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# Triggering of B lymphocytes through CD23: epitope mapping and studies using antibody derivatives indicate an allosteric mechanism of signalling

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## SUMMARY

By using five monoclonal antibodies in reciprocal cross-locking studies, a minimum of three epitope clusters have been defined for the B-cell restricted, activation-associated CD23 antigen. Two of the five antibodies were capable of replacing low molecular weight B-cell growth factor (BCGF) in B-cell co-stimulation assays. These two antibodies belonged to the same epitope group, while non-stimulatory antibodies fell outside this cluster. By prior coating of activated B lymphocytes at 4°, all five CD23 antibodies interfered with the subsequent uptake of BCGF activity onto the cells. However, only the two stimulatory antibodies were capable of inhibiting the absorption of BCGF completely. From one of these antibodies, $F(ab')_2$ and Fab fragments were generated and both were found to be equivalent to whole antibody in their ability to mimic BCGF. Immobilized antibody, however, failed to stimulate over a wide range of concentrations. These findings demonstrate that the ability of certain CD23 antibodies to deliver a growth-promoting signal to activated B cells is independent not only of the Fc portion of the molecule but also of receptor cross-linking. The latter observation is indicative of an allosteric mechanism of triggering, a notion supported by the epitope specificity of activation through CD23. The findings are discussed in relation to the putative natural ligands for CD23 and the way they may influence B-cell function through this receptor.

## INTRODUCTION

Following several years of considerable debate, the nature and actions of factors that influence B-cell function are now being resolved. This has been made possible by the purification of soluble factors to homogeneity and, more recently, by their gene cloning (Kinashi *et al.*, 1986; Hirano *et al.*, 1986; Noma *et al.*, 1986; Lee *et al.*, 1986; Yokota *et al.*, 1986). In contrast, the receptors through which these molecules transduce information to their target cells remain ill-defined. One approach has been to search for antibodies to cell surface structures whose effects mimic those associated with growth-promoting activities. From this, a number of antibodies have been identified that, when bound to the cell surface, deliver cell-cycle progression signals to activated human B cells. These include antibodies to a newly identified p50 antigen (Clark & Ledbetter, 1986), antibodies to the 135,000 molecular weight (MW) CD22 antigen (Dorken *et al.*, 1986; Pezzuto *et al.*, 1987) and an antibody to the 45,000 MW, B-cell restricted, activation-associated CD23 antigen (Gordon *et al.*, 1986a). We have shown that the ligation of CD23 at the B-cell surface by the monoclonal antibody MHM6 not only mimics the effects of a 12,000 MW T-cell derived B-cell growth factor (BCGF), but also interferes with the uptake of the BCGF onto CD23-positive cells (Gordon *et al.*, 1986b). These findings suggest an association between CD23 and the receptor for low molecular weight BCGF. Interestingly, the low affinity B-lymphocyte receptor for Fc of IgE has now been cloned (Kikutani *et al.*, 1986) and the expressed product has been shown to share identity with the CD23 molecule (reported by T. Kishimoto's group at the Third International Conference on Human Leucocyte Differentiation Antigens, Oxford, September, 1986).

In order to gain further insight into how this receptor controls B-lymphocyte growth, we have extended our studies to other CD23 antibodies and to the use of antibody fragments generated from MHM6. The results reported here demonstrate that while CD23 occupancy is sufficient for triggering B-cell growth, binding must occur within a specific site on the receptor for activation to proceed.

## MATERIALS AND METHODS

### *Antibodies*

EBVCS antibodies to CD23 were the generous gift of Dr B. Sugden (McArdle Laboratory, Madison, WI). Their generation

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and characterization have already been described in detail (Kintner & Sugden, 1981). With the exception of EBVCS4, which was an IgM, all these antibodies were of the IgG class. The generation of the MHM6 (IgG1) antibody to CD23 has also been described (Rowe *et al.*, 1982). The antibodies BK19.9 (IgG1) and 11EF7 (IgM) recognize, respectively, a non-lineage-specific 'proliferation' antigen and a novel 'late' B-cell surface antigen (Walker *et al.*, 1986). Apart from MHM6, which was purified by protein A affinity chromatography (Gordon *et al.*, 1986a), all other antibodies were prepared by ammonium sulphate precipitation followed by exhaustive dialysis into physiological buffered saline (PBS), pH 7·2. The EBVCS antibodies and MHM6 were also used conjugated to biotin. Briefly, 2 mg of antibody contained in 1·25 ml of 0·1 M $NaHCO_3$ (pH 8·4) were mixed with 180 $\mu$l of biotin-*N*-hydroxy succinimide (Miles-Yeda, Slough, Berks) dissolved in dimethylsulphoxide at 1 mg/ml and incubated at room temperature for 4 hr. At completion of the reaction, the conjugate was dialysed overnight against PBS with azide. Cell–surface staining with the conjugate was performed by incubating $5 \times 10^5$ cells with the antibody for 45 min on ice, followed by two washes and the addition of fluorescein–avidin (Miles-Yeda) at 10 $\mu$g/ml for a further 45 min on ice. Following a further two washes, cells were analysed on a FACS IV (Becton-Dickinson, Mountain View, CA). Conjugates were used at the concentration where a plateau level of staining of a CD23-positive Epstein–Barr virus (EBV)-transformed lymphoblastoid cell line (LCL) had been achieved in titration studies. For blocking studies, cells were preincubated with non-conjugated antibody (1 mg/ml) for 45 min on ice followed by washing prior to staining with the conjugate.

*Preparation of antibody derivatives*

$F(ab')_2$ fragments of MHM6 were prepared by mixing for 8 hr at 37°, 3·85 mg of IgG1 contained in citrate buffer (pH 3·5) with 48 $\mu$l of pepsin dissolved in citrate buffer at 2 mg/ml. Digestion was terminated by neutralization with 1 M NaOH. Following exhaustive dialysis against PBS, the digest was loaded onto a sepharose 4B–protein A column and 1·7 mg of material was collected in the wash.

In order to obtain Fab fragments, 10 mg of MHM6 IgG1 in 2·5 ml of 0·05 M phosphate buffer (pH 8·0) were mixed with papain to give an enzyme : IgG ratio of 1 : 100, and with cysteine to give a final molarity of 0·01 M. After 8·5 hr at 37° the reaction was terminated by exhaustive dialysis against 0·005 M phosphate, pH 8·0. A residual precipitate was removed by centrifugation and the remaining solution was passed down a calibrated G-100 column and material with the mobility of Fab monomers was collected yielding 2·3 mg of final product. Analysis of both Fab and $F(ab')_2$ preparations on 10% SDS–polyacrylamide gels followed by silver staining revealed single bands of material migrating with the expected mobility for both proteins with no evidence of contamination.

MHM6 IgG1 antibody was also used immobilized onto tissue culture plates and Affigel-10 beads (Bio-Rad, Richmond, CA). The former procedure has been described elsewhere (Gordon *et al.*, 1984), whereas the latter was performed according to manufacturer's instructions.

*B-lymphocyte activations*

Highly purified tonsillar B cells were prepared exactly as has been described in detail previously (Walker *et al.*, 1986). For co-stimulation assays, B cells of high buoyant density (i.e. those passing through > 62·5% Percoll) were cultured at $10^5$ per flat-bottomed microwell in 200 $\mu$l of culture medium with additions of the phorbol ester TPA at 1 ng/ml or fixed *Staphylococcus aureus* Cowan strain I (SAC) at 1 : $10^6$ as indicated in the text and as has been described in detail elsewhere (Gordon *et al.*, 1986a). DNA synthesis in these cultures was assessed by a [$^3$H]thymidine pulse (0·5 $\mu$Ci/well) during the last 16 hr. All results represent the mean of triplicate determinations, which were usually within 5% and never varied by more than 10% of each other. B-cell growth factor was purchased from Cellular Products Inc. (Buffalo, NY) and is a semi-purified preparation of the 12,000 MW T-cell product.

## RESULTS

### Effects of $F(ab')_2$ and Fab fragments of MHM6 on B-cell stimulation

Following the observation that the MHM6 IgG1 antibody was co-stimulatory with TPA for tonsillar B cells, $F(ab')_2$ and Fab fragments were prepared and introduced into the stimulation assay. As seen in Fig. 1a, the $F(ab')_2$ dimer was equal to whole antibody in promoting DNA synthesis in activated B cells, with the titration curves of the two following each other remarkably closely. In terms of titre, the Fab monomer was equivalent to both IgG antibody and $F(ab')_2$ in its stimulating capacity (Fig. 1b). The plateau stimulations achieved with the monomer were, however, slightly but reproducibly (three experiments) higher than those achieved with bivalent preparations.

### Effect of immobilized MHM6 on B-cell stimulation

For some receptors, delivery of a growth or activating signal is accomplished more efficiently by presenting antibody on a solid

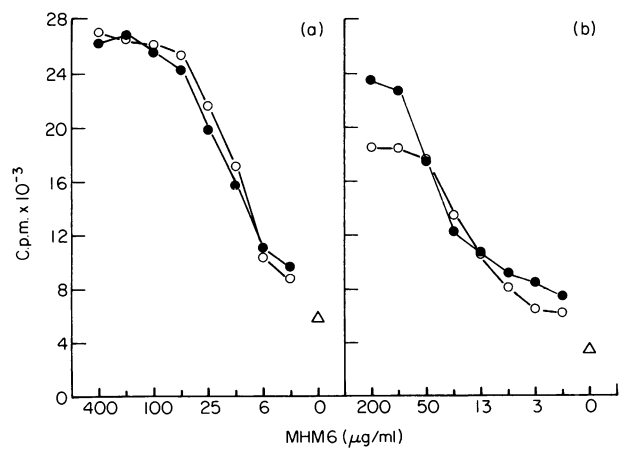


**Figure 1.** Influence of $F(ab')_2$ and Fab fragments of MHM6 on B-cell stimulations. High density tonsillar B cells were cultured for 68 hr with 1 ng/ml TPA at $10^5$ per microwell containing 200 $\mu$l of medium in the presence of MHM6 preparations as indicated. [$^3$H]TdR incorporation was assessed during the final 16 hr of culture. (a) IgG (●), $F(ab')_2$ (○); (b) Fab (●), $F(ab')_2$ (○). Stimulations in the absence of MHM6 preparations are also indicated (△). No MHM6 preparation was able to augment background DNA synthesis (237 c.p.m.) in the absence of TPA.

matrix. In order to explore this possibility for the CD23 antigen, the MHM6 antibody was coupled either to Affigel beads or directly onto the surfaces of tissue culture wells. It was found that in both procedures immobilization had nullified the stimulatory capacity of the MHM6 antibody (Table 1). There was even some indication that immobilized MHM6 might be reducing the stimulations occurring due to the effects of TPA in culture. Studies using anti-immunoglobulin bound to the tissue culture wells demonstrated that the coupling procedure did not simply destroy antibody activity as these wells were directly stimulatory for resting B cells. Furthermore, in both the wells and on the beads that had been coupled with MHM6, it was clear that the immobilized antibody was interacting with the TPA-activated cells as witnessed by the physical association of cells with the coated surfaces (not shown).

### Epitope mapping of CD23

MHM6 and four other monoclonal antibodies to CD23 were conjugated with biotin in order to perform cross-blocking studies by FACS analysis. Representative profiles from the three epitope clusters identified are shown in Fig. 2. Labelled EBVCS 1 antibody was blocked only by itself. EBVCS 2 binding was blocked by itself and by EBVCS 5. The binding of labelled EBVCS 4 was blocked by itself and by MHM6. These patterns of blocking were completely reciprocal. The three epitope groups are thus defined as: (i) EBVCS 1 (unique); (ii) EBVCS 2/5; and (iii) EBVCS 4/MHM6. It should be noted that EBVCS 2 and 5 gave partial blocking (approximately 20%) of EBVCS 4 binding (Fig. 2).

### Functional activity of EBVCS antibodies

Figure 3 shows the result of introducing the various EBVCS antibodies into the TPA co-stimulation assay. It is clear that only the EBVCS 4 antibody had the stimulatory properties already defined for MHM6. Both the unique EBVCS 1 antibody and EBVCS 2 and 5 failed totally to participate in stimulations of B cells with TPA. With respect to titre, the EBVCS 4 antibody was approximately 100-fold more potent than MHM6 in inducing DNA synthesis in activated B cells. Note that, for both stimulatory antibodies, whereas relatively high concentrations are required to reach plateau levels of stimulation, measurable stimulations can, nevertheless, be achieved with very low levels of antibody.

### Blocking of BCGF absorption by EBVCS antibodies

We have previously shown that the prior coating of activated B cells with an MHM6-containing ascites fluid interferes with the ability of these cells to absorb BCGF activity (Gordon *et al.*, 1986b). In the following experiments we have compared the ability of MHM6 IgG and the EBVCS antibody preparations to block BCGF uptake onto (preactivated) low buoyant density tonsillar B cells. As seen from Exp. 1 in Table 2, all four EBVCS antibodies significantly blocked the absorption of BCGF onto buoyant B cells. Only EBVCS 4, however, gave total blocking, a result repeated in two subsequent experiments. In these experiments, MHM6 was almost as efficient as EBVCS 4 in preventing BCGF uptake. Prior coating of cells with antibodies binding to two other structures on activated B cells failed to diminish the loss of BCGF activity through absorption (Table 2). As with EBVCS 4, one of these (11EF7) was an IgM antibody.

## DISCUSSION

The above results are highly suggestive of an allosteric mechanism being involved in the triggering of B lymphocytes through CD23. Only two of the five antibodies to CD23 were found to be stimulatory, and these were shown to bind their target molecule within the same topographical region. Furthermore, occupancy of that binding site was sufficient for signalling to occur — receptor cross-linking was not required. Intriguingly, immobilized antibody was unable to perform a stimulatory function.

**Table 1.** Effect of immobilized MHM6 on B-cell stimulation

| Antibody (μg/ml) | [$^3$H] TdR incorporation (c.p.m.) following exposure to: | | | |
|---|---|---|---|---|
| | Free MHM6 | Affigel MHM6 | Plate MHM6 | Plate anti-Ig |
| 1000 | ND | 3790 | 4900 | ND |
| 100 | 33,662 | 3647 | 5663 | 48,072 |
| 10 | 17,057 | 4506 | 6617 | 45,529 |
| 1 | 7338 | 4881 | 6345 | 17,823 |
| 0·1 | 6920 | 5276 | 6487 | 6687 |
| 0 | 5964 | 5444 | 6321 | 6321 |

High density tonsillar B cells were cultured with TPA as for Fig. 1 with either MHM6 IgG in solution (free) or coupled to Affigel beads or to the surfaces of tissue culture wells (plate) at the concentrations indicated. For the results given, MHM6-coated Affigel was used at 1/1000 (v/v), but was also tested over a range of 1/200 to 1/100,000 without any augmentation of stimulation being noted. Cells were also cultured in wells coated with anti-Ig as above but in the absence of TPA. DNA synthesis was measured between 52 hr and 68 hr from the onset of culture and results represent means of triplicate determinations. (ND, not determined.)

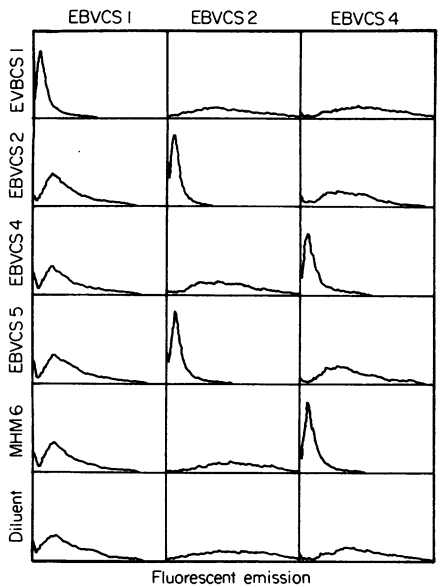


**Figure 2.** Blocking studies with EBVCS and MHM6 antibodies. LCL cells ($5 \times 10^5$) were first incubated with unconjugated antibodies or diluent as indicated on the left vertical axis. After washing off unbound antibody, cells were reacted with the biotin-labelled antibody indicated on the top horizontal axis. After staining with fluorescein–avidin, the data were collected from 50,000 cells and a linear fluorescent intensity histogram constructed as shown.

**Table 2.** Ability of different anti-CD23 to block BCGF absorption

| | % BCGF activity remaining | | |
|---|---|---|---|
| Pre-coating antibody | Exp. 1 | Exp. 2 | Exp. 3 |
| EBVCS 1 | 52 | ND | 36 |
| EBVCS 2 | 56 | ND | 49 |
| EBVCS 4 | 105 | 114 | 95 |
| EBVCS 5 | 63 | ND | ND |
| MHM6 | ND | 87 | 80 |
| 11EF7 | ND | 7 | ND |
| BK19.9 | ND | 0 | 0 |
| Control | 5 | 2 | 0 |

One ml of 20% BCGF was absorbed with $10^7$ low buoyant density B cells (<62·5% Percoll) for 2 hr on ice with constant shaking. Cells were pre-coated where indicated by incubating with antibody (1 mg/ml) for 2 hr on ice followed by thorough washing. Following absorption, cell-free, filtered supernatants were tested in the SAC co-stimulation assay and the percentage activity remaining determined. (ND, not determined.)

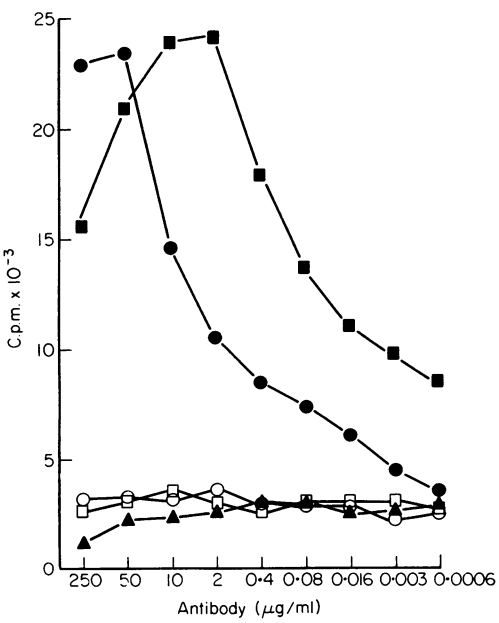


**Figure 3.** Stimulation with EBVCS antibodies. Cells were cultured with TPA as for Fig. 1 but in the presence of MHM6 or EBVCS antibodies at indicated concentrations: MHM6 (●), EBVCS 1 (○), EBVCS 2 (□), EBVCS 4 (■), EBVCS 5 (▲).

The behaviour of CD23 contrasts with that of immunoglobulin on B cells or T3/Ti on T cells where cross-linking of the antigen receptors, irrespective of where they are bound, either is a prerequisite for or leads to more potent activation. It does, however, parallel remarkably what is known of the triggering of murine B cells through the Lyb. 2 antigen. Here Fab monomers are as effective as whole antibody, and solid-phase antibody is ineffective (Subbarao & Mosier, 1984; Subbarao & Melchers, 1984). From their functional similarity, we have already suggested the possibility that the 45,000 MW Lyb. 2 antigen in mouse and CD23 in man may represent analogous structures (Gordon *et al.*, 1986a). The findings presented in this paper further support that notion.

The major question that remains unresolved for CD23 is with regard to the natural ligands for this molecule. We have previously shown that the signal delivered to B cells through CD23 is indistinguishable from that given by low molecular weight BCGF (Gordon *et al.*, 1986a). Furthermore, while BCGF down-regulates CD23 expression, coating of activated cells with MHM6 diminishes their capacity to absorb BCGF (Gordon *et al.*, 1986b). These observations demonstrate a close association between the actions of CD23 and those of BCGF, and suggest a possible identity between the former and the receptor for the latter. It was of interest that, in this study, the two stimulatory antibodies were the most effective at blocking BCGF uptake onto activated cells. At present, we have been unable to determine whether the increased efficiency of EBVCS 4 over MHM6 in stimulating DNA synthesis as well as in blocking BCGF absorption is due to it being an IgM as compared to an IgG1 for MHM6 or to it binding more precisely to the activating site on CD23.

It was recently reported (see Introduction) that the cloned product of the B-cell low affinity IgE receptor shares identity with CD23. Furthermore, it has been shown for Epstein–Barr virus (EBV)-transformed B cells that the 45,000 MW CD23 molecule is rapidly shed from the surface as a 33,000 MW product (Thorley-Lawson, Swendeman & Edson, 1986), and that this fragment possesses growth-promoting activity that

may be identical to autocrine BCGF (S. Swendeman, personal communication). It is also known that IgE can up-regulate its low affinity receptor on B cells (Suemura *et al.*, 1986), and this may occur by preventing such receptor shedding. One possible mode of action for the T-cell derived BCGF is that, through acting on the CD23 of normal activated B cells, it may generate a proteolytic activity in the receptor which results in its cleavage to the 33,000 MW form. It is this action that MHM6 and EBVCS 4 may be mimicking and, in effect, generating a growth factor cascade. In transformed cells, this process may be happening constitutively. The exploration of this intriguing possibility is the current focus of work in our laboratory.

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