



In vitro IgE inhibition in B cells by anti-CD23 monoclonal antibodies is functionally dependent on the immunoglobulin Fc domain

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Abstract

CD23, the low affinity receptor for IgE (FcεRII), is involved in regulation of IgE synthesis by B-lymphocytes. Five monoclonal antibodies to human CD23 were generated from cynomolgus macaques immunized with purified soluble CD23 (sCD23). Four of the five primate antibodies blocked the binding of IgE complexes to CD23 positive cells and also inhibited the production of IgE in vitro by IL-4 induced human peripheral blood mononuclear cells (PBMC). The variable domains of several primate antibodies were utilized to construct chimeric macaque/human (PRIMATIZED[®]) monoclonal antibodies. PRIMATIZED[®] p5E8G1, containing human gamma 1 constant region, inhibited IgE production in vitro as efficiently as the parent primate antibody, but the human gamma 4 constant version, PRIMATIZED[®] p5E8G4, was not as effective in IgE inhibition. An F(ab')₂ of p5E8G1 did not inhibit IgE production but did interfere with IgE inhibition by the intact anti-CD23 antibody in a dose dependent fashion. The murine monoclonal antibody MHM6 recognizes human CD23 at a different epitope than primate antibody 5E8, and inhibits IgE production by IL-4 induced PBMC. As with the F(ab')₂ of p5E8G1, the F(ab')₂ of MHM6 also failed to inhibit IgE production. These data imply that the mechanism by which anti-CD23 antibodies inhibit IgE production requires cross-linking of CD23 to an IgG receptor. These data also imply that neither bivalent cross-linking of CD23 alone or inhibition of CD23 binding to its natural ligands is sufficient to inhibit IgE production. © 2000 International Society for Immunopharmacology. Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

CD23 is a type II membrane glycoprotein of 45 kilodalton (kDa) molecular weight. The human CD23 gene has two different promoters resulting in two different messenger RNAs, and two forms of human protein (a and b) which differ only in the cytoplasmic amino terminal seven (a) or six (b) amino acids [1]. The “a” form is constitutively expressed at low levels only on B cells and possibly eosinophils [2,3] while the “b” form is IL-4 inducible and present on other cell types, including B cells, T cells, platelets, eosinophils, monocytes, natural killer cells, keratinocytes, Langerhans cells, and follicular dendritic cells [4–6].

CD23 forms a trimer at the cell surface, and binds to several ligands with a calcium dependent (C-type) lectin domain. Although the CD23-IgE binding region is a C-type lectin, CD23 does not bind to carbohydrate component of IgE [7]. Human CD23 binds to carbohydrate on human CD21, and may interact with carbohydrate in binding to human CD11b/CD18 and CD11c/CD18 [8,9].

CD23 is cleaved from the surface by membrane-associated metalloprotease and sCD23 forms of 37, 33 and 29, 25 and 16 kDa [10]. All of these fragments bind to the CD23 ligands, and specific forms are reported to have cytokine properties in humans, but not mice [11–13]. CD23 deficient mice have normal T cell and B cell development, but increased and sustained specific IgE levels compared to normal mice. This suggests that CD23 is involved in negative feedback inhibition of IgE synthesis [14–16].

Murine monoclonal antibodies to human CD23 have been reported to inhibit the production of IgE from IL-4 induced human PBMC [17–19], and inhibit the IL-4 and anti-IgM induced proliferation of human B cells [20]. The ligation of CD23 on an IgE producing B cell human myeloma by either allergen-IgE complexes or some murine monoclonal antibodies to CD23 is involved in feedback inhibition of the ongoing IgE response [21,22]. Murine monoclonal antibodies to human CD23 have also been reported to mobilize release of intracellular cal-

cium, and stimulate both polyphosphoinositide hydrolysis and cAMP generation in activated human B cells [23–25]. Furthermore, protease inhibitors that specifically block the cleavage of CD23 on the surface of IL-4 induced human PBMC have been shown to inhibit IgE production [26].

In vivo, polyclonal antibodies to CD23 have been reported to block an antigen-specific IgE response in the rat [27]. A rat monoclonal antibody to mouse CD23 blocked lung eosinophil infiltration in a murine asthma model, an effect that the authors suggest is mediated by a blockade of IgE-CD23 mediated allergen focusing [28].

In order to minimize immunogenicity of antibodies for therapy of chronic diseases, PRIMATIZED[®] monoclonal antibodies to human CD23 were constructed and evaluated in vitro. The antibodies were engineered as chimeras with either human gamma 1 or human gamma 4 constant regions, and evaluated for their potency in inhibiting IgE production. Monoclonal antibodies to human CD23 may be useful in downregulating IgE levels and in ameliorating the symptoms of allergic diseases in humans.

2. Materials and methods

2.1. Purification of sCD23 from cultures

The RPMI 8866 cell line was cultured in RPMI 1640 media containing 10% fetal bovine serum (FBS). Cells were concentrated 10-fold and resuspended in RPMI 1640 without FBS. After 2–3 days, the cell viability dramatically falls during which the CD23 antigen is shed into the culture medium. Cell debris was removed by filtration and the supernate was sterile filtered. Proteases in the supernate were inhibited by adding ethylenediaminetetraacetic acid and phenylmethylsulfonyl fluoride.

Supernate was concentrated using ultrafiltration (A/G Technology Corp., Needham, MA) and lipid material was removed with SM-2 Bio Beads (Bio-Rad Laboratories, Inc., Hercules, CA). For some lots of sCD23, protein was con-

centrated using a 70% ammonium sulfate salt cut followed by dialysis into PBS.

Soluble CD23 was purified by affinity chromatography using a murine anti-CD23 antibody (BU38, Binding Site Inc., San Diego, CA) and quantified using a commercially available ELISA kit (Binding Site Inc., San Diego, CA). SDS-PAGE analysis indicated two predominant protein bands in the molecular weight range of 25–35 kD (data not shown).

2.2. Immunization of cynomolgus macaques with sCD23

Macaque anti-human CD23 antibodies were generated from an adult cynomolgus monkey (White Sands New Mexico Primate Center) immunized intramuscularly with sCD23 every 3 weeks six times prior to isolation of B-lymphocytes for hybridoma fusion. The amounts of sCD23 used for immunization were 50, 50, 50, 225, 225, and 300 µg. Primate sera were analyzed for binding to sCD23 by ELISA following immunization. Titers of 1000, 6000, 16,000, 51,200, not bled, and 51,200 to human sCD23 were measured. Seven days after a final injection regional lymph nodes and spleen were excised. Heterohybridomas were generated using the cell line K6H6/b5, described by Carroll [29] as the fusion partner.

2.3. Isolation of primate monoclonals to human CD23

Fifty-six wells containing heterohybridomas were selected using an ELISA for binding to RPMI 8866 cells. Of those 56 wells, 21 were positive in an ELISA for sCD23. Five heterohybridomas were selected to produce primate monoclonal antibodies that were purified by protein A affinity chromatography for further characterization.

2.4. Inhibition of IgE-complex binding to RPMI 8866 cells (human CD23 positive cell line)

This was tested as described by Bonnefoy [18].

2.5. Binding affinity, Scatchard analysis

Monoclonal antibodies were radioiodinated using Iodo-Beads Iodination Reagent (Pierce, Rockford, Illinois). Scatchard analysis and estimations of apparent dissociation constants (Kd) were performed as described by Linsley [30].

2.6. In vitro IgE production from human PBMC

This was performed as described by Wu [31]. Human PBMCs were isolated by centrifugation of buffy coat cells (San Diego Blood Bank) over Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Piscataway, NJ). PBMCs were suspended at 10^6 cells per ml in HB101 Growth Media (Irvine Scientific, Irvine, CA) with 100 units of interleukin-4 (IL-4, Genzyme, Boston, MA) and either 10^{-6} M hydrocortisone (HC, Sigma, St Louis, MI) or 1 ng/ml of anti-CD40 monoclonal antibody (Genzyme, Cambridge, MA). These cells were cultured for 10 to 11 days, IgE released into the growth medium was assayed by ELISA.

2.7. Purification of $F(ab')_2$ from monoclonal antibodies

$F(ab')_2$ of PRIMATIZED[®] monoclonal antibody p5E8G1 were generated with immobilized pepsin (ImmunoPure $F(ab')_2$ Preparation Kit, Pierce, Rockford, IL). $F(ab')_2$ of murine monoclonal antibody MHM6 (Dako, Carpinteria, CA) were generated with immobilized ficin (ImmunoPure IgG, Fab, and $F(ab')_2$ Preparation Kit, Pierce, Rockford, IL).

2.8. Cloning of monkey immunoglobulin variable domains and expression of PRIMATIZED[®] monoclonal antibodies

mRNA was isolated from subcloned populations of heterohybridomas producing the primate anti-human CD23 monoclonal antibodies 5E8 and 6G5 by Micro-Fast Track[®], Invitrogen, San Diego, CA. Single stranded cDNA was synthesized from the mRNA by cDNA Cycle[®] Kit (Invitrogen, San Diego, CA).

PCR primers for heavy and light chain vari-

able region gene families were used to amplify single stranded cDNA (see Ref. [32] for primers). PCR products from two separate reactions were cloned into the Original TA Cloning[®] Kit (Invitrogen, San Diego, CA) and sequenced to show identity and eliminate potential PCR errors.

The variable domains isolated from primate monoclonal anti-human CD23 6G5 are similar to light chain human lambda family II, and heavy chain human family II and IV. The variable domains isolated from primate monoclonal anti-human CD23 5E8 are similar to human kappa family I, and human heavy chain family III. An asparagine-linked glycosylation site in the heavy chain variable region of 5E8 in framework three was eliminated by converting the asparagine (AAC) to a lysine (AAG). In addition, the natural leader sequence of the heavy chain of 5E8 was substituted by a synthetic leader.

Variable domains isolated from primate monoclonal antibody 5E8 were cloned into two immunoglobulin expression vectors, N5KG1 and N5KG4P. N5KG1 contain the human kappa constant region (allotype Km3) and the human gamma 1 constant region (allotype z+, a+). The antibody expressed using this vector was called PRIMATIZED[®] monoclonal p5E8G1. The expression vector N5KG4P is identical to N5KG1 except in place of human gamma 1 it contains a human gamma 4 constant region, with a single mutation. Amino acid 241 Kabat numbering, or 228 Eu numbering in the hinge has been changed from a (TCA) serine to a (CCA) proline [33]. The antibody expressed using this vector was called PRIMATIZED[®] monoclonal p5E8G4.

These expression vectors also contain a translation impaired intronic insertion modified neomycin phosphotransferase gene used for integration, and a murine dihydrofolate reductase gene used for amplification with methotrexate (MTX) [34].

Immunoglobulins were expressed in the CHO cell line DG44 [35], adapted to either serum free media (CHO SSFM II, GIBCO BRL, Gaithersburg, MD) or protein free media (CD CHO,

GIBCO BRL, Gaithersburg, MD) and suspension culture.

3. Results

3.1. Primate monoclonal antibodies to human CD23

Five primate monoclonal antibodies against human CD23 antigen were purified from heterohybridoma cultures. All five antibodies bound to the human CD23 positive cell line RPMI 8866 as well as to sCD23 immobilized on an ELISA plate. Four of the five monoclonal antibodies inhibited the binding of FITC-labeled IgE complexes to 8866 cells (see Fig. 1). The murine monoclonal antibody MHM6 to human CD23 [19] was used as a positive control. These data indicate that four of the five isolated primate antibodies to human CD23 would be expected to block IgE complexed with allergen binding to membrane CD23.

All monoclonal antibodies were tested for their ability to block IgE production in an in vitro assay using IL-4, HC induced human PBMC. As can be seen in Fig. 2, MHM6 and the four primate monoclonals that blocked IgE complex binding to CD23 inhibited IgE production in this assay. 3G12, the primate monoclonal that did not block IgE-complex binding to CD23 positive cells, failed to inhibit IgE production. None of the antibodies had any effect on IgG production in this assay (data not shown).

In order to determine whether the primate monoclonal antibodies recognized similar epitopes, MHM6 was labeled with FITC and a competition flow cytometry analysis was performed. The results in Fig. 3 show that primate antibodies B3B11 and 2C8, but not 5C8 or 6G5, compete effectively with murine MHM6 for binding to CD23 positive RPMI 8866 cells. To demonstrate whether 5E8 and 6G5 recognize similar epitopes, the monoclonal antibody 6G5 was labeled with biotin and competition for binding to CD23 with unlabeled 5E8 was assessed. The data in Fig. 3 show that unlabeled 5E8, effectively blocks 6G5 binding to RPMI 8866

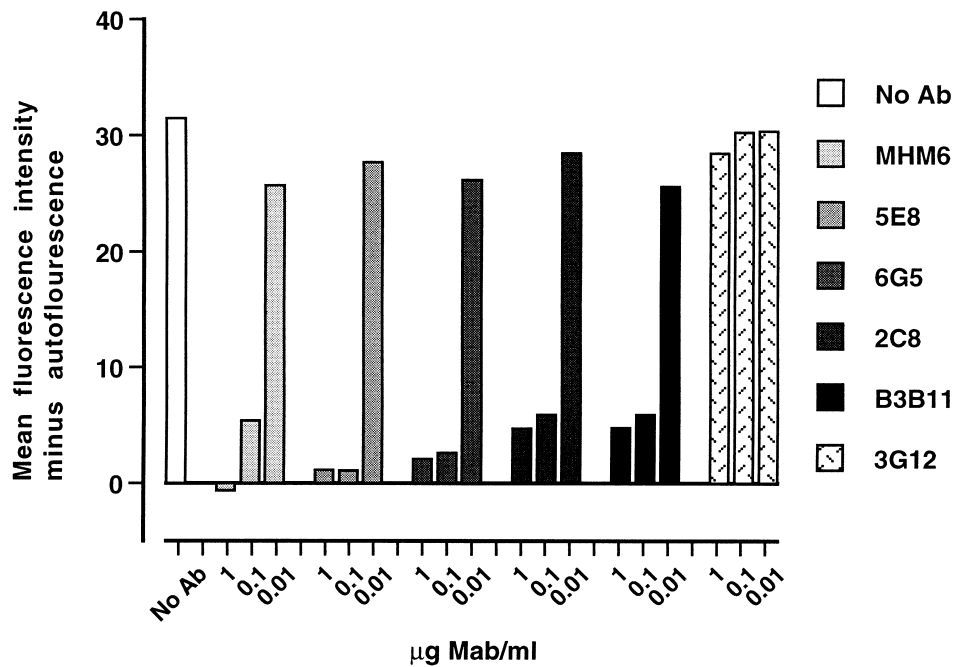


Fig. 1. Effects of anti-human CD23 monoclonal antibodies on IgE immune complex binding to CD23⁺ RPMI 8866 cells. Different concentrations of anti-human CD23 monoclonal antibodies were added to cells for 1 h at 37°C. The cells were washed and then incubated with FITC-labeled IgE immune complex for 1 h on ice, followed by washing and fixation. The results were analyzed by FACSscan. MHM6 is a murine monoclonal antibody, the other antibodies are primate monoclonals.

cells, indicating that the two antibodies recognize similar epitopes. These results define three epitopes recognized by the six monoclonal antibodies, one bound by 3G12, a second bound by MHM6, 2C8 and B3B11, and a third bound by 6G5 and 5E8.

3.2. PRIMATIZED[®] monoclonal antibodies to human CD23 and effects on IgE production

Human gamma 1 and gamma 4 versions of primate 5E8 were constructed, expressed in CHO cells, purified and evaluated in an IgE production assay. The results (Fig. 4) show that the PRIMATIZED[®] antibody p5E8G1 is as effective as the primate monoclonal antibody 5E8 in inhibiting IgE production, while the PRIMATIZED[®] antibody p5E8G4 is not as effective in IgE inhibition. Since both G1 and G4 versions

have similar K_d values (data not shown), and because human G1 has higher affinity for Fc receptors than human G4, these results indicate that the Fc portion of the antibody might be important in IgE inhibition. In addition, use of a PRIMATIZED[®] G1 version of the primate monoclonal antibody 6G5 also resulted in effective IgE inhibition while the PRIMATIZED[®] G4 version was weak (data not shown). Similar inhibition of IgE production by the PRIMATIZED[®] antibody p5E8G1 was obtained using human PBMC stimulated with IL-4 and a murine monoclonal against human CD40 instead of IL-4 and hydrocortisone (Fig. 5).

To confirm the role of antibody Fc in IgE inhibition, F(ab')₂ were made from PRIMATIZED[®] monoclonal antibody p5E8G1 and murine monoclonal antibody MHM6. Figs. 6 and 7 show that unlike the intact antibody, the F(ab')₂s do not

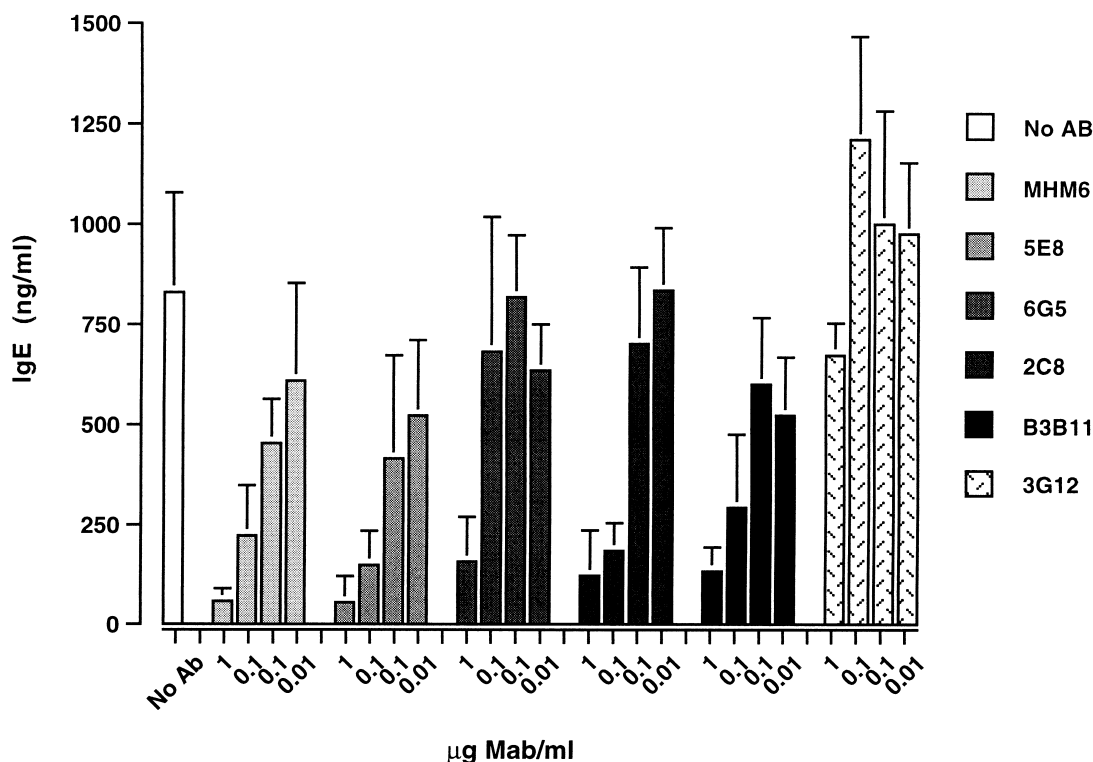


Fig. 2. Effects of anti-human CD23 monoclonal antibodies on IL4, HC induced IgE synthesis. Human PBMCs were cultured with IL4, HC, and anti-human CD23 monoclonal antibodies for 10 days at 37°C. An IgE ELISA was performed to measure IgE contents in the culture. MHM6 is a murine monoclonal antibody, the other antibodies are primate monoclonals.

suppress IgE production induced by IL-4 and hydrocortisone, but they do block suppression by intact antibodies in a dose-dependent fashion. Similar results were obtained using F(ab')₂ of p5E8G1 when IgE production was induced by IL-4 and anti-CD40 (Fig. 5). These data further indicate that the Fc region of the antibody is required for IgE inhibition of IL-4, HC stimulated human PBMC IgE production.

We have shown that primate monoclonal antibodies against two distinct epitopes on CD23 block binding of IgE immune complex to CD23 positive cells and inhibit IL-4 stimulated IgE production by human PBMCs. However, Fc receptor binding is likely to be important for such antibodies to exhibit this inhibitory function.

4. Discussion

There are several reports that F(ab')₂ of murine monoclonals to anti-CD23 were effective in inhibiting an IL-4 induced IgE response from human PBMC in vitro [17,36]. In addition, a polyclonal F(ab')₂ of anti-CD23 has been reported to inhibit antigen-specific IgE response in the rat [27].

We were therefore surprised when human gamma 4 versions of PRIMATIZED[®] antibodies 5E8 and 6G5 were significantly less effective than their human gamma 1 counterparts, and that F(ab')₂ of p5E8G1 and the murine monoclonal antibody MHM6 were inactive in IgE inhibition.

These results imply that the Fc regions of anti-

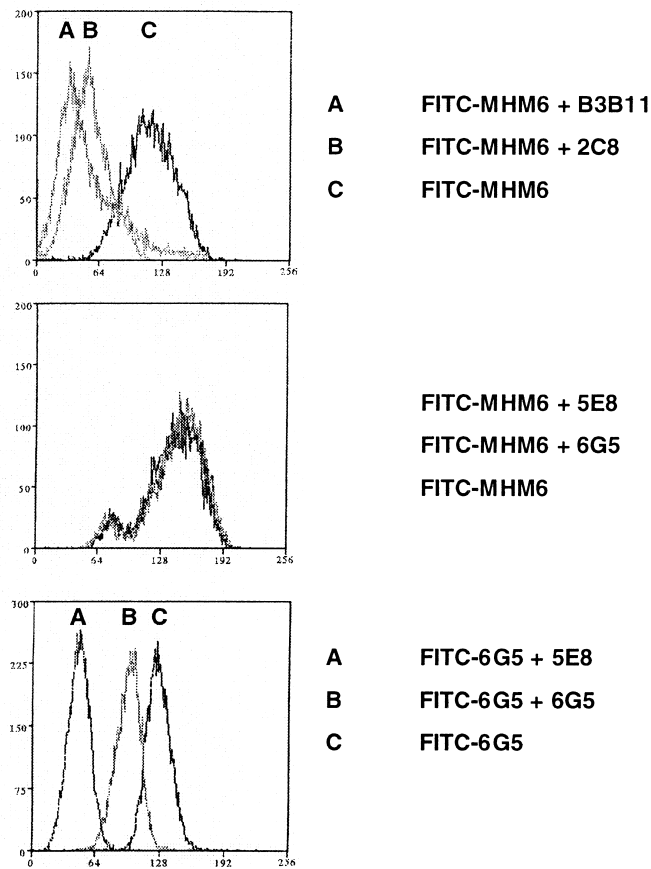


Fig. 3. Flow cytometry analysis for competition of anti-human CD23 antibodies. RPMI 8866 cells were incubated with various combinations of antibodies (shown on the right) for 1 h on ice. Cells were then washed, fixed and analyzed by flow cytometry.

CD23 antibodies are involved in IgE inhibition in vitro. Reports which show IgE inhibition in vitro by $F(ab')_2$ described PBMC induced by IL-4 to produce 3–15 ng/ml of IgE [17,36]. Our studies describe PBMC induced with the IL-4 and hydrocortisone or anti-CD40 monoclonal antibody to synthesize several 100 ng/ml to several 1000 ng/ml of IgE. Under these conditions, IgE synthesis is inhibited in vitro by anti-CD23 monoclonal antibodies, but not by their $F(ab')_2$. In addition, anti-CD23 $F(ab')_2$ block the inhibitory effect of anti-CD23 monoclonal antibodies on IgE synthesis in vitro, which indicates that

binding of the $F(ab')_2$ to the CD23 receptor is intact.

Ligation of CD23 on human B-lymphocytes triggers cyclic AMP generation [23,25]. How this signal is related to inhibition of IgE production is unknown. Our observations suggest binding of CD23 alone is insufficient to block IL-4 induced IgE production in vitro. It is likely that ligation of a Fc gamma receptor is also required to exert IgE suppression.

It is possible that clustering of CD23 molecules into RAFTS by coligation of the Fc portion of the anti-CD23 monoclonal antibody may induce

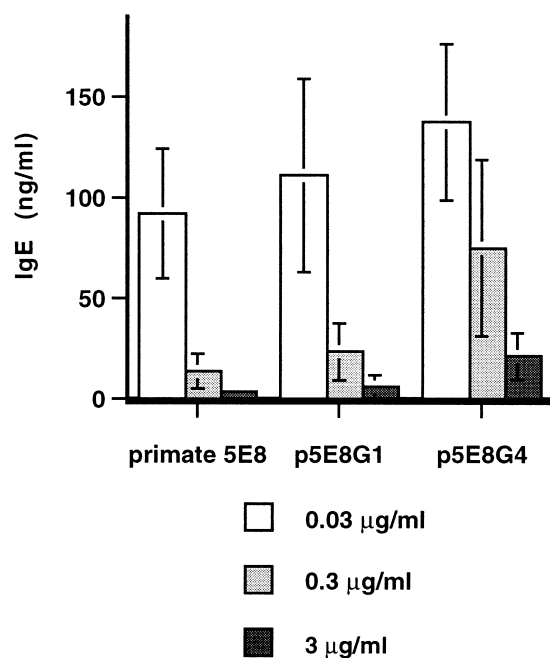


Fig. 4. Effects of primate 5E8 and PRIMATIZED[®] Versions of 5E8 on IL4, HC induced IgE synthesis. Human PBMCs were incubated in the presence of IL4, HC, and different monoclonal antibodies at concentrations ranging from 0.03 to 3 µg/ml for 10 days at 37°C. An IgE ELISA was performed to measure IgE contents in the culture.

negative signaling directly through the CD23 molecule. It is also possible that signals induced by FcγRIIb through its ITIM sequence may contribute to IgE inhibition by inducing apoptosis of B cells [37,38]. Experiments are ongoing to further investigate the signaling by anti-CD23 and Fc that is required for IgE inhibition in vitro. In conclusion, PRIMATIZED[®] anti-CD23 gamma 1 antibodies are effective in the inhibition of IgE production and may prove effective in treatment of IgE mediated allergic disease in humans.

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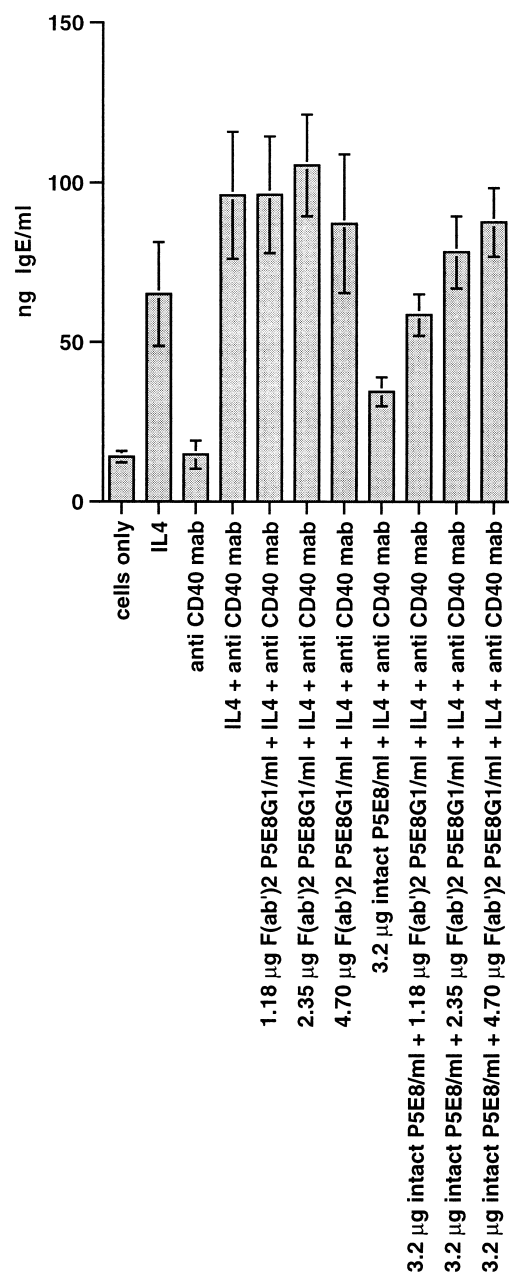


Fig. 5. Effects of PRIMATIZED[®] 5E8 on IL4, anti-CD40 induced IgE synthesis and F(ab')₂ effects on intact p5E8G1 suppression of IL4, anti-CD40 induced IgE synthesis. Human PBMCs were incubated in the presence IL4, anti-CD40 and either intact p5E8G1 or a mixture of intact p5E8G1 and F(ab')₂ p5E8G1 for 10 days at 37°C. An IgE ELISA was performed to measure IgE contents in the culture.

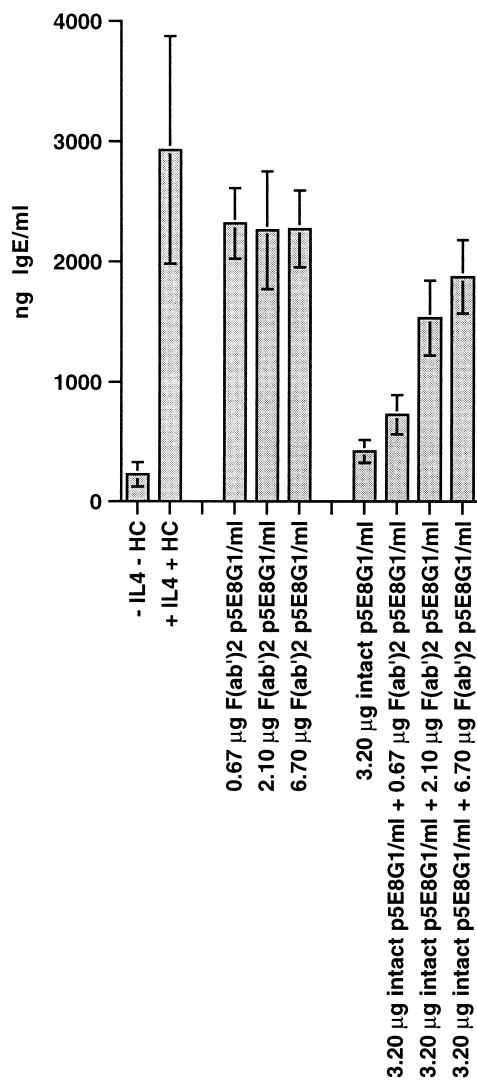


Fig. 6. F(ab')₂ effects on intact p5E8G1 suppression of IL4, HC induced IgE synthesis. Human PBMCs were incubated in the presence IL4, HC and either intact p5E8G1 or a mixture of intact p5E8G1 and F(ab')₂ p5E8G1 for 10 days at 37°C. An IgE ELISA was performed to measure IgE contents in the culture.

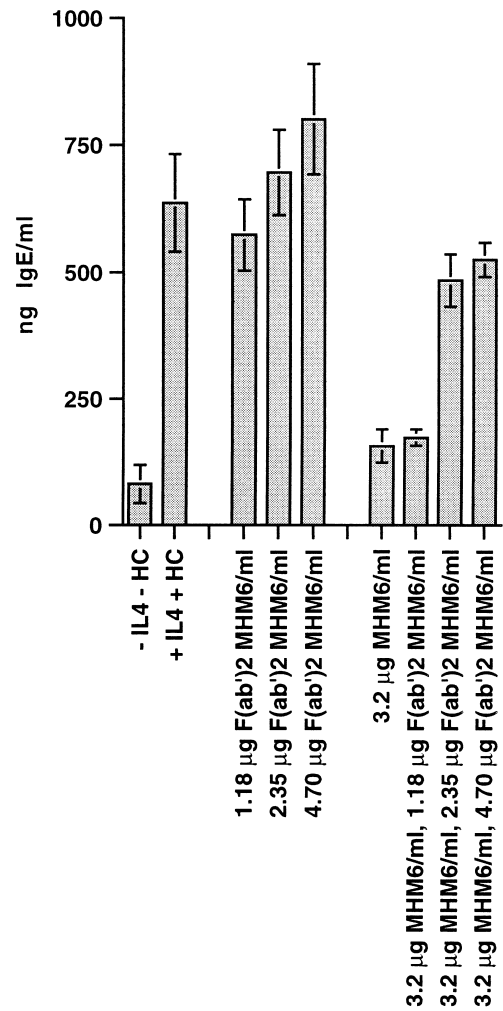


Fig. 7. F(ab')₂ effects on intact MHM6 suppression of IL4, HC induced IgE synthesis.

Human PBMCs were incubated in the presence IL4, HC and either intact MHM6 or a mixture of intact MHM6 and F(ab')₂ MHM6 for 10 days at 37°C. An IgE ELISA was performed to measure IgE contents in the culture.

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