, where it may fulfil a similar differentiating function as that observed *in vitro*.

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