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CD40 antibodies defining distinct epitopes display qualitative differences in their induction of B-cell differentiation

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SUMMARY

IgE production can be obtained *in vitro* by stimulating B lymphocytes with CD40 antibodies and interleukin-4 (IL-4). This stimulation also results in homotypic aggregation and cell proliferation. We have shown previously that IgE synthesis may be dependent on additional signals provided by the close cellular contact. Thus inhibition of the aggregation by lymphocyte function-associated antigen-1 (LFA-1) antibodies leads to a decrease in IgE production. In the present study we show that the inhibitory effect of LFA-1 antibodies is critically dependent on the CD40 antibody used for stimulation. Thus, while previously using the monoclonal antibody (mAb) S2C6, IgE production induced by the CD40 antibody mAb89 was generally higher and could be enhanced more than fivefold in the presence of LFA-1 antibodies. Similarly, the addition of the CD23 mAb MHM6, which blocked aggregation to a similar degree as the LFA-1 antibodies, inhibited S2C6-induced IgE production but enhanced that induced by mAb89. In contrast to these opposing effects on IgE synthesis, proliferation induced by the two CD40 antibodies was affected similarly by the blocking antibodies. As the interaction between CD23 and CD21 has been suggested to involve recognition of carbohydrate structures on CD21 by the lectin-like domain on CD23, we also tested the effect of some different sugars on IgE synthesis and proliferation. Addition of fucose-1-phosphate to anti-CD40 and IL-4-stimulated B cells completely inhibited IgE synthesis and proliferation. Inhibtion was also seen with mannose-6-phosphate but not with glucose-1-phosphate. In contrast to the MHM6 antibody, the effect of the sugars was similar irrespective of the CD40 antibody used for stimulation. The study shows that different antibodies to CD40 may give rise to qualitatively distinct signals depending on the epitope recognized.

INTRODUCTION

B-lymphocyte activation is a process that requires collaboration between several different cell types and involves interactions between a number of cell-surface molecules and soluble factors.[1,2] For many of the interactions, cells need to establish close cellular contact. This is made possible by the activation of different adhesion receptors and activated normal B cells as well as many B-cell lines are characterized by their growth in cell clusters. For instance, *in vitro* stimulation of resting B cells with CD40 antibodies and interleukin-4 (IL-4) leads not only to proliferation and IgE synthesis but also to the formation of large, spherical cell aggregates. These are caused by activation of the lymphocyte function-associated antigen-1/intracellular adhesion molecule-1 (LFA-1/ICAM-1) and CD23/CD21 adhesion molecules and may be inhibited by blocking antibodies to these proteins.[3,4] In a previous study we showed that the IgE production occurring in CD40/IL-4-stimulated cultures was connected intimately to the aggregated state of the cells. Thus, blocking of adhesion with antibodies to LFA-1 also led to reduced levels of IgE.[3] In the present study, we have used antibodies to both LFA-1 and CD23 to study further the relationship between aggregate formation and the capacity of B cells to differentiate and proliferate.

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MATERIALS AND METHODS

Reagents

Antibody MHM23 (IgG1) to the β-chain of LFA-1 (CD18) was obtained from Dakopatts a/s (Roskilde, Denmark). The CD23 monoclonal antibody (mAb) MHM6 (IgG1) was purchased from Dakopatts, and Epstein–Barr virus (EBV) CS-5 (IgG1)[5] against the same antigen was a kind gift from Dr B. Sugden (University of Wisconsin, Madison, WI). The CD40 antibody mAb89 (IgG1)[6] was generously provided by Dr H. Yssel (DNAX, Palo Alto, CA), while the CD40 mAb S2C6 (IgG1) was produced in our laboratory.[7] Purified human IgE and rabbit anti-IgE antibodies were obtained from Dr P. Larsson (National Institute of Occupational Health, Stockholm, Sweden) and the sugars fucose-1-phosphate, mannose-6-phosphate and glucose-1-phosphate were all from Sigma (St Louis, MO).

Table 1. IgE production by tonsillar B cells stimulated for 10 days with IL-4 and CD40 antibodies. Effect of antibodies to CD18 and CD23

	IgE production (pg/ml)*						
	Exp. 1		Exp. 2		Exp. 3		
	S2C6	mAb89	S2C6	mAb89	S2C6	mAb89	Aggregation
IL-4†	48	1400	20	10 000	15	200	+++
+ MHM23 (CD18)	15	35 000	< 2	53 000	4	8800	±
+ MHM6 (CD23)	11	3780	< 2	21 000	< 2	1200	±
+ EBV CS-5 (CD23)	432	602	529	5000	475	60	+++

* Standard errors were within 15%.

† The concentrations used were 500 U/ml (IL4), 1 μg/ml (S2C6 and mAb89) and 10 μg/ml (MHM23) or 60 μg/ml (MHM6 and EBV CS-5). No induction of IgE synthesis was seen with IL-4 or CD40 antibodies alone.

Cells

B cells were prepared from human tonsils as described elsewhere.[8] 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% fetal calf serum (FCS; Gibco, Paisley, UK) and monocytes were depleted by adherence to plastic tissue culture flasks for 1 hr at 37°. The non-adherent cells were then layered on a step gradient of 65%, 52·5% and 42·5% Percoll (Pharmacia) and centrifuged for 10 min at 800 *g*. The small dense B cells, used throughout the study and referred to as resting B cells, were collected from the 65–52·5% interphase. These cells contained 96–98% CD20-positive cells (B1; Coulter Electronics, Hialeah, FL), and less than 1% CD3- or CD11b-positive cells (OKT3 and OKM1, respectively; American Tissue Type Culture Collection, Rockville, MD), as determined by fluorescein-activated cell sorter (FACS) analysis.

Cell proliferation and aggregation

All cultures were carried out in HEPES-buffered RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, antibiotics and 5×10^{-5} M 2-mercaptoethanol. B cells were cultured at 2×10^6 cells/ml in half-area tissue culture plates (Costar, Cambridge, MA) for 4 days, with or without stimulatory agents. Optimally stimulatory doses of CD40 antibodies (1 μg/ml for both mAb89 and mAb S2C6) and IL-4 (500 U/ml) were determined by titration experiments. [^{3}H]thymidine (1 μCi/well; Amersham Int., Amersham, UK) was added during the final 18 hr of culture and cells were then harvested and measured in a liquid scintillation counter. Before harvesting, aggregation was scored under an inverted microscope according to a scale ranging from negative (−) to very strong (+++), reflecting both the number of cells involved in aggregation and the size and density of the aggregates.

IgE production

Resting, tonsillar B cells were cultured for 10 days in round-bottomed multi-well tissue culture plates (Linbro, Chemical Co., New Haven, CT) at a cell density of $0{\cdot}5 \times 10^5$ cells/ml, and supernatants were analysed for IgE content as described earlier.[8] Briefly, 96-well enzyme-linked immunosorbent assay (ELISA) plates (Costar) were coated with 10 μg/ml rabbit anti-human IgE overnight at 4° in phosphate-buffered salt solution (PBS), and then saturated with 0·5% bovine serum albumin (BSA) for 3 hr at 37°. After four washes in PBS containing 0·05% Tween-20, duplicates of test samples, 50 μl/well, were added and incubated over night at room temperature. After washing, biotinylated anti-IgE antibodies were added and incubated for 1·5 hr at 37°, followed by washing and the addition of streptavidin–alkaline phosphatase (ALP) (Mabtech, Stockholm, Sweden). After 1·5 hr at 37°, p-nitrophenyl substrate was added and the optical density at 405 nm (OD_{405}) was read in an ELISA reader. The IgE content of the supernatants was finally calculated by comparison against a calibrated IgE standard.

RESULTS

Cell adhesion and IgE synthesis

We have found previously that CD40/IL-4-induced IgE production in purified populations of resting B cells may be dependent on homotypic cell contact. Thus suppression of adhesion by antibodies to CD18 led to a significant decrease in IgE synthesis.[3] These results, which were obtained with the CD40 mAb S2C6, could be repeated in the present study (Table 1). However, when triggering cells with the CD40 antibody mAb89, which induces a much stronger signal for IgE synthesis, the addition of CD18 antibody had the opposite effect and resulted in a five–40-fold increase in IgE synthesis (Table 1). The CD23 mAb MHM6, which like the CD18 antibody is able to inhibit cell aggregation, also had a suppressive effect on S2C6-induced IgE synthesis, but enhanced production in cultures stimulated with mAb89 and IL-4.

This critical dependence on CD40 mAb for triggering was also observed with another CD23 mAb, EBV CS-5. This mAb, which in contrast to the MHM6 mAb did not affect aggregation, had a stimulatory effect on S2C6-induced IgE production while inhibiting IgE synthesis by more than 50% in mAb89-stimulated cultures.

Effects of different sugars on IgE production

As indicated from previous studies, the binding between CD23

Table 2. Effect of sugar phosphates on IgE production and aggregation of B cells stimulated for 10 days with IL-4 and CD40 antibodies

	IgE production (pg/ml)*				
	Exp.1		Exp. 2		
	S2C6	mAb89	S2C6	mAb89	Aggregation
IL-4†	4000	6400	500	10000	+++
+ fucose-1-phosphate	4	6	60	100	–
+ mannose-6-phosphate	6000	896	600	7000	++
+ glucose-1-phosphate	9000	12100	300	6000	+++

* Standard errors were within 15%.

† The concentrations used were 500 U/ml (IL-4), 1 mg/ml (S2C6 and mAb89) and 5 μM for the sugar phosphates.

and CD21 may reflect the recognition of a carbohydrate structure on CD21 by the lectin-like domain of CD23.[9] We therefore investigated the effect of different sugars on CD40/IL-4-induced aggregation and IgE synthesis. Besides fucose-1-phosphate, which is known to block the CD23–CD21 interaction, we also used the structurally similar mannose-6-phosphate, as well as glucose-1-phosphate.

Fucose-1-phosphate more or less abolished IgE synthesis, irrespective of the CD40 mAb used for stimulation (Table 2). Cell aggregation and the motile morphology, displayed by the stimulated cells, were also inhibited by this sugar. Mannose-6-phosphate had a similar, but less pronounced, effect on both IgE production and aggregation, whereas glucose-1-phosphate showed only minor effects on IgE production and did not affect aggregation (Table 2).

Cell adhesion and proliferation

In contrast to their different capacities to induce differentiation and IgE synthesis, both mAb89 and S2C6 gave very similar signals for proliferation (Table 3). The addition of CD18 antibody only marginally affected the proliferative response and could result either in a somewhat suppressed or, as often seen, in a slightly enhanced proliferation (Table 3). However, inhibition of cell contact by the CD23 mAb MHM6, more consistently affected proliferation in a negative manner, while addition of the CD23 mAb EBV CS-5 had either no or a slightly stimulatory effect. All of these effects were similar irrespective of whether mAb89 or S2C6 was used as the stimulatory agent.

The addition of fucose-1-phosphate to CD40/IL-4-stimulated cells led to an almost complete abrogation of proliferation (Fig. 1). Mannose-6-phosphate also led to decreased proliferation, but the effect was much less pronounced with inhibition rarely exceeding 20% of the normal response. Finally, glucose-1-phosphate did not affect or only slightly enhanced proliferation.

Table 3. Effect of CD18 and CD23 antibodies on IL-4/CD40-induced proliferation

	[^{3}H]thymidine incorporation (c.p.m.)	
Stimuli	Exp. 1	Exp. 2
Medium	1170 ± 100	3200 ± 310
IL-4*	3720 ± 10	7900 ± 810
mAb89*	5510 ± 50	7070 ± 250
IL4 + mAb89	30910 ± 670	32810 ± 5180
+ anti-CD18 (MHM23)	24480 ± 210	34270 ± 970
+ anti-CD23 (MHM6)	14800 ± 1880	25840 ± 2260
+ anti-CD23 (EBV CS-5)	45650 ± 2220	58240 ± 3550
S2C6	7640 ± 780	8020 ± 570
IL-4 + S2C6	57940 ± 6060	52430 ± 4920
+ anti-CD18 (MHM23)	51080 ± 2180	44080 ± 4290
+ anti-CD23 (MHM6)	38340 ± 1690	39180 ± 630
+ anti-CD23 (EBV CS-5)	50530 ± 3730	67220 ± 3400

* Doses of IL-4 and antibodies are as indicated in Table 1.

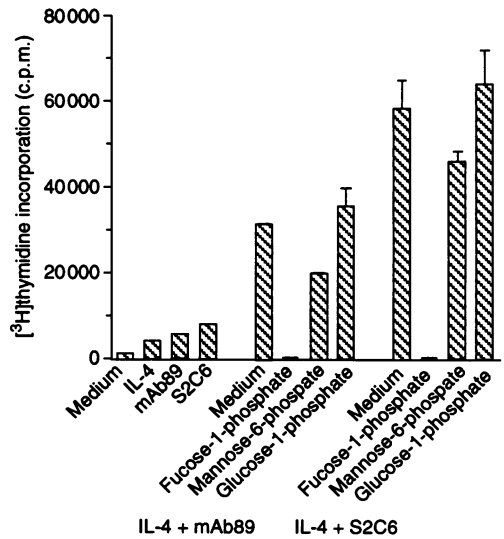

Figure 1. Effect of different sugars on CD40/IL-4-induced proliferation. High density, tonsillar B cells (1×10^6 cells/ml) were cultured with or without IL-4 (500 U/ml), CD40 antibody mAb89 (1 μg/ml) and different sugar phosphates (5 mM) for 4 days. [^{3}H]thymidine incorporation was measured during the last 18 hr of culture.

As with the antibodies, the effects on proliferation appeared to be independent of whether it was induced by mAb89 or mAb S2C6.

DISCUSSION

We have demonstrated previously that CD40/IL-4-induced IgE synthesis may require close cellular contact between the B cells, as inhibition of this contact with LFA-1 antibodies led to a significant decrease in the amount of IgE produced.[3] In apparent contradiction to this, Splawski *et al.*[10] recently reported an enhanced IgE production by LFA-1 antibodies in a very similar system using a different CD40 antibody. In the present study, we show that the effect of LFA-1 antibodies may be critically dependent on which CD40 antibody is used for the stimulation. Thus, when triggering cells with mAb89 instead of the previously used S2C6 antibody, blocking of aggregation with LFA-1 antibodies led to a more than fivefold increase in IgE synthesis. The same inverse relationship was seen when inhibiting aggregation with the CD23 mAb MHM6. Furthermore, the CD23 mAb, EBV CS-5, which did not affect the adhesion process and which, in contrast to MHM6, enhanced S2C6-induced IgE synthesis, displayed a clearly suppressive effect on IgE production induced by mAb89. Although one explanation of these results may be found in the different levels of IgE induced by the two CD40 antibodies, the same observation was also made in experiments in which mAb89 had been titrated to generate a smilar level of IgE synthesis as the S2C6 mAb. Thus it seems more likely that the signals given by the two CD40 antibodies differ qualitatively, and that these have different consequences when combined with other signals. We have previously shown that the two antibodies define distinct but partly overlapping epitopes on CD40[11] and that they also display a qualitative difference in their capacity to protect cells from apoptosis (E. Jakobson, P. Björck & S. Paulie, manuscript submitted for publication). As blocking experiments indicated that both antibodies react close to the ligand binding part of the molecule, we recently tested a soluble construct of the CD40 ligand, gp39, in an attempt to determine which one of the two CD40 antibodies corresponded best to the natural signal. In these preliminary experiments both the level of IgE production and the effect of the CD18 and CD23 antibodies resembled those obtained with the S2C6 antibody. However, as the CD40 ligand is normally expressed on the cell surface and in a trimeric form,[12] the use of a soluble dimer of gp39 may not be physiologically relevant. Significantly stronger signals have also been reported with cell-surface expressed gp39 compared with the soluble form.[13]

As we have recently found that CD40/IL-4-induced IgE synthesis is dependent on endogenouosly produced IL-6 (P. Björck, S. Larsson, M. Ancläng, L. Ärlund-Richler & S. Paulie, manuscript submitted for publication) and possibly also soluble CD23,[14] we also tested whether the two CD40 antibodies differed in their induction of these two factors. However, this appeared not to be the case, either when the antibodies were tested alone or in combination with IL-4 (P. Björck, unpublished observations). It was found recently that the CD40/IL-4-induced IgE secretion could also be enhanced by certain antibodies to CD21, as well as by soluble CD23.[15] The fact that CD21 is the receptor for C3dg/C3b, interferon-α (IFN-α) and also for EBV, suggests that complement factors and IFN-α may be involved in the natural regulation of IgE responses. It would be interesting to see whether the stimulatory CD21 antibodies as well as the natural ligands for this receptor also induce IL-6 production and if they, like antibodies to CD23 and LFA-1, display opposing effects on IgE production when combined with the two different CD40 mAb.

The observation that the sugars fucose-1-phosphate and mannose-6-phosphate inhibited aggregation as well as IgE synthesis is in line with previous data on the involvement of fucose-like carbohydrate structures in the binding between CD23 and CD21.[9,16] However, the fact that inhibition with the sugars was much more profound and also affected proliferation suggests that their action may not be confined strictly to CD23/CD21 but that they can also interfere with other molecular interactions.

In contrast to the apparent complexity of signals involved in regulating differentiation and IgE synthesis, induction of proliferation and aggregation seems to be more simple. Both the inducing capacity and the effects of LFA-1 or CD23 antibodies were very similar for the two CD40 antibodies. Moreover, we have previously seen that neither proliferation nor aggregation appear to be dependent on endogenous IL-6.[3] This indicates that the signals via CD40, as well as the cofactor/s if such are required, are different for proliferation and aggregation compared with the signals needed for differentiation and IgE synthesis.

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