

IgE-Dependent Activation of T cells by Allergen in Atopic Dermatitis: Pathophysiologic Relevance

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The importance of interactions between allergen and IgE in allergen-mediated activation of T lymphocytes from patients with atopic dermatitis (AD) is unclear. A role for this interaction is implied by past evidence for IgE-facilitated presentation of allergen to T cells, but this phenomenon has only been demonstrated in specific *in vitro* systems biased to maximize the effect. It is therefore not known whether the process is relevant in patients. We now show that the responses to allergen of unmodified peripheral blood mononuclear cells (PBMC) from individual AD patients are significantly greater in the presence of fresh, unheated, IgE-containing autologous serum than the same serum heated under IgE-denaturing conditions or specifically depleted of IgE by immunoprecipitation. In six independent experiments, 59%–67% of the maximal *in vitro* PBMC response to allergen was found to be dependent upon the presence of IgE in autologous serum used at 5% final concentration. These data provide the first evidence that sufficient amounts of allergen-specific IgE and allergen-reactive T cells occur concomitantly in the blood of individual AD patients to allow IgE-enhanced T cell responses to allergen. We conclude that IgE-enhanced T cell responses are pathophysiologically relevant and a therapeutic target in AD.

Key words: antigen presentation/atopic dermatitis/*Dermatophagoides pteronyssinus* allergens/immunoglobulin E/T lymphocytes

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The role of IgE-mediated cellular responses to common environmental allergens in the pathogenesis of atopic dermatitis (AD) has been controversial. Some have suggested that the raised serum IgE levels in patients with AD often appear incidental to the disease process (Holden and Parish, 1998). Although disputed by some (Bos, 2002), others have also introduced the concept of “intrinsic” AD as defining patients, including adults, with clinically typical skin disease but without evidence for raised serum levels of total or allergen-specific IgE (Walker *et al*, 1993). We have, however, found “intrinsic” AD to be rare in the British East Midlands region, at least in adults, in that total serum IgE and IgE specific for *Dermatophagoides pteronyssinus* (*Der p*) allergens were raised in all of 20 randomly selected adults with AD attending our clinics (Shah *et al*, 2002). A further finding in these studies was that *in vitro* peripheral blood mononuclear cell (PBMC) responses to *Der p* allergen were substantially and consistently lower when assays were carried out with heat-treated allogeneic AB serum rather than fresh, unheated autologous serum (Shah *et al*, 2002). This allows the speculation that *Der p*-specific IgE in autologous serum may play a key role in the generation of *in vitro* PBMC responses to this allergen in individual patients.

Abbreviations: AD, atopic dermatitis; cpm, counts per minute; *Der p*, *Dermatophagoides pteronyssinus*; FAP, facilitated allergen presentation; Fc ϵ RI, high-affinity IgE receptor; Fc ϵ RII/CD23, low-affinity IgE receptor; PBMC, peripheral blood mononuclear cells; SI, stimulation index

Indeed, evidence suggests that the focusing of allergens by IgE bound to Fc ϵ receptors on antigen-presenting cells (APC), may enhance the efficacy of allergen processing and presentation to T cells by up to 1000-fold (van der Heijden *et al*, 1993; Maurer *et al*, 1995). The phenomenon of IgE-facilitated allergen presentation (FAP) that may critically lower the threshold of atopic individuals to mount allergen-specific T cell responses, however, has only been demonstrated in *in vitro* systems that were wholly or partially biased to maximize the likelihood of demonstrating this process. Thus, IgE-FAP via high-affinity IgE receptors (Fc ϵ RI) on monocytes has been demonstrated in a system that used monocyte-enriched PBMC as APC, allergen-specific responder T cell clones, hapten-specific chimeric IgE, and purified, haptenized allergen (Maurer *et al*, 1995). A similar approach was used to demonstrate IgE-FAP via Fc ϵ RI on peripheral blood dendritic cells (Maurer *et al*, 1996). IgE-FAP via the low-affinity IgE receptor (Fc ϵ RII/CD23) on B cells has also been demonstrated by using selected Epstein–Barr virus-transformed B cell lines, tetanus toxoid-specific T cell clones, haptenized tetanus toxoid, and hapten-specific chimeric IgE (Pirron *et al*, 1990). In a system that employed more pathophysiologically relevant forms of allergen and IgE, Epstein–Barr virus-transformed B cell lines were used to demonstrate CD23-dependent enhancement of the responses of autologous *Der p* allergen II-specific T cell clones to unpurified protein extract of *Der p* by atopic sera containing *Der p* II-specific IgE (van der Heijden *et al*, 1993; van der Heijden *et al*, 1995). We are, however, unaware of any reports that confirm IgE-FAP in

in vitro systems that have not used enriched APC populations and allergen-specific T cell clones or lines. Therefore, the pathophysiologic relevance of IgE-FAP and the contribution of allergen-specific IgE to allergen-induced T cell responses in individual patients, as well as the potential of this process as a therapeutic target, all remain unclear.

Our previous finding that PBMC responses to *Der p* were greater in assays containing unheated autologous as opposed to heated allogeneic AB serum (Shah *et al*, 2002) suggests but does not confirm that sufficient numbers of allergen-reactive T cells and adequate amounts of corresponding allergen-specific IgE may be concomitantly available in individual atopic subjects to allow a significant level of IgE-FAP. In view of this and the nature of the previous experiments demonstrating IgE-FAP, which do not clarify the pathogenic relevance of this process in individual patients, we have asked whether the enhanced responses of PBMC to *Der p* extract in the presence of unheated autologous as opposed to allogeneic AB serum are due to the presence of IgE. To avoid the introduction of bias, we have used unpurified *Der p* extract in cultures containing autologous serum and density gradient-purified but otherwise unmodified PBMC from volunteers with AD, with the aim of obtaining an approximate quantification of the

contribution of allergen-specific IgE to T cell responses to allergen in individual AD patients.

Results

PBMC responses to *Der p* extract in the presence of unmodified and heated serum Nine adults with AD, whose PBMC showed concentration-related responses to *Der p* extract, donated venous blood samples for these studies. The PBMC of these donors were consistently and significantly more responsive to *Der p* extract in cultures containing fresh, unmodified autologous serum than in cultures containing autologous serum heated at 56°C for 60 min (Fig 1, Table I). These findings supported but did not prove a role for IgE in enhancing responses of atopic PBMC to *Der p*.

In contrast to the effects of unheated autologous atopic serum, unheated serum from non-atopic controls did not enhance PBMC responses to *Der p* above that seen with heated autologous atopic serum (Fig 2). Repeated assays with PBMC from different non-atopic controls failed to show responses to a full range of concentrations of *Der p* extract in the presence of 10% autologous serum ($n=9$, data not shown). Furthermore, responses of non-atopic PBMC remained at background levels (<600 cpm (counts per minute)) when 10% unheated atopic serum containing *Der p*-specific IgE was used instead of the autologous serum ($n=3$, data not shown).

Effects of serum IgE depletion Immunoreactive IgE levels in serum during depletion by immunoprecipitation are shown in Fig 3A. Serum from an AD patient with a high total IgE level was used in the experiment shown. Three

Table I. Maximum SI values obtained from proliferation assays with atopic PBMC, a range of concentrations of *Der p* extract and medium supplemented with 10% fresh autologous serum (either unmodified or heated at 56°C for 60 min)

Patient ^a	Unmodified serum	Heated serum
AD4	23.3 ^b	1.4
AD8	29.2	3.8
AD9	19.1	7.1
AD15	20.7	7.2
AD16	12.8	7.6
AD17	44.3	9.4
AD18	70.7	20.2
AD19	43.0	18.0
AD20	9.6	2.8
Mean \pm SD	30.3 \pm 19.3 ^c	8.6 \pm 6.5 ^c

^aResults of assays with PBMC and autologous serum from nine different AD patients are shown.

^bMaximum SI represents the highest response to *Der p* extract in cpm divided by cpm in the absence of allergen. In six cases, maximum SI values are obtained from data shown in Fig 1.

^c $p=0.002$, paired *t* test.

SI, stimulation index; PBMC, peripheral blood mononuclear cells; *Der p*, *Dermatophagoides pteronyssinus*; AD, atopic dermatitis; cpm, counts per minute.

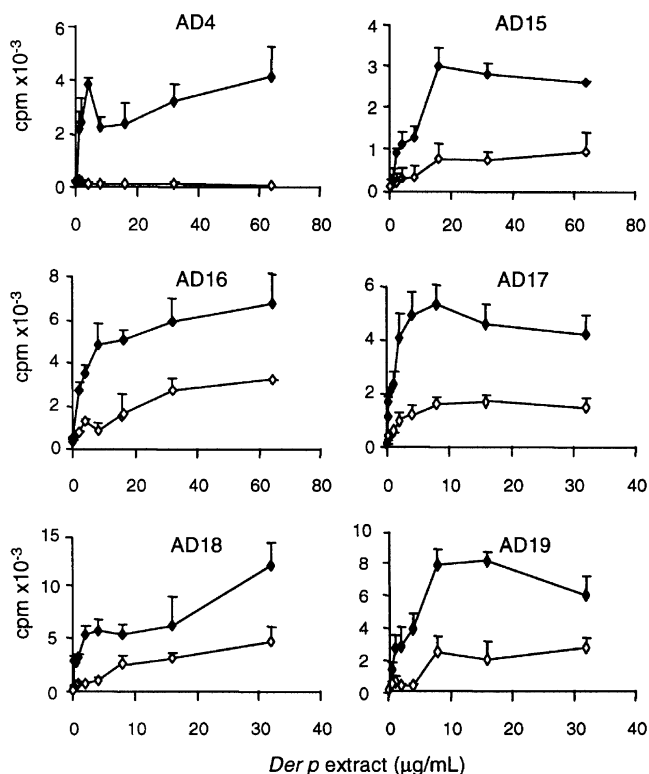


Figure 1
Proliferative responses of peripheral blood mononuclear cells (PBMC) from atopic dermatitis (AD) patients to *Dermatophagoides pteronyssinus* (*Der p*) allergen in the presence of unmodified or heated autologous serum. Responses in the presence of medium containing 10% fresh, unmodified autologous serum (shaded symbols) or 10% fresh, autologous serum that had been heated at 56°C for 60 min (open symbols) are shown. The results of independent experiments with PBMC and autologous sera from six different patients are given. Results are expressed as counts per minute (cpm), each point showing the mean of triplicate assays, with SD values. Where SD values are not shown, they are less than the diameter of the symbol.

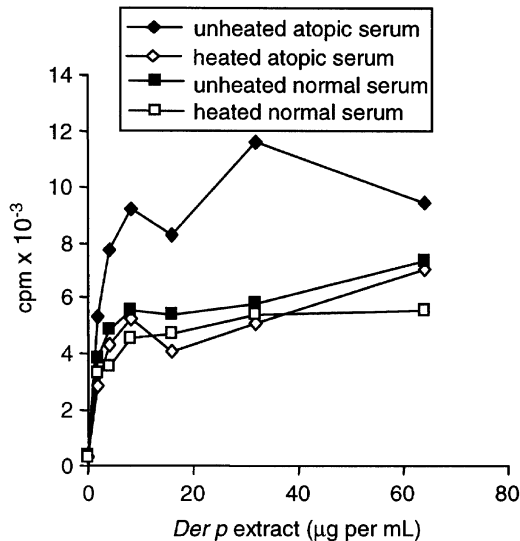
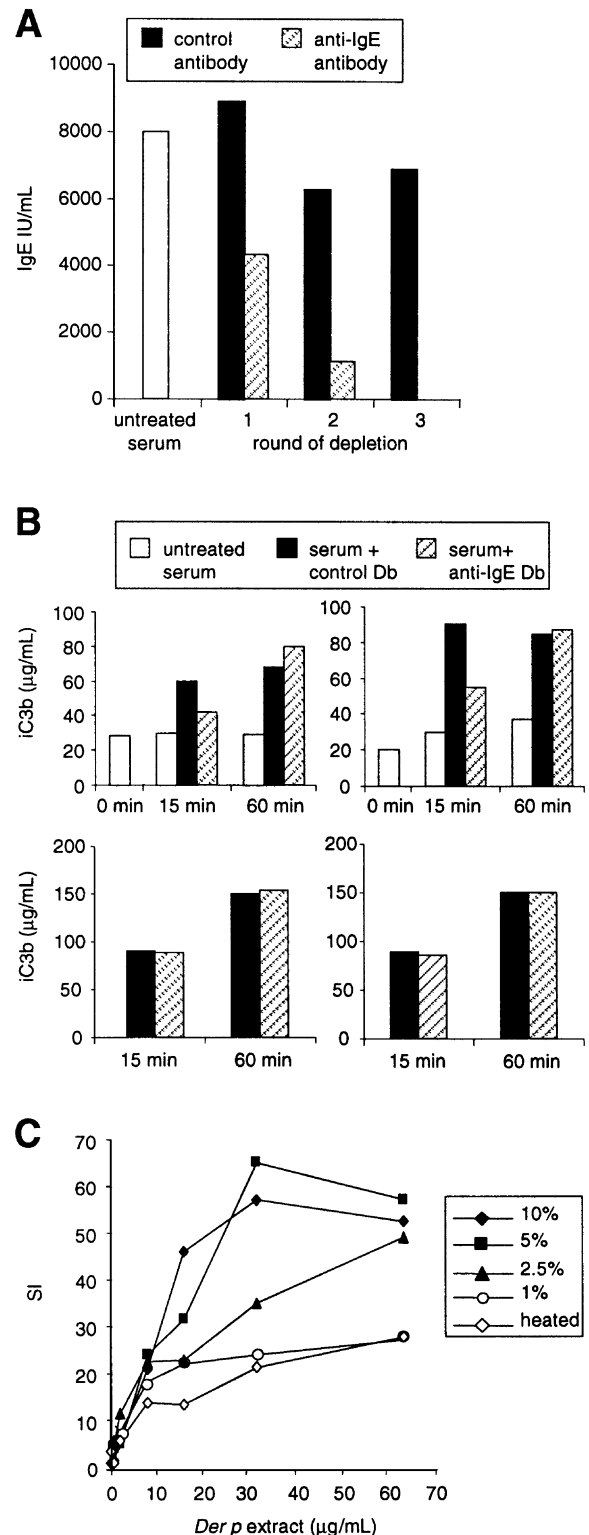


Figure 2
Effects of non-atopic serum on responses of peripheral blood mononuclear cells (PBMC) from an atopic dermatitis (AD) patient to *Dermatophagoides pteronyssinus* (*Der p*) allergen. The serum used was either autologous or from a healthy, non-atopic donor, and was either unmodified or heated at 56°C for 60 min. Symbols representing the different sera used are indicated in the figure. Results are expressed as counts per minute (cpm) and are the mean of triplicate assays. SD values were less than 30% of the means. Similar results were obtained in a second, independent experiment.

rounds of depletion by the described Dynabead method were necessary and sufficient to remove ELISA-detectable IgE from this atopic serum sample. Because of the possibility of complement activation during immunoprecipitation of IgE in unheated serum, and the unpredictable effects of complement products on T cell function (Dempsey *et al*, 1996; Kemper *et al*, 2003), ELISA measurements of the complement activation product, iC3b, were made during incubation of atopic serum with anti-IgE- and control antibody-coated Dynabeads. As shown in Fig 3B, immunoreactive iC3b levels were detectable in all samples

Figure 3
IgE depletion and complement activation in atopic serum, and dependence of peripheral blood mononuclear cell (PBMC) responses on autologous serum concentration. (A) Immunoreactive IgE levels in atopic serum before (*unshaded column*) and after depletion by the Dynabead immunoprecipitation method. IgE levels following each of three rounds of treatment with Dynabeads coupled to control antibody (*shaded columns*) or anti-IgE antibody (*hatched columns*), are shown. IgE levels were determined by ELISA after dilution of serum 2–100-fold with the sample diluent provided, and the mean results of duplicate assays are shown. The limit of detection of the ELISA was about 2 IU per mL. Results are representative of three independent experiments. (B) Immunoreactive iC3b levels in untreated atopic sera (*unshaded columns*) or sera treated with Dynabeads (Db) coupled to isotype control monoclonal antibody (*shaded columns*) or anti-IgE monoclonal antibody (*hatched columns*). Serum samples were freshly prepared on ice and antibody-coupled Dynabeads added at time zero. The samples were then kept on ice for 15 and 60 min prior to ELISA, which was carried out as described under Materials and Methods. Data represent the means of duplicate ELISA measurements, and are from four independent experiments. (C) Responses of PBMC from an AD patient to *Der p* allergen in medium supplemented with four different concentrations of fresh, unmodified autologous serum (1%, 2.5%, 5%, and 10%). The responses of PBMC to *Der p* allergen in the presence of 10% autologous serum heated at 56°C for 60 min are shown for comparison. Each point shows the mean of triplicate assays. SD values are less than 30% of the means. Results are representative of four independent experiments.

tested, including serum kept on ice in the absence of antibody-Dynabead conjugates, indicating a degree of spontaneous complement activation as has been reported previously in normal human serum (Manderson *et al*, 2001). Immunoreactive iC3b levels in untreated serum fell within the range (5–78 µg per mL) found following the analysis of 100 normal serum samples (information from Quidel Corporation, San Diego, California). Increased levels of iC3b were detectable in serum samples incubated with antibody-



Dynabead conjugates, but there was little or no difference in the levels in sera treated with anti-IgE or control antibody for 15 or 60 min (Fig 3B). Complement activation could therefore not explain the results obtained in subsequent experiments, in which the responses of atopic PBMC to *Der p* in the presence of IgE-depleted or control autologous serum were determined. Prior to the IgE-depletion experiments, the concentration of fresh, unmodified autologous serum required to support maximum responses of the atopic PBMC to *Der p* was determined. Here, 10% and 5% (vol/vol) final concentrations of autologous serum supported similar responses of the PBMC to *Der p*, whereas 2.5% and 1% serum gave lower responses (Fig 3C). For cost effectiveness, IgE-depleted or control sera at 5% final concentration were used in cultures with autologous PBMC from six different AD patients. Responses to *Der p* extract were consistently and significantly lower in cultures containing IgE-depleted versus control serum (Fig 4, Table II).

Discussion

Der p extract was chosen for use in these studies because of our previous work showing correlation between *in vitro*

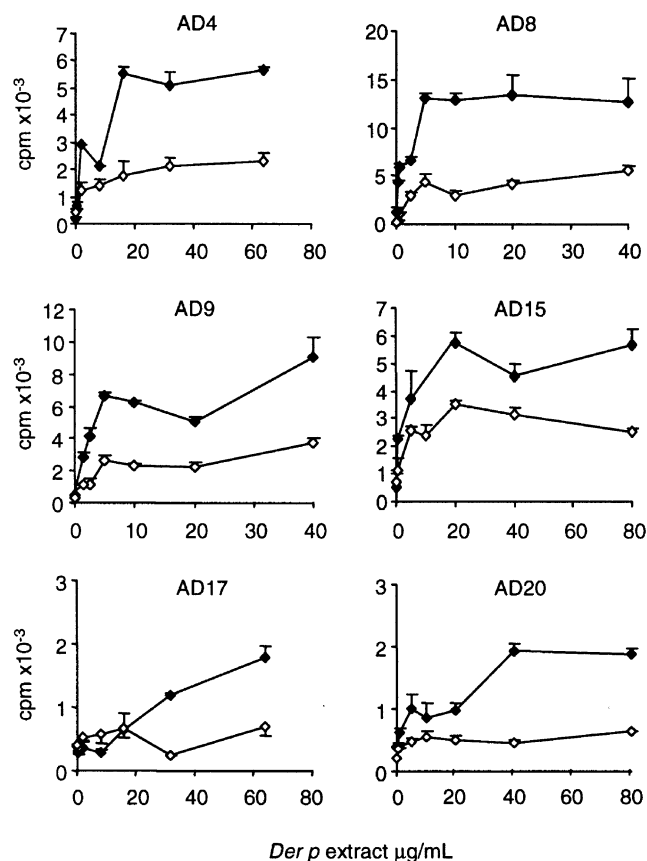


Figure 4
Proliferative responses of peripheral blood mononuclear cells (PBMC) from atopic dermatitis (AD) patients to *Dermatophagoides pteronyssinus* (*Der p*) allergen in the presence of IgE-depleted serum. Medium was supplemented either with 5% control autologous serum (shaded symbols) or 5% autologous serum depleted of IgE by immunoprecipitation (open symbols). Results are expressed as counts per minute (cpm) and are from independent experiments with PBMC and sera from six different patients. Each point shows the mean of triplicate assays with SD values.

Table II. Maximum SI values obtained from proliferation assays with atopic PBMC, a range of concentrations of *Der p* extract and medium supplemented with 5% fresh autologous serum (either IgE-depleted or control)

Patient	Control serum	IgE-depleted serum	Percentage reduction
AD4	28.1 ^a	11.5	59.1
AD8	13.4	5.6	58.2
AD9	53.3	24.8	53.5
AD15	12.0	5.4	55.0
AD17	4.7	1.7	63.8
AD20	12.0	4.0	66.7
Mean \pm SD	20.6 \pm 17.8 ^b	8.8 \pm 8.5 ^b	59.4

^aMaximum SI represents the highest response to *Der p* extract in cpm divided by cpm in the absence of allergen. Values are derived from the six assays shown in Fig 4.

^b $p = 0.027$, paired *t* test.

SI, stimulation index; PBMC, peripheral blood mononuclear cells; *Der p*, *Dermatophagoides pteronyssinus*; AD, atopic dermatitis; cpm, counts per minute.

PBMC responses to this allergen in the presence of 10% fresh, autologous serum and the ability to induce dermatitis by repeated topical challenge of the elbow flexures of patients with AD (Shah *et al*, 2002). This previous work showed that the major population of cells amongst PBMC from AD patients that undergo proliferation in response to *Der p* extract are CD4⁺ T cells (Shah *et al*, 2002). In this work, we have also found that the PBMC of non-atopic donors do not respond to *Der p* extract in the presence of 10% serum, either autologous or from AD patients, a finding that also indicates that the *Der p* extract was not contaminated by endotoxin or other non-specific mitogens. On this basis, we have concluded that the *Der p* extract is a clinically relevant allergen preparation with which to explore the pathophysiologic relevance of IgE in the responses of PBMC from AD patients. The assays were not biased to maximize IgE-enhanced T cell responses, unlike previous work with enriched APC populations, allergen-specific T cell clones and chimeric IgE molecules (Pirron *et al*, 1990; van der Heijden *et al*, 1993; Maurer *et al*, 1995, 1996; van der Heijden *et al*, 1995; van Neerven *et al*, 1999).

Our finding that the responses of PBMC from AD patients to *Der p* extract were strikingly greater in the presence of unmodified autologous serum than autologous serum containing IgE irreversibly denatured by heating, provided preliminary evidence for pathophysiologically relevant IgE-enhanced responses, but this was not conclusive as the heating method is likely to denature heat-labile molecules other than IgE. Subsequently, the use of sera specifically depleted of IgE by immunoprecipitation was found consistently to be associated with significantly lower responses of autologous atopic PBMC to *Der p* allergen than control autologous sera. Before concluding that the diminished responses to *Der p* allergen in assays with IgE-depleted serum were specifically due to lack of IgE, it was necessary to exclude effects of complement activation by IgE immune complexes during the depletion process. The earlier work of others indicates that fragments of the third component of

complement inhibit mitogen- and antigen-induced human lymphocyte blastogenesis (Ballas *et al*, 1983) and that the C3 fragment C3b suppresses monocyte-dependent T cell proliferation (Däubener *et al*, 1987). More recent research has also indicated a direct or indirect inhibitory effect of complement activation on T cell responses (Kemper *et al*, 2003; Sohn *et al*, 2003). In these experiments, however, there was no evidence for greater complement activation in IgE-containing serum incubated with anti-IgE-coated Dynabeads than in serum cultured with beads coated with control antibody. Consequently, the reduced atopic PBMC responses found in cultures containing autologous serum depleted of IgE by antibody treatment cannot be explained by the production of complement products that inhibit T cells.

Attempts were made to confirm the IgE-dependence of atopic PBMC responses to *Der p* extract by using Fc ϵ receptor blocking antibodies in assays containing fresh autologous serum. Isotype control murine monoclonal antibody, however, caused consistent inhibitory effects that were reproduced with a second isotype control antibody (data not shown). This non-specific inhibitory phenomenon was not investigated further, but led to abandonment of the use of blocking antibodies in the present work.

The use of IgE-depleted serum in the described PBMC proliferation assays shows that sufficient amounts of *Der p*-specific IgE and *Der p*-reactive T cells occur concomitantly in the blood of individual adult AD patients to allow enhancement of T cell responses to allergen by IgE-dependent mechanisms, probably via the process of IgE-FAP. As shown in Table II, 59%–67% of the maximal *in vitro* PBMC response to allergen was found to be dependent upon the presence of IgE in autologous serum used at 5% final concentration. We therefore conclude that IgE-dependent enhancement of T cell responses to allergen is a pathophysiologically relevant process in AD. The findings also justify further exploration of agents that abrogate IgE-FAP as a therapeutic measure in AD. Such agents are attractive as they would not cause indiscriminate immunosuppression.

Materials and Methods

Blood donors Following ethical committee approval, repeated heparinized venous blood samples were obtained from nine informed, consenting adults with AD. The patients (six males and three females aged 21–61 y), who satisfied standard diagnostic criteria for AD (Hanifin and Rajka, 1980) and had not received systemic immunosuppressant drugs or ultraviolet phototherapy for at least 3 mo, were recruited from the dermatology clinic of the Leicester Royal Infirmary. As is the case in the large majority of adults with AD attending this clinic (Shah *et al*, 2002), the patients recruited for the present study had raised serum levels of total IgE (691–40,960 U per mL, UniCAP 100 assay, Pharmacia Diagnostics, Milton Keynes, UK) and *Der p*-specific IgE (40–3064 U per mL, ImmunoCAP assay, Pharmacia Diagnostics). All had previously shown *in vitro* PBMC responses to *Der p* extract. Venous blood samples were also obtained from healthy adults donors with no personal or family history of atopic disorders.

Preparation of PBMC and sera Heparinized venous blood was diluted 1:1 in Earle's balanced salts solution (Life Technologies, Paisley, UK) and PBMC recovered by standard density-gradient centrifugation over Lymphoprep (Life Technologies). Interface cells were pelleted, washed in Earle's balanced salts solution and

resuspended in RPMI-1640 medium containing up to 10% fresh autologous serum. Sera were prepared by allowing blood to clot in glass tubes, aspiration and centrifugation to remove residual cells. In some experiments, the culture medium contained serum that was either unmodified or heated at 56°C for 60 min, conditions that irreversibly denature IgE amongst other molecules (Geha *et al*, 1985; Ishizaka *et al*, 1986), but not IgG (Shakib *et al*, 1992). Atopic sera specifically depleted of IgE were prepared by immunoprecipitation using protein G Dynabeads (Dynal, Bromborough, UK). Briefly, 100–500 μ L aliquots of Dynabeads from the supplied suspension were washed twice in ice-cold 0.1 M sodium phosphate buffer (pH 5.0) then reacted with an excess (100–500 μ g) of solid-phase-absorbed polyclonal rabbit anti-human IgE (product code A0094, DakoCytomation, Ely, UK) or 100–500 μ g control solid-phase-absorbed antibody from non-immunized rabbit serum (product code X0936, DakoCytomation) for 40 min at room temperature. The coated beads were washed twice in ice-cold 0.1 M sodium phosphate buffer (pH 5.0) and once in RPMI-1640 that was then removed and the isolated beads mixed with 100–500 μ L fresh atopic serum at 4°C for 60 min with continual rotation. Serum was recovered and subjected to two further rounds of treatment with fresh aliquots of antibody-coated Dynabeads. In preliminary IgE depletions, the serum level of total IgE was measured by ELISA (Autogen Bioclear, Calne, UK) after each round of treatment with antibody-coated Dynabeads. Evidence for complement activation during IgE depletion was also determined by incubating atopic serum with antibody-coated Dynabeads for 15 and 60 min at 4°C. After these time periods, sera were diluted 10-fold with ice-cold 0.1 M EDTA prior to a further 20-fold dilution with sample diluent and assay for the complement activation product iC3b by Quidel ELISA (Technoclone, Dorking, UK) according to the manufacturer's instructions.

PBMC proliferation assays Culture medium consisted of RPMI-1640 with penicillin and streptomycin (1% vol/vol of stock solutions containing 10 kU per mL and 10 mg per mL of the respective antibiotics). PBMC (5×10^5 per mL) were cultured in this medium, to which had been added up to 10% autologous serum (unmodified, heated or IgE-depleted) and *Der p* extract, for 5 d in microtiter plate wells. During the last 6 h each well was pulsed with 1 μ Ci tritiated thymidine, cells were harvested and incorporated radioactivity determined and expressed as cpm or alternatively as stimulation indices (SI, cpm in the presence of allergen divided by background cpm). In certain experiments, PBMC from AD patients were mixed with serum from non-atopic controls, and PBMC from non-atopic controls incubated with atopic serum. *Der p* extract used in the PBMC proliferation assays was prepared by dialysis of Soluprick SQ 10 Hep skin prick test solution (Alk-Abelló, Hungerford, UK) against RPMI-1640 containing penicillin and streptomycin. The protein concentration was determined by the Lowry method (Lowry *et al*, 1951) and aliquots stored at -80°C .

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