

Anti-CD23 Monoclonal Antibodies: Comparisons of Epitope Specificities and Modulating Capacities for IgE Binding and Production

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ABSTRACT

A large battery of anti-CD23 mAb were compared for their epitope specificities and for their abilities to alter both IgE binding to cell-associated CD23 and IgE production in vitro in response to three sets of stimulants. The nine mAb tested can be divided into four families which define four antigenic epitopes (A-D) of CD23. Of these four families, two bind antigenic sites, (A and D) that appear to lie outside the IgE ligand binding site and two bind sites (B and C) that appear to be located within or close to this site, as determined by the abilities of appropriate mAb to alter IgE binding to CD23.

The effects that these mAb had on IgE secretion by normal peripheral blood mononuclear cells (PBMNC) varied depending on the stimulant employed to induce IgE production. Interactions with epitope A, which was found to lie outside the ligand binding site and to be made more accessible by binding of mAb to other epitopes, had different effects on IgE production than interactions with the other epitopes. Indeed, mAb binding to this epitope lead to as much as a 10 fold enhancement in IgE biosynthesis induced by IL-4 alone or by IL-4 + hydrocortisone whereas interactions at the other sites resulted in almost complete inhibition of IgE production. In addition, mAb reactive with epitopes B and C had minimal effects on IgE production induced by IL-4 + anti-CD40 mAb whereas interactions at epitope A consistently enhanced IgE production. Finally, no apparent direct correlation was found between the ability of individual anti-CD23 mAb to alter IgE binding to cell-associated CD23 and their ability to modulate IgE production by PBMNC.

These studies suggest that IgE binding to cell-associated CD23 does not have a major role in the de novo synthesis of IgE that involves CD23 interactions. In addition, the different effects that binding to epitope A vs B or C have on IgE synthesis suggest that molecular interactions between distinct portions of the CD23 molecule and other cell surface molecules expressed on the same B cell or adjacent communicating cells may lead to

divergent cellular effects on IgE production. Finally these studies imply that only epitope A is involved in the generation of an IgE response through the CD40 pathway.

INTRODUCTION

The CD23 molecule can be expressed as a 45 kDa glycoprotein on the surface of lymphoid and non-lymphoid cells [1-5] or as a series of smaller, biologically active, soluble fragments that arise by proteolytic cleavage [6-9]. The parent molecule and its cleavage products are involved in several immunologic functions and effector mechanisms. For example, membrane-associated CD23 can function as a low affinity receptor for IgE (Fc ϵ RII; refs. 10-12) that leads to functional consequences when engaged. Thus, binding of IgE-antigen complexes to B cell surface membranes via CD23 can result in endocytosis and presentation of antigen to T cells [13,14]. In addition interactions with monocyte surface receptors result in secretion of immunologically relevant cytokines such as IL-1 β and TNF α [15]. Similar binding to eosinophil membrane-associated CD23 is believed to be involved in IgE-dependent cytotoxicity of parasites [16].

However, despite the initial definition of CD23 as a B cell surface molecule [17] and despite several years of extensive investigation [rev. in 18], the consequences of IgE binding to the B cell-associated form of CD23 and the role that this binding has in de novo IgE biosynthesis are still not completely understood. Monoclonal antibody crosslinking of CD23 on B cells can result in increases in intracytoplasmic calcium concentration, inositol phosphate generation [19], and depression of ongoing IgE synthesis [20]. In addition, studies of the human soluble CD23 cleavage fragments show that they can retain IgE binding capabilities [6,7] and in this form can exert regulatory effects for T and B cell growth and development [21-27]. However, in vivo murine models have failed to confirm an intergral role for B cell-associated and soluble CD23 in the physiologic regulation of IgE production [28-29].

The pleiotropic functional activities of CD23 are reflected in several unusual structural features of the molecule. First, the cell-associated form displays an inverse membrane orientation with a C-terminal extracellular domain and an N-terminal cytoplasmic domain [30-32]. Second, cell-associated CD23 is expressed as two separate forms that differ only at their cytoplasmic regions, generated by using two different transcriptional initiation sites and alternative RNA splicing [33]. The Fc ϵ RIIa form is constitutively expressed in B cells whereas the Fc ϵ RIIb form can be induced in B cells and monocytes by IL-4 [33]. Recently, the Fc ϵ RIIa form was shown to be involved with endocytosis and the Fc ϵ RIIb form with phagocytosis [34]. Thus, the different forms can mediate distinct functional pathways in B cells. Third, unlike other FcR, the Fc ϵ binding domain of CD23 is homologous to a carbohydrate-binding domain of C-type animal lectins [35], although binding to IgE through this domain is independent of lectin-like activity [36]. Finally, the parent molecule appears to have coded within its primary structure an autoproteolytic activity that helps in the generation of the soluble forms of the molecule [37].

The present study was undertaken in hopes of determining the roles that certain of these unusual features of CD23 structure play in the regulation of IgE synthesis. In particular, we were interested in understanding the role that IgE binding to the various portions of the lectin-like domain of CD23 has on IL-4 stimulated IgE production and also the possible significance that interactions of distinct epitopes of CD23 with other cell surface moieties might have on this process. To address these issues, we determined the ability of a battery of nine anti-CD23 mAb to alter IgE binding to CD23 and then correlated these findings with the effects that the mAb had on IgE synthesis induced by IL-4 alone or in conjunction with hydrocortisone or anti-CD40 mAb.

MATERIALS AND METHODS

Monoclonal antibodies (mAb).

Anti-CD23 mAb. mAb 48.20 is the product of a murine hybridoma produced in this laboratory by the fusion of BALB/c spleen cells primed to W11-2 cell line cells (gift of Dr. Hans Spiegelberg, La Jolla, CA) with the HGPRTase-deficient plasma cell variant SP2/0 AG-14 in the presence of 33% polyethylene glycol (mw @ 1,500; JT Baker Chemical Co., Phillipsburg, NJ). This IgG1 anti-CD23 mAb reacts only with human mature B cell lines and not with pre-B cell lines, plasma cell lines, or B cell lines from patients with Burkitt's lymphoma (Table 1); the mAb does not react with human cell lines of the T lymphocyte, myelomonocytic, or erythroblastoid lineages tested. SDS-PAGE analyses of the ¹²⁵I-labelled B cell surface molecules immunoprecipitated by this mAb (Fig. 1) demonstrate the 45kDa molecular mass pattern typical for the CD23 molecule [5]. In addition, pre-clearance of these labeled lysates with known anti-CD23 mAb prior to immunoprecipitation with 48.20 markedly diminish the immunoprecipitants obtained with 48.20 suggesting molecular identity between the molecules bound by these mAb (Fig. 1).

Other anti-CD23 mAb used in this study include: [1]. EBV-CS #'s 1, (IgG1), 2 (IgG1), 3 (IgM), and 5 (IgG) [17] provided by Dr. Bill Sugden (University of Wisconsin, Madison, WI); [2]. MHM6 (IgG1; ref. 38) from Drs. John Gordon and Margaret Goodall (University of Birmingham, UK); [3]. mAb 25 (IgG1; ref. 10) from Dr. Jean-Yves Bonnefoy (Glaxo Institute for Molecular Biology S.A., Geneva, Switzerland); and [4]. mAb 8-30 (IgM) and mAb 3-5 (IgG1; ref. 39) from Drs. Tadamitsu Kishimoto and Hitoshi Kikutani (Institute for Molecular and Cellular Biology, Osaka, Japan).

mAb reactive with non-CD23 antigens. These mAb included: [1]. anti-HLA-DR (clone 2.06) and anti-human β -2-microglobulin (BBM.1 clone E9) from the American Type Culture Collection (Rockville, MD); [2]. anti-human B1 (CD20) from Coulter Corporation (Hialeah, FL); [3]. anti-DNP (IgG1; ref. 40 from Dr. David H. Katz (Medical Biology Institute, La Jolla, CA); and [4]. anti-human IgE (clones 4.15 and 7.12; ref. 41) from Dr. Andrew Saxon (UCLA, Los Angeles, CA).

Immunofluorescent analyses.

A. Surface membrane phenotypes of various cell fractions were determined by indirect immunofluorescence using this battery of murine mAb and flow cytometry. For direct staining, cell fractions were incubated with saturating concentrations of fluorescein isothiocyanate (FITC)-labeled mAb at 4°C for approximately 40 minutes, washed with PBS-1% bovine serum albumin (PBS-BSA) solution three times, and then analyzed. For indirect staining, cells were incubated with saturating concentrations of unlabeled mAb, washed in PBS-BSA as above, and then counterstained with affinity-purified, FITC-conjugated F(ab')₂ fragments of goat anti-mouse Ig antibodies (Tago, Burlingame, CA) for an additional 40 minutes.

B. Epitope analyses of the various mAb were determined by initial incubation with various unlabeled mAb at varying concentrations for 40 minutes, washing, and then incubation with FITC-labeled anti-CD23 mAb as described [42].

C. The relative capacities of the various anti-CD23 mAb to inhibit IgE-binding to the CD23 molecule were determined by preincubation with the mAb for 40 minutes at 4°C, washing, and then incubating with unlabeled IgE PS myeloma (gift of Dr. Henry Metzger, NIH). Bound IgE was then detected using a FITC-labeled anti-human IgE.

D. Cytofluorographic analyses were performed on 10 x 10⁴ cells per sample using a FACS 440 (Becton-Dickinson, Mountain View, CA) interfaced with a microVax computer (Digital Equipment Co., Merrimack, NH).

Immunoprecipitation and SDS-PAGE analyses.

Surface membrane proteins were labeled with ¹²⁵I-Na (Amersham, Arlington Heights, IL) by the lactoperoxidase method [43], solubilized in 10mM Tris

buffer (pH 8.0) containing 0.1% Triton X-100 (Aldrich Chemical Co, Milwaukee, WI) and 100mM iodoacetamide (Sigma), and immunoprecipitated with the test mAb bound to rabbit anti-mouse Ig-coated protein A-Sepharose beads at 4°C. Complexes were eluted by boiling in loading buffer containing 2.5% SDS and analyzed under reducing conditions on 12% polyacrylamide gels.

Cell culture conditions.

All cell cultures were performed in medium consisting of RPMI 1640 (Flow Laboratories, McLean, VA) supplemented with 10% fetal bovine serum,

TABLE 1

Reactivities of mAb 48.20 with Cell Lines of Various Lineages

<u>Name of Cell Line</u>	<u>Stage of Differentiation</u>	<u>Ig Isotype Produced</u>	<u>Percent Reactivity</u>
Nalm	Pre B cell	μ	0
Josh 7	Pre B cell	μ	0
RPMI 1788	B cell	μ	45
SKW 6.4	B cell	μ	59
0467	B cell	μ	71
RPMI 8866	B cell	γ	80
IM-9	B cell	γ	93
Cess	B cell	γ	56
Dakiki	B cell	α	62
32A1	B cell	α	54
BJAB	B cell (Burkitt)	μ	0
EB 3	B cell (Burkitt)	μ	0
Raji	B cell (Burkitt)	μ	0
Daudi	B cell (Burkitt)	μ	0
Ramos	B cell (Burkitt)	μ	0
P3HR-1	B cell (Burkitt)	μ	0
NAMALVA	B cell (Burkitt)	μ	0
HS-Sultan	Plasma cell	γ	0
U266	Plasma cell	ϵ	0
CEM-CM 3	T cell	na	0
CCRF-HSB-2	T cell	na	0
Molt 4	T cell	na	0
GM 3639A	T cell	na	0
1301	T cell	na	0
Hut 102	T cell	na	0
U937	Monocyte	na	0
K562	Erythroblastoid	na	0

Cells were incubated with saturating concentrations of mAb 48.20 for 40 minutes at 0°C, followed by FITC-conjugated F(ab')₂ fragments of goat anti-mouse Ig and analyzed by flow cytometry.

na: not applicable

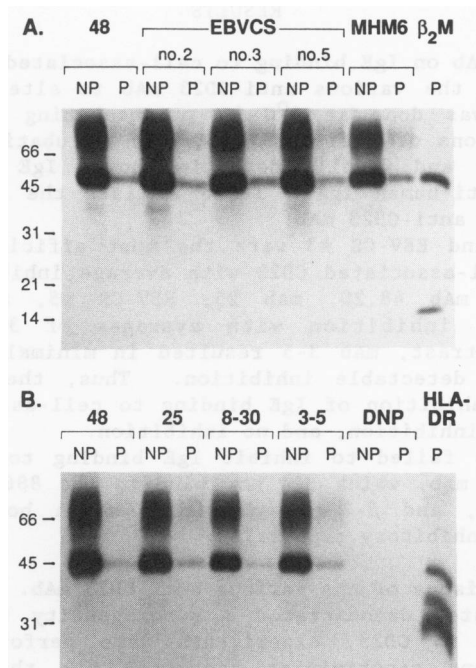


Figure 1. Preabsorption of Radiolabeled Cell Lysates with mAb 48.20 Diminishes the Immunoprecipitates Obtained with Known anti-CD23 mAb. ^{125} I-labeled RPMI 8866 cell lysates were precleared (P) or not precleared (NP) by mAb 48.20 and then the residual proteins again immunoprecipitated with either mAb 48.20 or the known anti-CD23 mAb, or Anti- β -2-microglobulin, anti-DR, and anti-DNP mAb as negative controls.

1% penicillin (100 u/ml)/streptomycin (100 ug/ml), and 4 mM glutamine (all from GIBCO, Chagrin Falls, OH). Three methods were used to induce IgE synthesis by PBMNC. These included: [1]. IL-4 (200 u/ml; Amgen Biologicals, Thousand Oaks, CA) alone; [2]. IL-4 + hydrocortisone (10^{-7} M; Sigma); and [3]. IL-4 + anti-CD40 mAb (mAb # 626.1, originally prepared by Gruber et al [44] and provided by Dr. Raif Geha, Children's Hospital, Boston, MA). In each instance, 1×10^6 mononuclear cells/ml were cultured in 24 well flat bottom culture plates (Linbro, Hamden, CT) at 37°C in a humidified 5% CO_2 -95% air atmosphere in the presence or absence of isolated anti-CD23 mAb (20 ug/ml). After 10 days, cells were removed by centrifugation and supernatants stored at -70°C until assayed for IgE content.

Determination of IgE levels by solid phase enzyme immunoassay (EIA).

IgE levels in culture supernatants were determined according to the method of Macy et al [41] using reagents provided through the National Institutes of Health, Bethesda, MD. Briefly, microtiter plates were coated with a mixture (1 ug/ml each) of two anti-human IgE mAb (CIA-E-7.12 and CIA-E-4.15). After blocking unoccupied sites with PBS-BSA, the test supernatants were incubated overnight at 4°C . Bound IgE was detected with purified F(ab') fragments of the CIA-E-7.12 mAb coupled to alkaline phosphatase, followed by substrate and ImmunoSelect ELISA amplification system (GIBCO BRL, Gaithersburg, MD; ref. 45). Myeloma IgE PS was used as a control in all assays to determine concentrations of IgE produced as well as to assure specificity of the EIA for IgE. No cross-reactions with control IgM, IgG, or IgA were noted in this system.

RESULTS

Effects of anti-CD23 mAb on IgE binding to cell-associated CD23.

The capacities of the various anti-CD23 mAb to alter IgE binding to CD23 were tested. This was done at 4°C by preincubating RPMI 8866 cells with saturating concentrations of anti-CD23 mAb, then incubating the cells with the human IgE myeloma PS, and finally detecting bound IgE by immunofluorescence using FITC-labeled anti-human IgE. Table 2 lists the results of three such experiments using nine anti-CD23 mAb.

mAb MHM6, 8-30, and EBV-CS #3 were the most efficient at inhibiting the binding of IgE to cell-associated CD23 with average inhibitions of 90, 88, and 85%, respectively. mAb 48.20, mAb 25, EBV-CS #5, and EBV-CS #2 showed mid-range levels of inhibition with averages of 36, 20, 17, and 16%, respectively. In contrast, mAb 3-5 resulted in minimal inhibition (10%) and EBV CS #1 showed no detectable inhibition. Thus, the mAb fell into three categories based on inhibition of IgE binding to cell-associated CD23: maximal inhibition, mid-range inhibition, and no inhibition.

Control antibodies failed to inhibit IgE binding to the RPMI 8866 cells; not only the anti-DNP mAb, which did not bind to the 8866 cells, but also mAb to CD20 (B1), HLA-DR, and β -2-microglobulin, which bound to virtually all the cells, showed no inhibitory capacities.

Epitope binding comparisons of the various anti-CD23 mAb.

Since the mAb tested demonstrated a heterogeneity in their abilities to inhibit IgE binding to CD23, experiments were performed to determine if differences in epitope reactivities accounted for the differences in IgE binding inhibition. These analyses were performed by incubating the RPMI 8866 cells with the various unlabeled mAb, washing, and then staining the cells with one of the FITC-labeled mAb mentioned above (Table 3).

EBV CS #2, #3, and #5, and mAb 48.20, 25, and 8-30 appear to recognize the same or spatially closely related epitope since each resulted in high levels of binding inhibition of the corresponding FITC-labeled anti-CD23 mAb tested. For instance, EBV CS #2, which is representative of the other mAb in this group, when inhibited by itself resulted in only 8% residual binding. This result is essentially indistinguishable from the data obtained when this mAb is inhibited by EBV CS #3 and #5, and mAb 48.20, 25, and 8-30.

In contrast, MHM6 and EBV CS #1 appear to represent two separate and distinct categories. For MHM6, efficient inhibition (78%) was seen only after pre-incubation with itself; the other mAb showed lesser, variable levels of inhibition ranging from 57 - 18%.

In the case of EBV CS #1 a clearly different result was observed. First, this mAb inhibited itself by only 60%; the use of supermaximal levels of unlabeled inhibitor did not alter this result (data not shown). In addition, binding of FITC-labeled EBV CS #1 was increased by pre-incubation with all the other anti-CD23 mAb studied, with the exception of 8-30.

Finally, mAb 3-5 appears to recognize another distinct epitope based on the inability of this mAb to cause high levels of inhibition of binding of any of the labeled anti-CD23 mAb tested. However, because of the limited amount of isolated mAb available for study, this could not be tested directly.

Thus, there appear to be at least three, and probably four, separate mAb binding epitopes on the CD23 molecule. Although these categories are similar to those seen for IgE binding inhibition, they do not correspond exactly.

Comparison of the abilities of anti-CD23 mAb to modulate IgE synthesis.

Since IgE synthesis can be induced in human B lymphocytes by at least three methods [46-50], the effects of the anti-CD23 mAb on IgE production by normal PBMNC were studied in each of these systems. In the experiments reported below, each anti-CD23 mAb was used at a concentration of 20 ug/ml since preliminary dose-response experiments had demonstrated that optimal and reproducible effects were obtained at this concentration (data not shown).

TABLE 2

Inhibition of IgE Binding to the CD23 Molecule by Various anti-CD23 mAb

Inhibiting Monoclonal <u>Antibody</u>	Percent IgE-binding Cells		
	<u>Expt. #1</u>	<u>Expt. #2</u>	<u>Expt. #3</u>
None	91.5	97.0	96.1
Anti-CD23 mAb:			
MHM 6	15.6 (83)	10.6 (89)	2.4 (98)
8-30	15.9 (83)	ND	7.4 (92)
EBV-CS #3	10.5 (89)	15.0 (85)	17.1 (82)
48.20	43.2 (53)	65.1 (33)	74.7 (22)
25	58.4 (36)	80.3 (17)	90.0 (6)
EBV-CS #5	66.8 (27)	79.1 (18)	89.1 (7)
EBV-CS #2	67.7 (26)	83.8 (14)	88.4 (8)
3-5	69.2 (24)	94.2 (3)	92.6 (4)
EBV-CS #1	93.5 (0)	95.9 (0)	95.7 (0)
Irrelevant mAb:			
anti-DNP	90.1 (2)	99.0 (0)	98.7 (0)
anti-B1	ND	98.0 (0)	98.8 (0)
anti-DR	ND	99.4 (0)	98.8 (0)
anti-B ₂ M	ND	99.9 (0)	96.9 (0)

1. RPMI 8866 cells were incubated with saturating concentrations of the inhibitor mAb for 40 minutes at 4°C, washed, and then incubated with IgE (20 ug/ml) for 40 minutes at 4°C. Bound IgE was detected by counterstaining with FITC-labeled murine anti-human IgE mAb.

2. Numbers in parentheses represent percentage inhibition of IgE-binding to the RPMI 8866 cells calculated by the formula:

$$\% = \frac{(\% \text{ IgE}^+ \text{ cells without inhibitor}) - (\% \text{ IgE}^+ \text{ cells with inhibitor})}{(\% \text{ IgE}^+ \text{ cells without inhibitor})}$$

TABLE 3

Comparison of Epitope Specificities of Various anti-CD23 mAb

Percent of Control Binding of:

<u>Inhibitor</u>	<u>FITC- EBV CS #1</u>	<u>FITC- EBV CS #2</u>	<u>FITC- EBV CVS #3</u>	<u>FITC- EBV CS #5</u>	<u>FITC- 48.20</u>	<u>FITC- MHM6</u>
None	100.0	100.0	100.0	100.0	100.0	100.0
EBV-CS #1	44.4	90.8	109.2	130.3	105.9	81.6
EBV-CS #2	123.4	7.8	14.7	8.4	10.6	63.3
EBV-CS #3	116.9	8.6	14.7	9.9	26.0	49.5
EBV-CS #5	120.8	12.6	15.0	13.4	8.1	58.7
48.20	114.6	11.7	18.0	13.4	24.9	52.0
25	179.0	9.3	25.8	10.0	11.0	43.6
8-30	86.2	11.8	24.7	13.0	5.6	57.7
3-5	123.3	85.9	103.5	65.9	91.4	76.9
MHM6	130.9	90.9	95.7	82.8	77.1	22.0
anti-DNP	106.2	104.6	107.2	93.0	108.5	106.6

1. RPMI 8866 cells were incubated with various concentrations of the inhibitor mAb for 40 minutes at 4°C, washed extensively, and then stained with FITC-conjugated anti-CD23 mAb (25 ug/ml) for 45 minutes at 4°C. In separate experiments, all of the inhibitor mAb were shown to bind to >98% of the RPMI 8866 cells when counterstained with FITC-conjugated goat anti-mouse Ig (data not shown).

2. Numbers (the means of three separate experiments) represent the percent of the normalized linear values of the control binding to RPMI 8866 cells.

TABLE 4

Effects of Various anti-CD23 mAb on
IL-4-induced IgE Antibody Production

<u>anti-CD23 mAb added</u>	<u>IgE (ng/ml)</u>		
	<u>Exp. #1</u>	<u>Exp. #2</u>	<u>Exp. #3</u>
None	0.92	0.38	0.20
EBV-CS #1	0.90	0.31	0.36
EBV-CS #2	0.38	0.30	0.37
EBV-CS #3	0.27	0.18	0.13
EBV-CS #5	0.27	0.10	0.14
48.20	0.28	0.15	0.13
25	n.d.	n.d.	n.d.
MHM6	0.59	0.27	0.13

PBMC were cultured for 10 days with IL-4 (200 u/ml) in the presence or absence of 20 ug/ml of the various anti-CD23 mAb.

n.d.: not determined.

IL-4 induced IgE production. The results of three experiments of this type are listed in Table 4. In these three studies involving three separate, selected normal individuals, IL-4 was able to induce detectable levels of IgE after 10 days of culture ranging from 0.2 - 0.9 ng/ml. When the six anti-CD23 mAb used in these studies (EBV-CS #'s 1,2,3,5, 48.20, and MHM6) were added at the culture initiation, all but one (EBV CS #1) resulted in appreciable, although variable levels of suppression. For instance, EBV CS #5 and 48.20 were very potent in inhibiting IL-4 induced IgE antibody synthesis, whereas MHM6 resulted in lesser levels of suppression and EBV CS #2 had variable effects. As mentioned, EBV CS #1 had essentially no effect on the levels of IgE produced in these studies.

Hydrocortisone + IL-4 induced IgE production. The results of these experiments are shown in Table 5. Hydrocortisone (10^{-7} M) + IL-4 proved to be a much more effective stimulus to IgE production than IL-4 alone (Table 4) or IL-4 + anti CD40 mAb (Table 6). The addition of anti CD23 mAb to these cultures had very potent effects on this process. EBV CS # 2, 3, and 5, 48.20, and MHM6 resulted in highly significant suppression, with inhibitions of IgE synthesis approaching 100% in most instances. In contrast, EBV CS #1 markedly augmented IgE production, with levels of enhancement ranging from 4 - 10 fold. mAb 25 had variable effects on IgE production (minimal suppression to almost 2 fold enhancement) depending on the individual tested. This is in contrast to the published suppression of IL-4 induced IgE synthesis [51].

Anti-CD40 + IL-4 induced IgE production. In the three experiments listed in Table 6, supplementation of the cultures with optimal concentrations of anti-CD40 mAb + IL-4 resulted in a ten-fold higher levels of IgE production than seen with IL-4 alone (2.0 - 7.5 ng/ml), but not as high as those induced by hydrocortisone + IL-4 (14 - 70 ng/ml). Again, the addition of mAb EBV CS #1

TABLE 5

Effects of Various anti-CD23 mAb on
Hydrocortisone + IL-4-induced IgE Antibody Production

anti-CD23 mAb added	IgE (ng/ml)		
	<u>Exp. #1</u>	<u>Exp. #2</u>	<u>Exp. #3</u>
None	70.0	40.0	14.0
EBV-CS #1	300.0	400.0	90.0
EBV-CS #2	11.0	<0.3	<0.3
EBV-CS #3	17.0	2.0	<0.3
EBV-CS #5	0.4	0.3	<0.3
48.20	13.0	0.3	<0.3
25	130.0	70.0	10.0
MHM6	<0.3	0.8	<0.3

PBMC were cultured for 10 days with hydrocortisone (10^{-7} M) and IL-4 (200u/ml) with or without 20 ug/ml of anti-CD23 mAb.

TABLE 6

Effects of anti-CD23 mAb on
anti-CD40 + IL-4-induced IgE Antibody Production

anti-CD23 mAb added	IgE (ng/ml)		
	<u>Exp. #1</u>	<u>Exp. #2</u>	<u>Exp. #3</u>
None	5.0	7.5	2.0
EBV-CS #1	15.0	30.0	8.0
EBV-CS #2	2.5	5.0	1.6
EBV-CS #3	2.3	8.0	1.5
EBV-CS #5	5.0	7.5	2.0
48.20	5.4	6.0	1.5
25	7.5	7.0	1.8
MHM6	5.4	6.0	3.5

PBMC were cultured for 10 days with anti-CD40 mAb and IL-4 (200 u/ml) with or without 20 ug/ml of anti-CD23 mAb.

to these cultures resulted in a reproducible and appreciable enhancement of IgE production (3-4 fold). In contrast to the studies obtained with the other IgE-inducing stimulants (Tables 4 and 5), the other anti-CD23 mAb tested resulted in variable or minimal alterations in the levels of IgE produced.

DISCUSSION

In this study a series of anti-CD23 mAb have been compared as to their epitope specificities, abilities to inhibit IgE binding to CD23, and abilities to modulate IgE production in three experimental systems (summarized in Table 7). These characteristics were determined in hopes of defining unique features of the mAb and inferring information about the role of CD23 in IgE binding and biosynthesis.

The mAb can be grouped into four families that appear to define distinct antigenic epitopes of membrane-associated CD23. For convenience of discussion, letters have been assigned to these epitopes. Monoclonal antibody EBV CS #1 defines a unique epitope designated "A"; mAb EBV CS #s 2, 3, and 5, mAb 48.20, 25, and 8-30 appear to define epitope "B"; MHM6 defines epitope "C"; and mAb

TABLE 7

Summary of Characteristics of Various anti-CD23 mAb

<u>mAb</u>	<u>Apparent Epitope Specificity</u>	<u>Inhibition of IgE Binding</u>	<u>Modulation of IgE Production Induced by</u>		
			<u>Il-4 Alone</u>	<u>HC + Il-4</u>	<u>anti-CD40 + Il-4</u>
EBV CS 1	A	No Effect	No Effect	+++	++
EBV CS 2	B	+/-	Variable	---	+/-
EBV CS 3	B	+++	--	--	+/-
EBV CS 5	B	+/-	--	---	No Effect
48.20	B	++	--	---	+/-
25	B	+ Tested	Not	Variable Effect	No
8-30	B	+++	Not Tested	Not Tested	Not Tested
3-5	D	No Effect	Not Tested	Not Tested	Not Tested
MHM6	C	+++	-	---	No Effect

3-5 may define a distinct epitope "D". These data are in agreement with those of other investigators who have compared smaller numbers of anti-CD23 mAb in more limited studies [17,52]. However, this present study is unique in that it utilizes a larger battery of anti-CD23 and analyzes these both for antigen-binding and functional characteristics.

There is limited information available on the precise portion of the CD23 molecule bound by anti-CD23 mAb. Bettler et al [53] recently showed that MHM6 and mAb 25 react with the lectin-homology domain that the CD23 molecule has in common with certain lymphocyte homing receptors [54], thus mapping the molecular location of epitope C to this broad region of CD23 that encompasses approximately 123 amino acids. Of interest is the observation that mAb 3-5 appears to bind outside this domain, most likely to the C-terminal 20 amino acids of the molecule [53]. This observation, and those suggesting that 3-5 binds denatured CD23 [39], support our data and contention that this mAb recognizes a distinct epitope (epitope D).

The various anti-CD23 mAb can be divided into three categories based on and 7): complete (EBV CS #3, 8-30, and MHM6), partial (mAb 48.20, 25, and EBV CS #s 2 and 5), and negligible (EBV CS #1 and 3-5) inhibition. Although these functional categories do not correspond exactly with the mAb epitope binding categories, certain generalizations can be made.

First, interactions with epitopes A and D have no effect on IgE binding to CD23, thus suggesting that these two epitopes are situated outside the ligand binding site. Second, binding to epitopes B and C clearly alters IgE - CD23 interactions. Interaction of MHM6 with epitope C results in essentially complete inhibition of IgE binding to CD23 suggesting that this epitope lies within the IgE binding site. However, since binding of certain mAb (EBV CS #3 and 8-30) to epitope B also result in almost complete inhibition of ligand binding whereas certain others (mAb 48.20, 25, and EBV CS # 2 and 5) result in lesser degrees of inhibition, it is difficult to strictly assign functional categories to the epitope designations. Our data suggest that either EBV CS #3 and 8-30 bind to an epitope distinct from epitope B that also lies at/within the IgE binding site on CD23 or that they result in more appreciable inhibition than the other anti-epitope B mAb due to their larger pentameric IgM structure or due to possible differences in affinity for the epitope. Further studies will be necessary to evaluate the possibility that EBV CS #3 and 8-30 react with another IgE binding site related epitope on CD23 distinct from epitope C.

Although the effects of the various anti-CD23 mAb on IgE production are more difficult to decipher because of the involvement of multiple cell types (some of which express CD23), and the length of the in vitro period, again certain generalizations can be made (summarized in Table 7). First, regardless of the stimulant used to induce IgE production, binding of EBV CS #1 to epitope A has different effects on IgE production than mAb reacting with all other CD23 epitopes tested. Specifically, EBV CS #1 had no consistent effect on IgE synthesis induced by IL-4 alone (Table 4) but augmented quite significantly (3 - 10 fold) the level of IgE produced in response to HC + IL-4 (Table 5) and anti-CD40 mAb + IL-4 (Table 6).

Second, mAb to the B and C epitopes consistently suppressed IgE production induced by IL-4 alone and HC + IL-4 (Tables 5 and 6) as well as the enhanced IgE production induced by EBV CS #1 (data not shown). In contrast, these mAb had minimal and less consistent effects on IgE production induced by anti-CD40 + IL-4. These findings suggest that interactions only at epitope A (and not B or C) are involved in the CD40 pathway of IgE antibody production whereas all three epitopes are involved in the IL-4 +/- hydrocortisone pathways. In addition, they demonstrate that the inhibitory interactions at epitopes B and C override the stimulatory effects of interactions at epitope A.

Third, there was no clear, direct relationship between the ability of individual anti-CD23 mAb to alter IgE binding to CD23 and the ability of these mAb to alter IgE production by MNC. For instance, even though certain mAb that react with epitopes B and C (EBV CS #3 and MHM6) block IgE binding to CD23 with maximal efficiency, their level of inhibition of IgE synthesis was no different than that seen with other mAb reactive with these same epitopes (EBV CS # 2 and 5 and mAb 25) that demonstrated much less ability to block IgE

binding to the receptor. Thus, their abilities to block IgE binding to the receptor (or their potential abilities to mimic this interaction) did not correlate with the level of IgE synthesis inhibition observed. In addition, interactions with epitope A by EBV CS #1, which had no effect on ligand binding to cell-associated CD23 and therefore could not mimic its effects, had dramatic enhancing effects on IgE production. Unfortunately the effects of mAb 3-5, that reacts with epitope D but does not alter IgE binding, could not be determined because of a lack of sufficient quantities of mAb. Nevertheless, it appears that epitope reactivity rather than level of IgE binding inhibition correlates best with the type of alteration in IgE synthesis induced by these mAb.

These data suggest that crosslinking of cell-associated CD23 per se is not the critical determinant for the effects on IgE production since binding to all CD23 epitopes would be expected to result in crosslinking. Rather these data suggest important, distinct roles for individual epitopes of this surface molecule. It is unclear why binding to different epitopes on the CD23 molecule should result in different effects on IgE production. However, there are other examples of distinct functional consequences as a result of interactions by different mAb with different sites on CD23.

For example, regarding epitope A, we have shown in this study that EBV CS #1 is unique since binding of this mAb to cell-associated CD23 is substantially increased by preincubation of B cells with all of the other anti-CD23 mAb tested with the possible exception of mAb 8-30 (Table 3). Possibly distortion of the usual three dimensional structure of CD23 by other mAb may more readily expose epitope A, thereby allowing greater amounts of EBV CS #1 to bind, and/or possibly the distorted epitope may have higher affinity for the EBV CS #1 combining site. This latter possibility is supported by the finding that EBV CS #1 has a limited ability to inhibit its own binding to cell associated CD23 (59%; Table 3). Presumably, this increased availability or expression of epitope A occurs in vivo from interactions of IgE (or another ligand) at the lectin binding domain. It should be kept in mind that simultaneous interactions at epitopes A and B or A and C result in inhibition of IgE synthesis. Thus, the interplay of interactions at multiple epitopes may be complex. Furthermore, Bonnefoy et al [51] have shown that EBV CS #1 differs from other anti-CD23 mAb by its inability to upregulate CD23 expression on IL-4-stimulated tonsillar mononuclear cells. Based on this observation and the varying abilities of anti-CD23 mAb to modulate IgE synthesis, these investigators have suggested [51] that binding to different epitopes may alter the autoprolysis that the CD23 molecule undergoes [37], thereby resulting in different biosynthetic effects.

Regarding epitope C, MHM6 in conjunction with phorbol ester (TPA) has been found to be a co-stimulant for the induction of proliferation of tonsillar B cells whereas other mAb (reactive with other epitopes) failed to have this property [55]. Although this was initially thought to represent mimetic effects of low molecular weight B cell growth factor binding [52,55], more recent studies have failed to confirm this explanation [56,57]. However, MHM6 and IgE itself, both of which bind to the lectin-homology region [22], but not other anti-CD23 mAb, have been found to inhibit allogeneic mixed lymphocyte reactions [58]. Relevant to these studies are the observations that HLA-DR molecules appear to be spatially associated with CD23 since certain anti-DR mAb (eg. mAb 135; ref #59) can alter IgE-CD23 interactions and can also co-precipitate CD23 and HLA-DR under appropriate experimental conditions [59]. Similarly, in the murine system chemical crosslinking experiments have revealed coprecipitation of membrane Ig and CD23 [60].

Collectively, these studies suggest at least two mechanisms whereby reactivity with individual epitopes could have distinct effects on IgE biosynthesis: effecting CD23 autoprolysis and/or effecting CD23 interactions with other cell surface molecules (such as, but not limited to, HLA-DR and/or mIg).

Autoprolysis of CD23 has been shown to be upregulated by IL-4 [61] and downregulated by IgE binding [62] and it has been postulated that enhanced levels of soluble CD23 promote IgE biosynthesis [54,63]. It is conceivable

that interactions at epitopes B and C reduce the levels of proteolysis that IL-4 should induce and thereby suppress the formation of soluble CD23 and the eventual production of IgE. However, since our studies fail to demonstrate an obvious direct correlation between the ability of various mAb to interact with the IgE binding site on CD23 (and to block or presumably mimic IgE binding) and the ability of the mAb to enhance or suppress IgE production induced by different stimuli, these data suggest that IgE binding to CD23 is not have a major role in the de novo synthesis of IgE that involves CD23 interactions.

However, one could envision that different epitopes of the CD23 molecule interact with different cell surface molecules, either on the same or interacting cells, and thus different combinations of protein-protein interactions involving the constitutive and IL-4-responsive forms of CD23 might occur. These different sets of molecular interactions might result in different combinations of transmembrane signals and divergent cellular effects, especially in light of the fact that the two isoforms of CD23, which differ in their N-terminal intracytoplasmic portions [33], have been shown to mediate different functional effects [34]. In this model, masking or altering individual CD23 epitopes that interact with different proteins would lead to a different set of triggers and a distinct set of functional consequences. The studies reported here, and those recently reported [64] demonstrating that cell surface interactions between CD23 and CD21 occur which can alter IgE responses, may support this model.

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