Circulating IgG autoanti-IgE antibodies in atopic patients block the binding of IgE to its low affinity receptor (CD23)

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Abstract

Aims—To investigate the ability of circulating IgG autoanti-IgE antibodies from atopic rhinitis patients to block the binding of IgE to its low affinity receptor (FceRII), also termed CD23.

Methods—This involved the use of a well validated flow cytometric method to detect inhibition of FITC labelled IgE binding to human B cells expressing CD23 (RPMI 8866 cell line).

Results—Taking inhibition values greater than 20% as being significant, 15 out of 20 IgG anti-IgE containing sera inhibited the binding of IgE-FITC to the RPMI 8866 cells. The inhibitory effect was recoverable in the IgG fraction of serum, but was not related to the titre of either IgG1 anti-IgE or IgG4 anti-IgE, thus suggesting that it might be related to epitope specificity. No such inhibition was demonstrable with rheumatoid sera containing autoanti-IgG (that is, rheumatoid factor), but lacking autoanti-IgE.

Conclusions—The capacity of anti-IgE to block the binding of IgE to CD23 has important implications, particularly in terms of upregulation of IgE synthesis and the consequent perpetuation of the inflammatory response.

Keywords: Atopy, autoanti-IgE, CD23, IgE.
and Biophysics Group, The Randall Institute, King's College, London, UK. The single IgE domains were expressed in E. coli and refolded; the IgE-Fc fragment (Cα2–Cα4) was expressed in NSO cells and was glycated as described elsewhere.12

MAPPING THE EPITOPE LOCATION OF mAB ANTI-IgE
A range of chimaeric mouse-human IgE antibodies was used for mapping the epitope location of anti-IgE antibody. The chimaeric antibodies were constructed, as described elsewhere,13 by interchanging constant region domains between human and mouse IgE. Wells of Nunc Maxisorp microtitre plates were coated with 100 μl of 1 mg/ml NIP-BSA (bovine serum albumin) in 0.05 M carbonate-bicarbonate buffer pH 9.6 and incubated overnight at 4°C. The plate was washed three times with phosphate buffered saline (PBS)/Tween20 pH 7.2 buffer and 100 μl aliquots of 1:10 dilution of the chimaeric mouse-human IgE anti-NIP antibodies were added to the appropriate wells and the plate incubated for one hour at 37°C. The plate was then washed and 100 μl of mAb anti-human IgE was added to each well and the plate incubated for one hour at 37°C. The plate was washed three times, 100 μl of alkaline phosphatase conjugated goat anti-mouse IgG (1/500) was added to each well, and the enzymatic colour reaction was developed as described elsewhere.37

PURIFICATION OF HUMAN IgE (WT)
IgE was isolated from plasma by euglobulin precipitation and affinity chromatography. The purity of the IgE preparation was assessed by immunoelectrophoresis, and its concentration determined by a Milenia IgE kinetic EIA (DPC Ltd, Gwynedd, UK).

FITC LABELLING OF IgE
One millilitre of the IgE protein (2.5 mg/ml) was incubated for three hours at 37°C with FITC isomer 1 on 10% Celite (Calbiochem, Nottingham, UK) in 0.15 M sodium chloride containing 0.1 M sodium hydrogen carbonate. The samples were passed through a Sephadex G-25 column (Pharmacia, Milton Keynes, UK) to remove unbound FITC. Fractions containing FITC labelled IgE were collected and pooled, and the FITC to protein ratio was determined by measuring optical density at 280 nm and 495 nm.

IgG SUBCLASS ANTI-IgE MEASUREMENT
IgG1 and IgG4 anti-IgE antibodies were measured in serum samples using a previously described enzyme linked immunosassay (ELISA).57 Briefly, the procedure involved incubating a 1/5 dilution of serum on IgE or IgE-Fc coated microtitre plates and detecting bound IgG1 and IgG4 anti-IgE with mouse monoclonal anti-human IgG1 and IgG4, followed by alkaline phosphatase conjugated goat anti-mouse IgG. Results were expressed in OD405 units by taking the mean value of duplicate estimations, corrected for non-specific binding (that is, samples added to wells not coated with IgE).

TOTAL IgE ASSAY
Total IgE levels were measured in serum using a Milenia IgE kinetic EIA (DPC Ltd, Gwynedd, UK).

SERUM SAMPLES
Blood samples were obtained, as detailed elsewhere,6 from atopic rhinitis patients attending the ENT outpatient clinic at University Hospital, Queen’s Medical Centre, Nottingham. Samples which were found to be positive for IgG anti-IgE were used in the IgE blocking experiments (see below). Sera from rheumatoid arthritis patients containing autoantibodies to IgG (that is, rheumatoid factor) were used as controls. Such rheumatoid factor positive sera did not have detectable levels of IgG anti-IgE.

CELL CULTURES
RPMI 8866 (kindly provided by Prof J Gordon, University of Birmingham, UK) and Raji (European Collection of Animal Cell Cultures, Porton Down, Salisbury, UK) were cultured in RPMI 1640 containing 10% heat inactivated fetal calf serum (FCS) (Sigma) and 100 U/ml penicillin + 100 μg/ml streptomycin. Both cell lines were cultured and split 1/3 every 2 d. Binding studies were carried out in PBS containing 1% BSA, 0.1% azide (NaN3), and 1 mM CaCl2 at a cell concentration of 106/ml.

EXPRESSION OF CD23 ON THE RPMI 8866 CELLS
A 100 μl volume of 10 μg/ml FITC labelled IgE was incubated for one hour at 37°C, with 100 μl of either RPMI 8866 (with or without treatment with 2 μg of anti-CD23 mAb) or Raji cells (106 cells/ml) and 100 μl of incubation buffer, and then washed with 2 ml of PBS containing 0.1% azide (to prevent capping). Unlabelled IgE was used to assess autofluorescence. The cells were resuspended and fixed in 0.3 ml Sysmex cellpack containing 0.5% methanal. Cells were collected on a FACScan (Becton Dickinson, Oxford, UK) with a linear fluorescence setting of 660 V. The fluorescence (FL1) profile versus forward scatter (FSC) was used to monitor the cells, the amplification scale was altered according to the level of fluorescence. For each sample 4000 events were collected and then analysed using the flowMATE program (Dako, High Wycombe, UK).
DEMONSTRATION OF THE IgE BINDING SPECIFICITY OF THE RPMI 8866 CELLS

A 50 μl volume of 20 μg/ml (that is, 5.32 x 10^{-8} M) FITC labelled IgE (WT) was mixed with 50 μl of 10^{-6} to 1 x 10^{-8} M of either unlabelled human IgE or recombinant human epsilon constant region domains (Ce2-Ce4, Ce2, Ce3) before incubation for one hour at 37°C, with 100 μl of RPMI 8866 cells (10^6 cells/ml) and 100 μl of PBS containing 0.1% BSA. The cells were subsequently prepared for FACS analysis, as described above. Furthermore, to ascertain the IgE binding specificity of RPMI 8866 cells, blocking experiments were performed whereby 100 μl volume of 10 μg/ml FITC labelled IgE (WT) was incubated for one hour at 37°C, with 1-0 or 0.5 μg of a range of anti-human IgE mAb and four anti-human IgG subclass mAb (controls). Each mixture was then incubated for one hour at 37°C with 100 μl of RPMI 8866 cells (10^6 cells/ml). The cells were subsequently prepared for FACS analysis as described previously.

THE USE OF IgG ANTI-IgE CONTAINING SERA TO BLOCK IgE-FITC UPTAKE BY RPMI 8866 CELLS.

Serum (100 μl) was incubated for 1 h at 37°C with 100 μl of IgE-FITC (5 μg/ml) and the mixture was then incubated for one hour at 37°C with 100 μl of RPMI 8866 cells (10^6 cells/ml), after which the cells were prepared for FACS analysis as described earlier. In subsequent blocking assays IgE-FITC was used at a concentration of 0.5 μg/ml.

PURIFICATION OF AUTOANTI-IgE CONTAINING IgG FRACTIONS

Euglobulin fractions were prepared from sera of rhinitis patients with high titres of IgG anti-IgE by precipitation with 33% saturated solution of ammonium sulphate. The IgG fraction was isolated on protein G-Sepharose and the preparations were tested for anti-IgE activity by ELISA.

INVESTIGATION OF THE EFFECT OF ANTI-IgE mAb AND HUMAN IgG ANTI-IgE CONTAINING SERA ON RPMI 8866 BOUND IgE-FITC

A 100 μl volume of 5 μg/ml FITC labelled IgE was incubated for one hour at 37°C with 100 μl of RPMI 8866 cells (10^6 cells/ml); cells were washed and then treated for 30 minutes at 37°C with 1 μg of mAb anti-IgE or 100 μl of serum. The cells were then prepared for FACS analysis as described earlier.

STATISTICAL ANALYSIS

The Spearman correlation was used to test for the existence of any correlation between IgG subclass anti-IgE titres, or IgE levels, and the ability to inhibit the binding of FITC labelled IgE to the RPMI 8866 cells.

**Results**

IgE BINDING SPECIFICITY OF RPMI 8866 CELLS

A dose-response curve was produced from a plot of the median channel number against the concentration of IgE-FITC per 10^3 RPMI 8866 cells. The observed increase in fluorescence was due to the binding of FITC-IgE to the cells, as no such increase in fluorescence was seen with unlabelled IgE. There was no significant binding of IgE-FITC to the Raji cells at antibody concentrations below 1 μg/ml per 10^3 cells. In addition, the binding of IgE-FITC to the RPMI 8866 cells was shown to be due to uptake by CD23, as this effect was completely blocked by previous treatment of the cells with anti-CD23 mAb (fig 1).

The specificity of the interaction of IgE with the RPMI 8866 cells was further ascertained in a competitive assay using purified human IgE and recombinant epsilon constant region domains. The binding of IgE-FITC to the RPMI 8866 cells was inhibited in a dose-dependent manner by unlabelled human IgE (WT) and by a recombinant fragment spanning...
Anti-IgE blocks the binding of IgE to CD23

### Table 1
The effects of anti-IgE mAb and anti-IgGl-4 mAb (i.e. tested at two concentrations) on IgE-FITC binding to the RPMI 8866 cells. The results show inhibition (−) and enhancement (+) of IgE-FITC binding.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Domain specificity</th>
<th>1-0 μg</th>
<th>0-5 μg</th>
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<tbody>
<tr>
<td>HE-69B</td>
<td>Cc2</td>
<td>+93</td>
<td>+212</td>
</tr>
<tr>
<td>HE-5B</td>
<td>Cc2</td>
<td>+140</td>
<td>-187</td>
</tr>
<tr>
<td>BSW17</td>
<td>Cc2</td>
<td>-93</td>
<td>-90</td>
</tr>
<tr>
<td>HE-35A</td>
<td>Cc3</td>
<td>-94</td>
<td>-91</td>
</tr>
<tr>
<td>E3</td>
<td>Cc4</td>
<td>-54</td>
<td>-10</td>
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<tr>
<td>Le27</td>
<td>Cc4</td>
<td>+164</td>
<td>+117</td>
</tr>
<tr>
<td>HE-50H</td>
<td>Cc4</td>
<td>+33</td>
<td>+73</td>
</tr>
<tr>
<td>HE-56A</td>
<td>Cc4</td>
<td>+74</td>
<td>+122</td>
</tr>
<tr>
<td>E1</td>
<td>Cc4</td>
<td>+74</td>
<td>+69</td>
</tr>
<tr>
<td>NL16</td>
<td>Cc2-Cy3</td>
<td>-10</td>
<td>-8</td>
</tr>
<tr>
<td>GOM1</td>
<td>Cc2-Cy3</td>
<td>-4</td>
<td>-4</td>
</tr>
<tr>
<td>ZG4</td>
<td>Cc2-Cy3</td>
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<td>-3</td>
</tr>
<tr>
<td>GB7B</td>
<td>Cc2-Cy3</td>
<td>-5</td>
<td>-3</td>
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### Table 2
Percentage inhibition of IgE-FITC binding to RPMI 8866 cells with IgG anti-IgE containing rhinitis sera

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgGl anti-IgE (OD_{405})</th>
<th>IgG4 anti-IgE (OD_{405})</th>
<th>IgE (IU ml⁻¹)</th>
<th>Inhibition of IgE-FITC binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>0.690</td>
<td>0.000</td>
<td>84.1</td>
<td>29</td>
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<tr>
<td>PF</td>
<td>0.176</td>
<td>0.053</td>
<td>34.2</td>
<td>10</td>
</tr>
<tr>
<td>JK</td>
<td>0.042</td>
<td>0.359</td>
<td>103.1</td>
<td>20</td>
</tr>
<tr>
<td>SJ</td>
<td>0.080</td>
<td>0.272</td>
<td>20.1</td>
<td>35</td>
</tr>
<tr>
<td>SS</td>
<td>0.028</td>
<td>0.125</td>
<td>395.3</td>
<td>38</td>
</tr>
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<td>SJ</td>
<td>0.191</td>
<td>0.425</td>
<td>53.0</td>
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<tr>
<td>DM</td>
<td>0.120</td>
<td>0.130</td>
<td>27.7</td>
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</tr>
<tr>
<td>CD</td>
<td>0.135</td>
<td>0.354</td>
<td>44.4</td>
<td>48</td>
</tr>
<tr>
<td>KM</td>
<td>0.038</td>
<td>0.152</td>
<td>56.5</td>
<td>38</td>
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<tr>
<td>JC</td>
<td>0.057</td>
<td>0.215</td>
<td>20.1</td>
<td>12</td>
</tr>
<tr>
<td>LY</td>
<td>0.459</td>
<td>0.046</td>
<td>216.1</td>
<td>48</td>
</tr>
<tr>
<td>MC</td>
<td>0.424</td>
<td>0.222</td>
<td>195.8</td>
<td>15</td>
</tr>
<tr>
<td>ZK</td>
<td>0.042</td>
<td>0.239</td>
<td>20.1</td>
<td>12</td>
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<tr>
<td>RZ</td>
<td>1.033</td>
<td>0.114</td>
<td>385.8</td>
<td>26</td>
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<tr>
<td>HG</td>
<td>0.158</td>
<td>0.218</td>
<td>97.1</td>
<td>46</td>
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<tr>
<td>RG</td>
<td>0.166</td>
<td>0.355</td>
<td>45.2</td>
<td>26</td>
</tr>
<tr>
<td>CC</td>
<td>0.108</td>
<td>0.051</td>
<td>125.5</td>
<td>44</td>
</tr>
<tr>
<td>DG</td>
<td>0.229</td>
<td>0.239</td>
<td>401.0</td>
<td>45</td>
</tr>
<tr>
<td>AS</td>
<td>0.225</td>
<td>0.270</td>
<td>41.1</td>
<td>42</td>
</tr>
<tr>
<td>JR</td>
<td>0.286</td>
<td>0.015</td>
<td>56.6</td>
<td>3</td>
</tr>
</tbody>
</table>

### Discussion

#### BLOCKING OF IGGl-FITC BINDING TO RPMI 8866 CELLS WITH SERA CONTAINING IgG ANTI-IgE

IgE-FITC was routinely used at a concentration of 0.5 μg/ml per 10⁵ cells. This concentration was chosen because it falls within the linear part of the binding curve (fig 1), thus ensuring optimal sensitivity of the assay.

#### EFFECT OF ANTI-IgE mAb AND HUMAN IgG ANTI-IgE CONTAINING SERA ON CELL BOUND IGGl-FITC

Addition of mAb anti-IgE to RPMI 8866 cells which had already taken up IgE-FITC resulted in increased fluorescence of the cells. Such increase in detection of cell bound IgE-FITC was shown by all six (HE-56A, HE-5B, HE-50H, HE-69A, HE-35A, and E3) anti-IgG mAb that were tested (range 54–168%; mean 119%). However, the level of cell bound IgE-FITC was not affected by any of the eight IgG anti-IgE containing sera that were tested.

#### MODULATION OF IgE-FITC BINDING TO RPMI 8866 CELLS WITH ANTI-IgG mAb

The monoclonal anti-human IgE antibodies were tested for their reactivity with a range of chimaeric IgE anti-NIP, constructed by interchanging constant region domains between human and mouse IgE molecules. This has shown that the anti-IgG mAb recognise epitopes located within the Cc2 (HE-69B, HE-5B, and BSW17), Cc3 (HE-35A and E3), or Cc4 (E1, Le27, and HE-50H) domains respectively (table 1).

Anti-IgE mAb showed two opposing effects on IgE-FITC binding to the RPMI 8866 cells (table 1): three inhibited (HE-35A, E3, and BSW17) and five enhanced (E1, Le27, HE-50H, HE-69B, and HE-5B) the binding of IgE. These modulatory effects appeared to be related to the IgE domain specificity of the antibodies, with the inhibitory property being exhibited mainly by those recognising epitopes within Cc3. None of the four anti-human IgG subclass mAb affected the binding of IgE-FITC to the RPMI 8866 cells.

#### DISCUSSION

The close proximity between the site on IgE to which CD23 binds (Cc3 domain) and those recognised by circulating IgG anti-IgE...
antibodies (Cc2-Cc3 interface and Cc4 domain) has prompted us to examine the effect of this autoantibody on the binding of IgE to CD23. These interactions were explored by flow cytometry using a human B cell line (RPMI 8866) expressing CD23.

We have initially demonstrated the IgE binding specificity of the B cell line. In addition, we have shown that the binding of IgE to CD23 occurs through the Fc portion of IgE, as evidenced by the competitive inhibition experiment using recombinant human IgE-Fc. As expected, such inhibition was not demonstrable with recombinant Cc2 or Cc3 domains because, although the CD23 binding site is located within the Cc3 domain, the structural integrity of the entire Fc region is apparently required for receptor engagement. The IgE binding specificity of the B cells was further ascertained by showing that a range of mouse monoclonal anti-human IgE, but not anti-human IgG, was able to inhibit this interaction.

Monoclonal anti-human IgE showed two different effects on IgE binding to the B cells: three inhibited and five enhanced the binding of IgE. These modulatory effects appeared to be related to the domain specificity of the anti-IgE, the inhibitory property being shown mainly by antibodies recognizing epitopes within Cc3, which contains the CD23 binding site. Presumably the inhibitory effect was a result of direct or steric blocking of the CD23 binding site on IgE, whereas the enhancing effect was caused by the formation of IgE anti-IgE complexes favouring binding of IgE to CD23. The latter effect may also have been responsible for the enhancement seen when the anti-IgE mAb were added to B cells that had already taken up IgE-FITC.

Having established the validity of our experimental system, we tested 20 serum samples, taken from rhinitis patients with positive titres of IgG1 or IgG4 anti-IgE, or both, for their ability to block the binding of IgE-FITC to the B cells. This revealed significant inhibition (greater than 20%) in 15 out of 20 sera tested. The inhibitory effect was recoverable in the IgG fraction of three out of four serum samples tested. However, the inhibitory effect was not related to the titres of IgG1 anti-IgE, IgG4 anti-IgE, or IgE, thus suggesting that it might be related to epitope specificity of the anti-IgE. None of the 15 rheumatoid sera tested, containing anti-IgG but lacking anti-IgE, was able to cause more than 20% inhibition of IgE binding to the B cells.

Although the IgG anti-IgE containing patients' sera caused modest inhibition (up to 48%) of IgE binding to the B cells, we believe that the experimental conditions used in this study underestimate the IgE blocking effect of anti-IgE in vivo. Inhibition values presented in this paper were obtained using relatively high concentrations of IgE, thereby making the ratio of IgE to anti-IgE much greater than that in the circulation.

The demonstration that human autoanti-IgE blocks the binding of IgE to CD23 in vitro is of pathological significance, since this action will render CD23 available for enzymatic cleavage. This will lead to upregulation of IgE synthesis through both sCD23 release and disruption of the negative IgE regulatory signal delivered by membrane CD23 to the B lymphocyte. The net effect of such excessive IgE production is the perpetuation of an ongoing allergic inflammation. A positive correlation has been reported between circulating levels of IgE and anti-IgE, providing in vivo evidence in support of a positive IgE regulatory role for autoanti-IgE.

We have recently shown that IgG autoanti-IgE blocks the binding of IgE to its high affinity receptor (FceRI), found on mast cells, basophils, and eosinophils. In this context, IgG anti-IgE appears to have an anti-allergic effect, which is in marked contrast to the pro-allergic effect reported here. However, we have previously argued that the existence of epitope specific subpopulations of autoanti-IgE is clearly suggestive of a multiple role in immunological events involving IgE.

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