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Regulation of IgE Production Requires Oligomerization of CD23¹

Michelle A. Kilmon,^{2*} Rodolfo Ghirlando,^{3†} Marie-Paule Strub,^{4†} Rebecca L. Beavil,[‡] Hannah J. Gould,[‡] and Daniel H. Conrad*

Here we describe the production of a rabbit polyclonal Ab (RAS1) raised against the stalk of murine CD23. RAS1 inhibits release of CD23 from the surface of both M12 and B cells resulting in an increase of CD23 on the cell surface. Despite this increase, these cells are unable to bind IgE as determined by FACS. CD23 has previously been shown to bind IgE with both a high ($4\text{--}10 \times 10^7 \text{ M}^{-1}$) and low ($4\text{--}10 \times 10^6 \text{ M}^{-1}$) affinity. Closer examination by direct binding of ¹²⁵I-IgE revealed that RAS1 blocks high affinity binding while having no effect on low affinity binding. These data support the model proposing that oligomers of CD23 mediate high affinity IgE binding. These experiments suggest that RAS1 binding to cell surface CD23 results in a shift from oligomers to monomers, which, according to the model, only bind IgE with low affinity. These experiments also suggest that high affinity binding of IgE is required for IgE regulation by CD23 and is demonstrated by the fact that treatment of Ag/Alum-immunized mice treated with RAS1 results in a significant increase in IgE production similar to the levels seen in CD23-deficient mice. These mice also had significantly decreased levels of serum soluble CD23 and Ag-specific IgG1. RAS1 had no effect on IgE or Ag-specific IgG1 production in CD23-deficient mice. *The Journal of Immunology*, 2001, 167: 3139–3145.

The low affinity receptor for IgE, CD23, is a type II glycoprotein. The carboxyl terminus of CD23 contains a lectin domain, making it a member of the c-type lectin family (1). The lectin head is the site of interaction with the Cε3 domain of IgE (2, 3) and like other members of this family, this binding is calcium dependent (4, 5).

CD23 is found on a variety of cell types in humans including B cells, monocytes, eosinophils, and Langerhans cells (reviewed in Ref. 6). Two isoforms of CD23 exist in the human, CD23a and CD23b, which result from two different transcription initiation sites. The extensive distribution of CD23 on human cells is due to the use of the CD23b promoter. Only one isoform is found in the mouse that most closely resembles human CD23a (7). Therefore, expression of CD23 is relatively limited in the mouse and is found only on B cells and follicular dendritic cells (8).

CD23 is initially expressed as a membrane-bound protein, but it is cleaved by an unknown metalloprotease (9, 10) releasing the

majority of the protein as a soluble protein (sCD23),⁵ which contains the stalk and lectin domain (11, 12). The initial fragment that is released has a molecular mass of 37 kDa in the human and 38 kDa in the mouse. Smaller fragments of sizes ranging from 33 to 12 kDa in the human and from 35 to 25 kDa in the mouse, all containing the lectin domain, are also seen.

The stalk region of CD23 is located between the lectin domain and the transmembrane region. The stalk of murine CD23 contains four 21-aa repeats (7), whereas the human has three (13). Beavil et al. (14) noted a periodic heptad repeat containing a hydrophobic amino acid (usually leucine) in the stalk, which is characteristic of a leucine zipper motif, and suggested that CD23 might form an α helical coiled coil. Cross-linking of CD23 resulted in a molecule with a molecular mass consistent with trimer formation (15), and use of reversible cross-linkers showed that only the 49-kDa form of CD23 was present in the oligomers (16). Membrane CD23 has been shown to bind IgE with a dual affinity, from $4\text{--}10 \times 10^6 \text{ M}^{-1}$ to $4\text{--}10 \times 10^7 \text{ M}^{-1}$ (16). These observations led Gould et al. (1) to suggest a model where CD23 forms trimers and that oligomerization of CD23 mediates high affinity binding to IgE. A mutated form of murine CD23, containing only one of the 21-aa repeats, was capable of binding IgE with only a low affinity (16). These data indicate that in the oligomeric form, CD23 binds IgE with a high affinity ($4\text{--}10 \times 10^7 \text{ M}^{-1}$), and that the monomeric form of CD23 can only bind IgE with a low affinity ($4\text{--}10 \times 10^6 \text{ M}^{-1}$). More recently a chimeric protein was made which consisted of an isoleucine zipper (17) attached to the extracellular portion of CD23 (LZ-C1M-CD23), allowing the formation of a stable soluble trimer (18). This molecule bound IgE with a comparable affinity seen with membrane CD23, presumably because the leucine zipper enhances trimer formation. These data further suggest that CD23 must form a trimer to bind IgE with a high affinity.

This study used a polyclonal Ab made against the stalk region of CD23 to examine the role oligomerization of CD23 plays in IgE

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⁵ Abbreviations used in this paper: sCD23, soluble CD23; RAS1, polyclonal rabbit anti-CD23 stalk Ab; nRIgG, normal rabbit IgG; muCD23_{50–175}, murine CD23_{50–175}; KLH, keyhole limpet hemocyanin; CHO, Chinese hamster ovary.

production. The results support the hypothesis that only oligomers of CD23 can bind IgE with high affinity. Surprisingly, these studies also indicate that only membrane CD23 in the form of oligomers and not monomers can regulate IgE production. These data also suggest that it is the trimeric form of CD23 that plays a role in enhancing Ag processing and presentation of IgE/Ag complexes.

Materials and Methods

Reagents and animals

Baculovirus supernatant containing recombinant murine IL-4 was a generous gift from Dr. W. Paul (National Institutes of Health, Bethesda, MD); recombinant murine IL-5 was purchased from R&D Systems (Minneapolis, MN). rCD40 ligand trimer and M15 (mouse IgG1 anti-CD40 ligand trimer) (19) were obtained from Immunex (Seattle, WA). FITC-anti-CD23 (B3B4) was obtained from BD PharMingen (San Diego, CA) and FITC-goat anti-rabbit IgG was purchased from Cappel (Durham, NC). Monoclonal mouse IgE anti-DNP (20) was purified from ascites as described (21). BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). CD23^{-/-} mice (22) were a gift from Dr. H. Van der Putten (Novartis Pharma, Basel, Switzerland). All animals used in experiments were between 6 and 10 wk of age and were kept in an accredited animal facility.

Cloning, expression, and purification of muCD23₅₀₋₁₇₅

DNA for the expression of muCD23₅₀₋₁₇₅ was amplified by PCR from a muCD23 cDNA containing plasmid (sFsCD23 in pcDNA-1-amp; Ref. 7) using the following primers: CGCCTTTCCATATGGAACGGAGAA GAATCTAAA and CGGATCCTTATATCTCTATCCACAGCTTTGC. The PCR fragment was digested with *Nde*I and *Bam*HI, gel purified, and cloned into the *Nde*I and *Bam*HI sites of pET15b (Novagen, Madison, WI). This yields the plasmid pRG15b:muCD23:50-175(1), which codes for HT⁺-muCD23₅₀₋₁₇₅, namely, the muCD23₅₀₋₁₇₅ peptide containing an N-terminal histidine tag.

Recombinant HT⁺-muCD23₅₀₋₁₇₅ was expressed in BL21(DE3) *Escherichia coli*. Transformants were grown overnight at 37°C in Luria-Bertani medium containing 100 µg/ml of ampicillin and diluted 40-fold into 4 liters of Ecpm1 medium containing 100 µg/ml ampicillin. The *E. coli* were grown with aeration at a pH of 7.0 and 37°C for 5 h in a 4-liter fermenter (Maestro; LSL Biolafitte, Saint Germain en Laye, France) as described (23). Protein expression was induced with 1.0 mM isopropyl β-D-thiogalactopyranoside, and the cells were grown for an additional 3 h. The cells were harvested by centrifugation and stored at -80°C.

Cells (100 g) obtained from a 4-liter fermentation were resuspended in 200 ml of 300 mM NaCl and 50 mM sodium phosphate (pH 8.0). The suspension was homogenized (Ultra-Turrax T25; Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) and sonicated on ice (VibraCell 72408 sonicator; Bioblock Scientific, Illkirch, France) for 5 min to disrupt the cells. Insoluble material was pelleted by centrifugation for 30 min at 40,500 × *g*. The pellet was resuspended in 90 ml of 5 M guanidine hydrochloride and 50 mM sodium phosphate (final pH = 6.0) and allowed to stand at room temperature for 24 h. The solution was clarified by centrifugation, and the pellet was extracted a second time to yield 200 ml of a solution containing HT⁺-muCD23₅₀₋₁₇₅.

The recombinant protein was purified by nickel affinity chromatography. The HT⁺-muCD23₅₀₋₁₇₅ in 5 M guanidine hydrochloride and 50 mM sodium phosphate (final pH = 6.0) was loaded at 0.5 ml/min on 40 ml of an agarose Ni(II)-NTA resin (Qiagen, Valencia, CA), which had been equilibrated with the same buffer. The column was rinsed with 250 ml of the same buffer, and the HT⁺-muCD23₅₀₋₁₇₅ was eluted with 100 ml of 5 M guanidine hydrochloride and 50 mM sodium phosphate (final pH = 4.0). The fractions containing the HT⁺-muCD23₅₀₋₁₇₅ were pooled (50 ml) and purified in 12.5-ml batches by gel filtration on a Sephacryl S-200 HR 100-cm XK26 column (Pharmacia, Peapack, NJ) with 5 M guanidine hydrochloride and 50 mM sodium phosphate (final pH = 6.0). Fractions containing the HT⁺-muCD23₅₀₋₁₇₅ were identified by SDS-PAGE and pooled to yield 125 ml of an ~1 mg/ml solution of HT⁺-muCD23₅₀₋₁₇₅ that was at least 95% pure.

The pooled fractions were dialyzed exhaustively (SpectraPor 7, molecular weight cutoff 2000 membranes) at 4°C against 2 liters of 50 mM sodium phosphate (pH 8.0). In the course of the dialysis a white precipitate of the HT⁺-muCD23₅₀₋₁₇₅ formed. Following dialysis, the dialysate was made up to a total volume of 350 ml with water to dissolve the precipitate.

The purified HT⁺-muCD23₅₀₋₁₇₅ was cleaved with bovine thrombin (56 NIH U/mg of total solids; Sigma, St. Louis, MO) to remove the histidine tag and yield HT⁻-muCD23₅₀₋₁₇₅. The 350 ml of HT⁺-muCD23₅₀₋₁₇₅ were equilibrated to room temperature and stirred with 35 ml of 10× PBS

(pH 7.4) followed by 3.5 ml of a 10 mg/ml bovine thrombin stock solution. Cleavage was complete and quenched after 22 min with 6.0 ml of a 0.5 M benzamidine hydrochloride solution. The HT⁻-muCD23₅₀₋₁₇₅ solution was concentrated to a final volume of 25 ml on an Amicon (Beverly, MA) concentrator using a YM1 membrane (Amicon) and purified in two batches by gel filtration (Sephacryl S-200 HR, XK26 100-cm column; Pharmacia) with 5 M guanidine hydrochloride and 50 mM sodium phosphate (final pH = 6.0). The fractions containing the HT⁻-muCD23₅₀₋₁₇₅ were identified by SDS-PAGE, pooled, and dialyzed exhaustively (SpectraPor 7, molecular weight cutoff 2000 membranes) against 2 liters of water at 4°C. The peptide was then lyophilized and stored as a white powder at -20°C. Analysis by 15% SDS-PAGE showed that the HT⁻-muCD23₅₀₋₁₇₅, comprising muCD23₅₀₋₁₇₅ and an N-terminal GSH, was >99% pure. A 4-liter fermentation yielded ~75 mg of the peptide.

Preparation of anti-stalk rabbit polyclonal Ab

The immunogen (mouse stalk) was emulsified in CFA and injected into a New Zealand White rabbit. Subsequent boosts were given in IFA. The resulting antiserum tested positive by ELISA. The IgG from the antisera was purified by precipitation with 40% ammonium sulfate and ion exchange chromatography on DE-52 cellulose performed with 0.175 M phosphate, pH 6.3. The polyclonal anti-stalk Ab will be further referred to as RAS1 (rabbit anti-stalk 1).

Cell culture and B cell preparation

CD23-expressing Chinese hamster ovary (CHO) K1 cells, Fc1.7, were made as previously described (16) and grown in DMEM-glutamate synthetase (24), and parental CHOK1 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and streptomycin, and 2 mM glutamine. M12.4.5 (M12), a murine B cell lymphoma (25), was purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium with 10% FBS, 100 U/ml penicillin and streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM oxaloacetic acid, and 5×10^{-5} M mercaptoethanol (M12 medium). M12 cells were activated using 50,000 U/ml IL-4 and 50 mg/ml LPS (Sigma). B cells were purified as previously described (26, 27). Briefly, single cell suspensions were made by crushing spleens between frosted glass slides. B cells were negatively selected with anti-CD5 (Lyt-1), anti-CD8 (Lyt-2) (both from Dr. W. Paul), anti-Thy1.1 (TiB99) (28) and guinea pig complement (Life Technologies, Gaithersburg, MD). Resting B cells were obtained by fractionation on a discontinuous Percoll gradient; cells at the 66–70% interface were considered to be resting. B cells were cultured in M12 medium except that FBS was heat inactivated and 1% nonessential amino acids were also present (B cell medium). B cells were stimulated in B cell medium containing 50,000 U/ml IL-4, 5 ng/ml IL-5, 0.1 mg/ml recombinant CD40 ligand trimer, and 0.1 mg/ml M15 (anti-CD40 ligand trimer mAb) (19). To determine the effect of RAS1 on surface CD23 and sCD23 release, 1×10^5 M12 or 1×10^6 B cells were stimulated as indicated above in 24-well plates (Corning Costar, Cambridge, MA) in a final volume of 1 ml. On day 2, cells were washed and resuspended in medium with stimulators, and indicated concentrations of RAS1 or normal rabbit IgG (nRIgG; Sigma) as an isotype control. The cells were also cultured with or without IgE (100 µg/ml). Eighteen hours later, cells and supernatants were harvested. CD23 surface levels were determined by FACS analysis, and sCD23 release was measured by ELISA. IgE binding capability was determined by incubating cells with mouse IgE (10 µg/ml) for 30 min and then detecting bound IgE with FITC-rat anti-mouse IgE (BD PharMingen).

Scatchard analysis

The affinity for CD23 with or without the presence of RAS1 was determined as previously described (16). Briefly, 5×10^5 Fc1.7 cells were added to tubes containing increasing amounts of cold IgE and RAS1 or nRIgG at a concentration of 1.5 mg/ml. After a 30-min incubation, ¹²⁵I-labeled IgE (0.5 or 5.0 µg) was added, resulting in a final concentration range of 1.0–400 µg/ml. These tubes were incubated for 60 min on ice, and the free and cell-bound ¹²⁵I-IgE was separated on a phthalate oil mixture (29). Nonspecific binding was determined by adding 100-fold excess cold IgE, and the value was subtracted to obtain specific binding. Addition of nRIgG did not inhibit binding of IgE. Binding affinities were determined by linear regression analysis.

In vivo studies

BALB/c mice were treated with 2 mg RAS1 in PBS i.p. on days -2, 0, and 7. Another group of mice received nRIgG (Sigma) as a control. On day 0, mice were immunized s.c. with 100 µg keyhole limpet hemocyanin

(KLH)-DNP in an adjuvant consisting of 4 mg Alum and 110 ng pertussis toxin (Sigma). Mice were bled on days 14 and 20. Levels of total IgE as well as KLH-DNP-specific IgG1 in the serum were determined by ELISA. sCD23 levels in the serum were also determined by ELISA. Serum IgE, KLH-DNP-specific IgG1, and sCD23 levels of experimental mice were normalized to the levels in control (BALB/c) mice. Statistical differences were determined by Student's *t* test.

ELISAs

sCD23 was determined as previously described (18). Immulon ELISA plates (Dynex Technologies, Chantilly, VA) were coated with 2G8 (anti-CD23). EC-CD23 was used as the standard (18). EC-CD23 consists of the extracellular portion of CD23 and was made in *E. coli* as a denatured protein. EC-CD23 was renatured as described (18), and the concentration was determined by spectrophotometry. Samples and standards were detected using a rabbit polyclonal anti-mouse CD23 followed by goat anti-rabbit IgG-HRP (Southern Biotechnology Associates, Birmingham, AL).

IgE was determined as previously described (30) using a pair of rat anti-mouse mAbs, B1E3 and R1E4 (both purified from ascites), as the capture and biotinylated secondary Ab, respectively. IgG₁ (30) was determined using an unlabeled primary goat anti-mouse IgG1 Ab and detected using a goat anti-mouse IgG1 coupled to alkaline phosphatase (both obtained from Southern Biotechnology Associates). All ELISAs were performed in Immulon ELISA plates (Dynex Technologies).

Results

Analysis of a polyclonal anti-mouse CD23 stalk Ab

A portion of the stalk of CD23 corresponding to amino acids 50–175 and produced in *E. coli* was used as the immunogen to produce a polyclonal antiserum. The purified IgG fraction from the rabbit antiserum (RAS1) was tested for CD23 binding to Fc1.7 cells, CD23-overexpressing CHO cells. As shown in Fig. 1, RAS1 did bind to the Fc1.7 cells but not to control CHO cells. To further confirm specificity of RAS1 for CD23, cell lysates from ¹²⁵I-labeled Fc1.7 and CHO cells were immunoprecipitated with RAS1. RAS1 specifically bound CD23 in the Fc1.7 cells but not proteins of any other size and did not immunoprecipitate any proteins from the control CHOK1 cells (Fig. 1, inset).

Effect of anti-stalk Ab on sCD23 release

To determine whether RAS1 binding to CD23 had any effect on CD23 cleavage, M12 cells, a B cell lymphoma, were stimulated with IL-4 and LPS to induce CD23 expression. Activated cells were then cultured in the presence of RAS1 overnight. Fig. 2A shows that M12 cells cultured alone or with nRIgG (control) both show equal amounts of CD23 released into the supernatant. When added at 200 μg/ml, RAS1 had no effect on CD23 cleavage, and at 400 μg/ml RAS1 inhibited cleavage. Lee et al. (31) showed that when IgE is added to the cells, CD23 is protected from cleavage. This effect of stabilization of CD23 by IgE was repeatable in these

experiments. When RAS1 was added to activated B cells, the results were similar to that seen with the M12 cells (Fig. 2B).

Because addition of RAS1 decreased the amount of sCD23 released by CD23-expressing cells, the amount of CD23 on the surface of these cells was examined. As shown in Fig. 3A, nRIgG had no effect on the amount of CD23 on the surface of B cells, nor did the addition of 10 μg/ml RAS1 to the cells (data not shown). However, when RAS1 was added at 100, 200, or 400 μg/ml, CD23 expression was increased on the surface of these cells dose dependently.

Increased cell surface CD23 levels do not correspond to increased IgE binding

Because B cells cultured with increasing amounts of RAS1 exhibited increased CD23 on the cell surface, we examined whether the increased CD23 would correspond to an increased ability of these cells to bind IgE. B cells cultured with nRIgG were able to bind IgE (Fig. 3B). Addition of 10 μg/ml RAS1 did not affect IgE binding to the B cells (data not shown). However, cells treated with 100 μg/ml RAS1 exhibited less IgE binding, and cells cultured with either 200 or 400 μg/ml RAS1 demonstrated no IgE binding. Thus, even though cells cultured with at least 100 μg/ml RAS1 have increased levels of CD23 on their surface, these cells show a decreased ability to bind IgE.

Cells cultured with RAS1 exhibit only low affinity IgE binding

To further examine the effect RAS1 had on IgE binding to CD23, saturation analysis was performed. Fig. 4 shows that Fc1.7 cells incubated with a large range of IgE concentrations exhibited the same dual-affinity IgE binding ($K_a = 9.9 \times 10^7 \text{ M}^{-1}$ and $1.4 \times 10^6 \text{ M}^{-1}$) previously seen with cells expressing CD23 (16). However, addition of RAS1 to these same cells resulted in a single low affinity binding ($K_a = 2.1 \times 10^6 \text{ M}^{-1}$). Inhibition of high affinity IgE binding to CD23 by RAS1 was also temperature sensitive. At 37°C, 10-fold less RAS1 was needed to give 100% inhibition of IgE binding as compared with the amount needed to inhibit IgE binding at 4°C (data not shown). These data suggest that RAS1 binds to the stalk of CD23 and causes disassociation of CD23 trimers allowing only low affinity binding of IgE to monomers of CD23. The presence of nRIgG did not affect IgE binding.

Effects of RAS1 on IgE production in vivo

To determine whether RAS1 would have an effect on IgE production in vivo, mice were immunized with KLH-DNP in alum with pertussis toxin. This treatment stimulates a strong IgE response. Mice were also treated with either nRIgG or RAS1 on days –2, 0, and 7, and serum was collected on days 14 and 20. BALB/c mice

FIGURE 1. RAS1 recognizes an epitope on CD23-expressing CHOK1 cells (Fc1.7) but not control CHOK1 cells. Fc1.7 (dotted line) and CHOK1 cells (solid line) were incubated with RAS1 and then goat anti-rabbit-FITC, and then analyzed by FACS analysis. Inset, Cell lysates from ¹²⁵I-labeled CHOK1 (lane 1) and Fc1.7 cells (lane 2) were immunoprecipitated with RAS1 and run on SDS-PAGE and exposed to film.

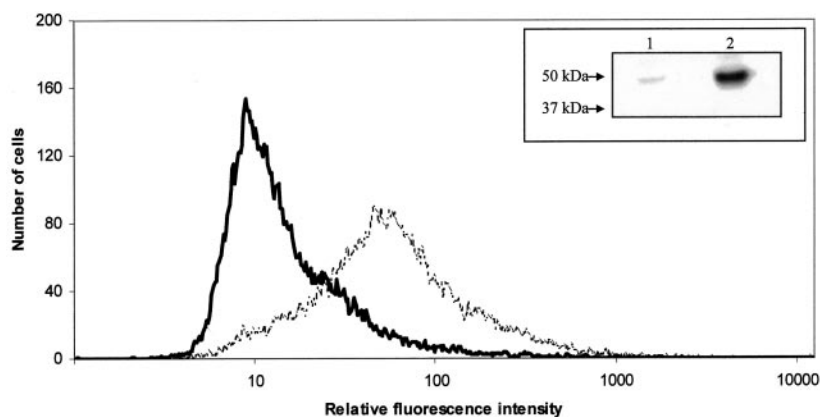
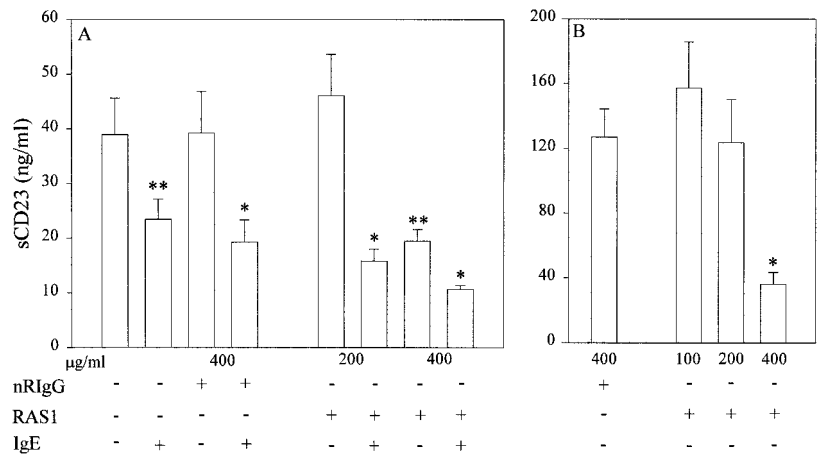


FIGURE 2. Effect of RAS1 on sCD23 release by M12 and B cells. *A*, Stimulated M12 cells were cultured with nRIgG or varying concentrations of RAS1 with or without IgE (100 μ g/ml) for 18 h. Supernatants were collected and sCD23 was determined by ELISA. *B*, B cells were stimulated as in *Materials and Methods* and then cultured with indicated amount of RAS1 for 18 h, and sCD23 was determined as in *A*. The numbers under the graphs represent the amount of nRIgG or RAS1 added to each sample in micrograms per milliliter. Statistical analysis was performed using Student's *t* test. Samples treated with nRIgG or RAS1 alone were compared with cells alone, and IgE-containing cultures were compared with identical cultures without IgE (*, $p \leq 0.05$; **, $p \leq 0.001$).



treated with 2 mg RAS1 during the IgE induction phase produced significantly higher amounts of IgE than mice that received nRIgG (Fig. 5). Lower doses were tested for their ability to influence IgE production, and RAS1 was found to be effective at increasing IgE levels at a dose as low as 0.5 mg/mouse; however, lower doses (0.01 and 0.1 mg/mouse) did not change IgE levels as compared with the control group (data not shown). There was no difference in the amount of IgE produced by CD23^{-/-} mice whether or not they received RAS1, proving that the increase in IgE production seen in BALB/c mice was mediated by CD23. Also of importance is that the CD23^{-/-} mice produced higher amounts of IgE than the BALB/c mice, 4313.1 $\pm$ 1478.1 and 1887.1 $\pm$ 518.1 ng/ml (day 14), respectively, which is consistent with a previous report (32) where the lack of CD23 results in higher IgE synthesis. KLH-DNP-specific IgG1 was decreased in BALB/c mice that received RAS1 as compared with nRIgG-treated mice. There was no difference in the amount of Ag-specific IgG1 produced by the two groups of CD23^{-/-} mice. Interestingly, BALB/c mice treated with RAS1 had significantly lower levels of serum sCD23 than nRIgG-treated mice (4.2 $\pm$ 1.9 vs 12.8 $\pm$ 0.7 ng/ml, respectively). IgE and sCD23 levels were examined as far out as day 50 in one experiment, and both remained suppressed in the mice treated with RAS1.

Discussion

This study examines the effect of using a polyclonal Ab directed against the stalk region of mouse CD23 on IgE binding to CD23

and IgE regulation by CD23. RAS1 specifically binds an epitope on CD23-transfected cells, but not on control CHOK1 cells. When added to cultures of activated M12 or B cells, RAS1 inhibits sCD23 release by both types of cells. This suggests that the cleavage site is protected by RAS1. The protection from cleavage that RAS1 provides corresponds to an increase of CD23 on the cell surface. However, the increase in surface CD23 does not lead to an increase in IgE binding. In fact, the cells treated with the highest doses of RAS1 exhibit negligible IgE binding when measured by FACS analysis.

CD23 binds IgE with both a high and low affinity. This dual affinity was explained by Gould et al. (1) to be related to the oligomerization of CD23. According to this model, low affinity binding occurs when one molecule of IgE is bound to one CD23 molecule, and high affinity binding of IgE to CD23 occurs when a molecule of IgE is bound by multiple CD23 receptors at the same time. The higher affinity binding seen in this case is really a measure of higher avidity because multiple receptors are involved in binding to one molecule of IgE. We further examined the lack of IgE binding when RAS1 is bound to CD23 by Scatchard analysis. Fc1.7 cells, expressing a native form of CD23, exhibited the expected dual affinity binding to IgE ($K_a = 9.9 \times 10^7$ M⁻¹ and 1.4×10^6 M⁻¹) previously seen. The addition of RAS1 to these cells resulted in only low affinity binding. Because RAS1 recognizes the stalk of CD23, which mediates oligomerization of CD23, it is most

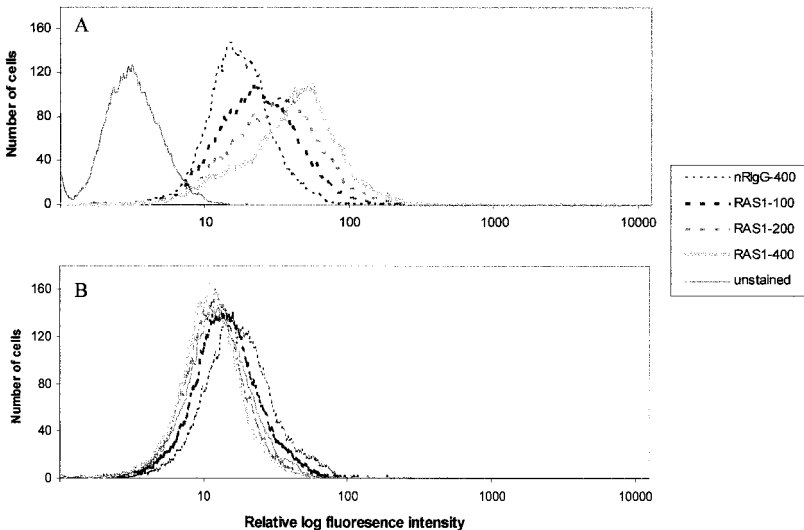


FIGURE 3. RAS1 dose dependently increases CD23 surface levels while decreasing IgE binding ability. Resting B cells were stimulated as in *Materials and Methods* and then cultured in the presence of the indicated concentrations of RAS1 or nRIgG for 18 h. The cells were then harvested and stained for CD23 expression (*A*), or IgE (10 μ g/ml) was added to determine the amount of IgE that was able to bind the cells and was detected using FITC-rat anti-mouse IgE (*B*).

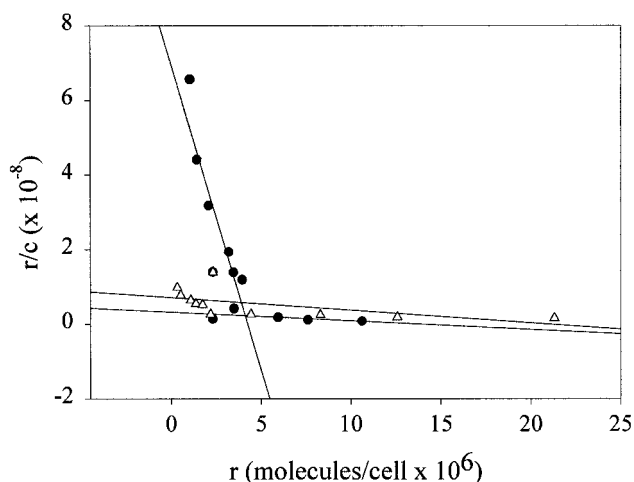


FIGURE 4. Scatchard analysis for IgE binding to Fc1.7 in the presence of RAS1. Fc1.7 cells were incubated with increasing concentrations of cold IgE with or without RAS1 for 30 min, followed by ^{125}I -labeled IgE. After 1 h, cell-bound cpm was determined on duplicate aliquots of cells. ●, IgE binding to Fc1.7; △, binding to Fc1.7 in the presence of RAS1. The lines represent regression analysis.

likely inhibiting high affinity binding by destabilizing CD23 trimers. Inhibition of IgE binding to CD23 by RAS1 was shown to be temperature dependent in that 10-fold less RAS1 was required to completely inhibit high affinity binding at 37°C as compared with 4°C. This suggests that the stalk must uncoil for some of the epitopes to become available for RAS1 to bind and inhibit IgE binding.

CD23 has been proposed to have several functions. Among these include IgE regulation and enhancement of processing and presentation. The first suggestion that CD23 might be a regulator of IgE came from the observation that IL-4 stimulates both B cell switching to IgE and production of CD23 (reviewed in Ref. 33). However, only recently has more substantial data in support of this concept been reported. Culture of B cells with CHO cells expressing high levels of membrane CD23 resulted in a decrease in IgE production by those B cells (34). The availability of CD23 transgenic and knockout mice further supports this idea of CD23 as a negative regulator. CD23-deficient mice produced more Ag-specific IgE in response to Ag/alum treatment (32). The decrease in Ig production was limited to the IgE isotype. One line of CD23 transgenic mice with CD23 expression under the control of the Thy1.1 promoter showed a decrease of IgE production when challenged with Ag/alum (35). A second line of transgenics, with CD23 expression under the control of the MHC class I promoter with expression limited to lymphocytes, produced barely detectable levels of IgE in response to Ag and alum and infection with *Nippostrongylus brasiliensis* (36). Adoptive transfer studies using these mice suggest that it is the CD23 on nonlymphoid cells, possibly on follicular dendritic cells, that is important in regulating IgE (37). These data show that membrane CD23 functions as a negative regulator for IgE production.

Several studies have examined the effect of anti-CD23 Abs on IgE binding to CD23 and their influence on the regulation of IgE. In the human, Abs that inhibit IgE binding and, therefore, most likely bind to the lectin domain have been shown to inhibit IgE production by PBMC stimulated with IL-4 alone or in combination with anti-CD40 mAb or hydrocortisone (38). An in vivo study by Flores-Romo et al. (39) examined the effect of a polyclonal Ab to the lectin domain of CD23 on IgE production. Rats immunized with OVA and injected with the anti-lectin domain Ab produced

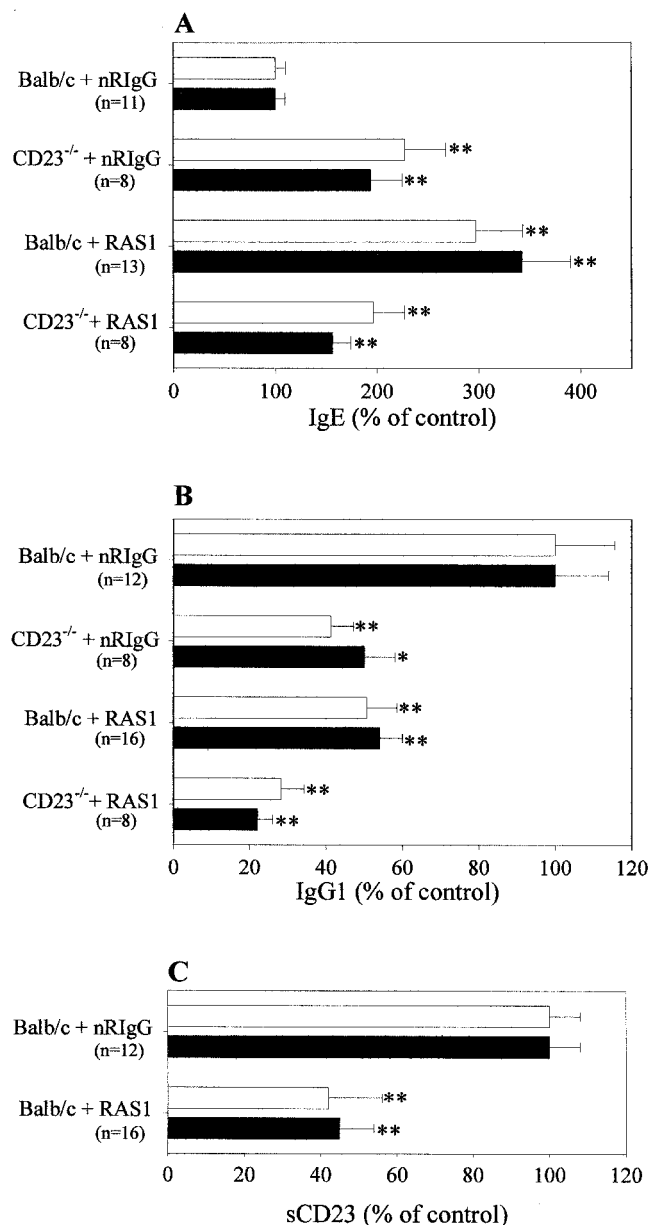


FIGURE 5. In vivo treatment of mice with RAS1 results in augmented IgE production and is mediated by CD23. BALB/c or CD23-deficient mice were injected with 2 mg RAS1 or nRIgG on days -2, 0, and 7 and immunized with KLH-DNP in alum on day 0. Mice were bled on day 14 (□) and day 20 (■). Total IgE, KLH-DNP-specific IgG1, and sCD23 levels were determined by ELISA. Total amounts of IgE, KLH-DNP-specific IgG1, and sCD23 varied between experiments: IgE, 1.04–8.1 $\mu\text{g}/\text{ml}$; IgG1, 73–876 $\mu\text{g}/\text{ml}$; sCD23, 2.61–190 ng/ml . Statistical analysis was performed using Student's *t* test and represents significant differences between the nRIgG-treated BALB/c mice and the other groups. Values of *p* were ≥ 0.2 when CD23 $^{-/-}$ mice (plus or minus RAS1 treatment) were compared with BALB/c mice that received RAS1 (*, $p \leq 0.05$; **, $p \leq 0.01$).

90% less total IgE as well as less Ag-specific IgE. The inhibition observed was also isotype specific in that only IgE was affected. Both intact and Fab anti-lectin were capable of inhibiting IgE production. However, in another study, only the intact anti-CD23 mAb was capable of inhibiting IgE production and not the F(ab')₂, suggesting that the Fc region of the anti-CD23 mAb may be important (40). In contrast to the results obtained with the Abs directed against the lectin domain, an Ab directed against the stalk

portion of human CD23 (EBVCS-1) enhanced IgE production by PBMC stimulated with a combination of either IL-4 plus anti-CD40 mAb or hydrocortisone (38). This Ab did not inhibit IgE binding to CD23 (38), but IgE did inhibit binding of EBVCS-1 to CD23. This same Ab was later shown to enhance cleavage of CD23. The enhancement of proteolysis by EBVCS-1 could be decreased by the addition of IgE (41).

The effects of RAS1 on IgE production were studied using an *in vivo* model. Treatment of mice with RAS1 results in an increased IgE production in response to Ag/alum treatment. RAS1 also greatly reduces sCD23 found in the serum of these same mice suggesting that it is again stabilizing CD23 on the cell surface similar to the phenomenon seen with M12 and B cells incubated with high levels of RAS1. This is particularly interesting, because even though these mice have higher levels of surface CD23, it can only bind IgE with a low affinity. The resultant increases in IgE levels in these mice suggest that a high affinity interaction between CD23 and IgE is necessary for IgE regulation by CD23. These experiments suggest that by using RAS1 to abrogate CD23 oligomerization, therefore, high affinity binding to IgE, these mice have been made to resemble CD23-deficient mice, which produce more IgE than control mice.

Work by Kehry et al. (42) showed that when Ag was complexed with IgE, it was processed by murine B cells 100 times more efficiently than Ag alone. In the human system, targeting of Ag to the B cell through CD23 also results in increased Ag processing and presentation (43). It has also been shown that Ag complexed with anti-CD23 Abs could replace Ag-IgE complexes *in vitro* to target Ag complexes to B cells, enhancing processing and Ag presentation (44). These studies imply a role for CD23 in allergies due to its role in Ag processing and presentation. Work done in CD23-deficient animals showed that the absence of CD23 resulted in no enhancement of Ag-specific IgG when these mice were injected with IgE anti-hapten/hapten-Ag complexes (45). More recently, Heyman et al. (46) published that it is the CD23 on B cells that is responsible for the enhancement of IgE-mediated Ag presentation. Interestingly, mice treated with RAS1 produce less Ag-specific IgG1 than control animals. The levels of KLH-DNP-specific IgG1 are similar to those produced by CD23-deficient mice. Therefore, it seems likely that high affinity binding to IgE is needed not only to regulate IgE production but also for CD23 to play a role in enhancement of Ag presentation of IgE/Ag complexes.

These data support the work presented by Cousin et al. (41) who proposed that the mAb EBVCS1 enhanced cleavage of CD23 by interfering in oligomerization. This manuscript also supports the hypothesis of Cousin et al. that any molecule that could destabilize CD23 trimers would cause an increase in IgE production.

CD23 is being considered as a potential target for treatment of allergic diseases (9). The principle behind the idea is that by increasing CD23 expression on the cell surface, IgE production would be decreased. This work suggests that not only is it important for there to be high levels of surface CD23, but the oligomeric conformation of CD23 must be present to allow for high affinity IgE binding for IgE production to be regulated.

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