

Analysis of cytokine signaling in patients with extrinsic asthma and hyperimmunoglobulin E

Rachel L. Miller, MD,^a Thomas M. Eppinger, MD,^b David McConnell, MD,^a Charlotte Cunningham-Rundles, MD, PhD,^c and Paul Rothman, MD^d New York, NY

Background: Recent data suggest that the regulation of class switching to IgE by cytokines is mediated by STAT transcription factors. The induction of IgE by IL-4 and IL-13 occurs through the activation of the intracellular signal-transducing protein Stat6, whereas the inhibition of IgE class switching by interferon- γ (IFN- γ) occurs through the activation of Stat1.

Objective: We hypothesized that in extrinsic asthma or in cases of markedly elevated IgE (ie, hyperimmunoglobulin E [HIE]) increased levels of IgE may be associated with alterations in the cytokine levels or the activation of Stat6.

Methods: PBMCs and sera from 8 patients with extrinsic asthma (mean IgE, 285 ± 100 IU/mL), 3 patients with HIE (mean IgE, 7050 ± 1122 IU/mL), and 14 nonatopic control subjects (mean IgE, 112 ± 28 IU/mL) were analyzed.

Results: The mean IL-4 level detected by ELISA was much greater in patients with HIE than control subjects (88.6 ± 11.5 pg/mL vs 11.5 ± 7.1 pg/mL, $P = .005$), and increased IL-4 levels among patients with both asthma and HIE correlated with the increased IgE levels. In contrast, IL-13 levels were not elevated. Levels of Stat6 protein present in PBMCs did not differ in the patients and control subjects. Examination of Stat6 DNA-binding activity demonstrated no activation of IL-4 signaling in patients with either HIE or acute asthma. Interestingly, evidence for the presence of B cells that have already switched to IgE was seen in PBMCs of several patients with asthma or HIE.

Conclusion: These results indicate that (1) IgE production in asthma and HIE usually is associated with elevated levels of IL-4, but not IL-13, in the peripheral blood; (2) the increased sera IL-4 levels in asthma and HIE are not sufficient to induce Stat6 activation in PBMCs; and (3) evidence of switch recombination to ϵ may be detected in isolated cases of elevated IgE. This implies that high levels of IgE in these patients either results from B cells that have already undergone class switching, from Ig class switching that is localized to target tissues, or both. (J Allergy Clin Immunol 1998;102:503-11.)

Key words: IL-4, IL-13, hyperimmunoglobulin E, extrinsic asthma, Stat6, switch recombination

Abbreviations used

EMSA:	Electrophoretic mobility shift assay
GAS:	γ -Activating site
HIE:	Hyperimmunoglobulin E
HIES:	Hyperimmunoglobulin E syndrome
IRF-1:	Interferon response factor-1
Jak:	Janus kinase
NS:	Nonsignificant

The T_{H2} cytokine IL-4 has been shown to be a key mediator in Ig class switching to IgE by B cells.^{1,2} The requirement of IL-4 for switching to IgE in mice has also been confirmed in vivo. For example, IgE levels in mice homozygous for a mutation in the IL-4 gene are undetectable or strongly reduced, even after infection with the nematode *Nippostrongylus brasiliensis*.^{3,4} More recently, a second cytokine secreted by human T_{H2} cells, IL-13, has been shown to share biologic function with IL-4, including induction of IgE synthesis.⁵⁻⁷ The effects of these T_{H2} cytokines can be inhibited by the T_{H1} cytokine IFN- γ , which has been shown to nearly abrogate IL-4-mediated induction of IgE by B cells.^{1,2} The actions of these cytokines appear to result from their modulation of germline- ϵ transcription, a requisite step before class switching to IgE. These observations have prompted a model in which T_{H1} and T_{H2} cells mediate class switching to IgE through cytokines that antagonistically regulate transcriptional activity at the germline- ϵ promoter.^{8,9}

The regulation of transcription by the cytokines IL-4, IL-13, and IFN- γ occurs through the intracellular signaling pathway termed JAK-STAT pathway.¹⁰⁻¹⁴ In hematopoietic cells, IL-4 binds to a cell surface receptor on resting IgM⁺ B cells that consists of a heterodimer of the ligand-specific α -chain (IL-4R α) and the common γ -chain (γ C). Dimerization of the receptor leads to the activation, likely by transphosphorylation, of Janus kinase (Jak) 1, which is physically associated with IL-4R α , and Jak3, which is associated with the γ C chain.¹⁵ The activation of these Jaks leads to the phosphorylation of several tyrosine residues contained within the cytoplasmic tail of IL-4R α , which in turn serve as docking sites for the binding of the intracellular transducing protein Stat6 by means of interactions between one of several phosphotyrosyl motifs of the IL-4R α and src homology domains of Stat6. Once recruited to the receptor complex, Stat6 becomes tyrosine phosphorylated, which allows it to form homodimers that translocate to the nucle-

From ^athe Department of Medicine and ^dthe Department of Medicine and Microbiology, College of Physicians and Surgeons, Columbia University, New York; and ^bthe Department of Medicine and ^cDepartment of Pediatrics and Biochemistry, Mt Sinai Hospital, New York.

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Reprint requests: Rachel L. Miller, MD, College of Physicians and Surgeons, Columbia University, 630 W 168th St, New York, NY 10032.

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us and bind to IL-4 response elements that contain the sequence TTCN(4)GAA present in the promoters of IL-4-inducible genes, such as CD23 and IgE.^{8,11,16-18} Because the IL-4R α chain is a component of the IL-13 receptor,¹⁹⁻²⁰ the downstream signaling initiated by IL-13, including the activation of Stat6, overlaps with the signaling activated by IL-4.

Proof of the importance of Stat6 to cytokine-mediated signal transduction was provided from recently generated Stat6 knockout mice. These mice, which lacked expression of Stat6, are unable to initiate T_{H2} proliferative responses, increase CD23 and MHC class II expression on B cells in response to IL-4, or elicit a heightened IgE response after helminth infection or anti-IgD.²¹⁻²³ Stat6 knockout mice also are unable to induce MHC class II expression on peritoneal macrophages or decrease the nitric oxide production in response to IL-13.²⁴

The production of high levels of IgE in response to antigen requires the occurrence of several events. The first key step appears to be the stimulation of T_H cells capable of producing type-2 cytokines. In the absence of type-1 cytokines, type-2 cytokines induce germline- ϵ transcription in IgM+ B cells.^{9,16,17,25} Both IL-4 and IL-13 can induce these transcripts in human B cells.⁵ Another key step, the deletional recombination event that is critical to Ig class switching, requires both germline- ϵ transcription and a B-cell activation signal after contact between the B-cell surface molecule CD40 and its ligand CD40L, which is expressed by activated T_H cells.^{8,17,25-27} Subsequently, deletional recombination occurs between regions of repetitive DNA (switch [S] regions) present upstream from each heavy-chain locus, juxtaposing the fully assembled variable region (composed of rearranged VDJ gene segments) directly upstream of different heavy-chain genes that encode for a particular Ig isotype. The switched B cell then differentiates further into an antibody-secreting cell. For example, in the case of Ig class switching from IgM to IgE, the C μ locus and downstream genes are deleted so that the C ϵ gene becomes juxtaposed directly 3' to the VDJ region, and VDJ-C ϵ transcripts are encoded.⁸

The identification of signaling molecules essential for specific class switching to IgE may enable further definition of the mechanisms underlying human disease states characterized by high levels of IgE. Hyperimmunoglobulin E syndrome (HIES) is a rare congenital immunodeficiency characterized by markedly elevated serum levels of IgE, characteristic facial features, chronic dermatitis, and lifelong recurrent severe infections.^{28,29} Variants of this disease (ie, hyperimmunoglobulin E [HIE]) also have been described and are characterized by markedly elevated IgE in the absence of severe infections. Extrinsic asthma is a far more common disease characterized by increased serum levels of IgE. Our aim was to study the role of cytokine-induced intracellular signaling critical to IgE regulation in these 2 disorders, one characterized by symptoms localized to the airways and the other by extremely elevated levels of IgE. We hypothesized that some patients with acute exacerbations of extrinsic asthma

or HIE may demonstrate measurable systemic alterations in the molecular regulation of IgE production. More specifically, we hypothesized that some subjects with extrinsic asthma or HIE may demonstrate increased systemic expression of T_{H2} cytokines in association with the activation of Stat6.

METHODS

Patient selection

Eight adult subjects with extrinsic asthma were recruited on presentation to the Presbyterian Hospital Emergency Department complaining of acute exacerbation of their asthma. All subjects with asthma met the clinical definition for asthma proposed by the American Thoracic Society.³⁰ Exclusion criteria included smoking, parenteral or oral corticosteroids taken within 4 weeks of entry into the study, and asthma or bronchospasm due to nonallergic causes. Three adult subjects with HIE were recruited, 2 from the Mt Sinai Hospital Immunology Clinic (New York) and 1 from a private referral. All 3 of these subjects were given diagnoses of variants of HIES characterized by markedly elevated IgE levels in the absence of severe lifelong systemic infections and the characteristic facial features. Nonatopic adults first seen at the Presbyterian Hospital Emergency Department or Clinic for other medical reasons served as control subjects. Institutional review board approval had been obtained, and all of their guidelines were followed.

Peripheral blood was drawn from all patients and nonatopic control subjects. Serum was separated by centrifugation immediately and stored at -70° for subsequent ELISA and RIA. PBMCs were isolated by Ficoll-Hypaque (Sigma Chemical Co, St. Louis, Mo) gradient as previously described.³¹

Cell culture and in vitro cytokine stimulation

Ramos 266 (human B cells) cells used as positive controls for several assays were grown in Iscove's modified Dulbecco's medium supplemented with 10% FCS (Sigma Chemical Co, St. Louis, Mo), 100 U/mL penicillin, and 100 μ g/mL streptomycin and incubated at 37° C and 5% CO₂. Positive control Ramos cells or PBMCs were stimulated with human rIL-4 (gift from Dr Satwant Narula, Schering-Plough Corp., Kenilworth, NJ) for 15 minutes at a final concentration of 100 U/mL before generation of RNA or whole cell extracts. U266 (IgE-secreting myeloma) cells were grown in RPMI-1640 medium supplemented with 15% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin and incubated as described above before extraction of DNA.

Generation of whole cell extracts

Whole cell extracts were prepared by using a detergent-free buffer as previously described.³² Briefly, pelleted cells were incubated in RSB lysis buffer (5 mmol/L Tris [pH 7.4], 5 mmol/L NaCl, 3 mmol/L MgCl₂, 100 μ mol/L Na₃VO₄, 1 mmol/L dithiothreitol, and 0.2 mmol/L phenylmethylsulfonyl fluoride) and rapidly resuspended in 30 mL of lysis buffer (Buffer C; 20% glycerol, 20 mmol/L Hepes [pH 7.9], 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 100 μ mol/L Na₃VO₄, 3 μ mol/L aprotinin, 1 μ mol/L pepstatin, and 1 μ mol/L leupeptin). The supernatant fraction (containing DNA-binding proteins) was removed by centrifugation and stored at -70° C.

Cytokine ELISAs

Human IL-4, IL-13, and IFN- γ sera levels were measured by using a commercially available ELISA kit (Immunotech, Inc., Westbrook, Me) according to the procedure indicated by the man-

ufacturer. Performance characteristics included a sensitivity of 5 pg/mL and a standard range of 0 to 1000 pg/mL of sera for IL-4, a sensitivity of 1.5 pg/mL and a standard range of 0 to 500 pg/mL for IL-13, and a sensitivity of 4 pg/mL and a standard range of 0 to 1250 pg/mL for IFN- γ . All specimens were analyzed in duplicate.

Western blot analysis

Five micrograms of whole cell extracts from subjects were resuspended in 1.3 $\times$ SDS loading buffer (20% glycerol, 2% SDS, 0.025 mg/mL bromophenol blue, 0.125 mol/L Tris [pH 6.8], and 0.5% β -mercaptoethanol) and analyzed by SDS-PAGE (7% gel) as previously described.³³ Nitrocellulose membranes were blotted with an antibody to Stat6 (M-20; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) at 1:1000 dilution, analyzed after incubation with horseradish peroxidase-linked rabbit Ig secondary antibody (Amersham Corp., Arlington Heights, Ill), and detected with enhanced chemiluminescence (Amersham International plc, Buckinghamshire, England). The blots were then stripped and reprobed with an antibody to β -actin (Sigma Immunochemicals, St. Louis, Mo) at 1:1000 dilution.

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed as previously described.^{11,34} Briefly, DNA binding was done in 40 mmol/L KCl, 1 mmol/L MgCl₂, 0.1 mmol/L EGTA, 20 mmol/L HEPES (pH 7.9), 4% Ficoll, 0.5 mmol/L dithiothreitol, 1.2 mg/mL BSA, 200 μ g/mL poly(dI-dC:dI-dC), 5 to 10 μ g of whole cell extract, and 1 ng of probe. The P³²-labeled probe was to interferon response factor-1 (IRF-1) γ -activating site (GAS) element with the following sequence: 5' gatcGATTCCCCGAAAT 3'. We have previously shown that several activated Stat proteins bind the GAS element.³⁴ The complexes were resolved on 0.22 $\times$ Tris-buffered EDTA/4.5% acrylamide (29/1) gels.

DNA extraction, PCR, and Southern blot analysis

Total DNA was extracted from the whole cell extract pellet after incubation at 55 $^{\circ}$ C for 12 to 24 hours in lysis buffer (50 mmol/L Tris HCl [pH 6.8], 5 mmol/L EDTA, 0.2% SDS, and 200 mmol/L NaCl) and 0.2 mg of proteinase K. Samples were then mixed with equal volume phenol and SEVAG (chloroform:isoamyl alcohol 24:1). The supernatant was precipitated in 100% ethanol and dissolved in TE buffer. Switch junction fragments (S μ /S ϵ) were amplified from the DNA by means of nested PCR. The first round of PCR was performed with primers S6 (5'-CTGCA-GACTCAGAAGGGAGGGGA TGCTCCG-3') and S4 (3'-ACT-GATCCAAGA CAGGAGTGTGGCGGATGT-5') on 200 ng DNA, and the second round was performed on a 5- μ L aliquot from the first round of PCR with primers S7 (5'-GAGGGTG-GTAATGATTGGTAATGCTTTGGA-3') and S9 (3'-GAACCT-GAGG TCCGGTCCCCGCTTCCCGG-5') as previously described.^{35,36} The PCR products were Southern blotted and incubated with a P³²-labeled probe derived from a 2.3 kb BAMHI fragment containing S ϵ .

RIA

Total human IgE was measured by a solid-phase immunoradiometric assay by using a commercial kit (Coat-A-Count Total IgE IRMA; Diagnostics Products, Corp, Los Angeles, Calif). The assay has a sensitivity of 0.5 IU/mL. All specimens were analyzed in duplicate.

Data analysis

Comparisons between mean sera levels of individual cytokines or IgE were made with Student's *t* test for means assuming equal

variance. Regression analysis was performed to determine the degree of correlation between cytokine and IgE levels. Results are displayed as $\pm$ SE. A *P* value of less than .05 was considered statistically significant.

RESULTS

IgE determinations

To study the regulation of STATs in human patients with high IgE levels, we recruited patients with HIE and patients with extrinsic asthma. The 8 patients recruited with extrinsic asthma had mean IgE levels of 285 $\pm$ 100 IU/mL (range, 99 to 972 IU/mL). Three others initially recruited were excluded from further study because of IgE levels less than 99 IU/mL. The 3 patients referred for evaluation of HIE had mean IgE levels of 7050 $\pm$ 1122 IU/mL (range, 5828 to 9268 IU/mL). The 14 nonatopic control subjects had mean IgE levels of 112 $\pm$ 28 IU/mL (range, 5 to 364 IU/mL). These results confirm that our selection criteria provided subjects with abnormally elevated IgE levels that were increased over the levels of nonatopic control subjects.

Cytokine analysis

Most allergen-specific CD4⁺ T-cell clones derived from atopic patients' peripheral blood lymphocytes secrete elevated levels of IL-4 and IL-5.^{37,28} Spontaneous levels of IL-4 and IL-5 also have been shown to be increased in the sera of subjects with allergic asthma.^{39,40} In comparison, the number of CD4⁺ T-cell clones capable of producing IL-4 do not differ among patients with HIES, patients with atopic dermatitis, and healthy control subjects in one report,⁴¹ nor does the extent of IL-4 production after in vitro stimulation of PBMCs with mitogens differ.^{41,42} To characterize more precisely and to compare spontaneous sera IL-4 levels in extrinsic asthma, which is associated with primarily pulmonary symptoms and moderately elevated IgE levels, and in HIE, a variant of a systemic disorder that is associated with extremely elevated IgE levels but not necessarily target organ-related symptoms or severe infections, we measured sera IL-4 levels in all patients and nonatopic control subjects. Sera IL-4 levels are much greater in patients with HIE compared with control subjects (88.6 $\pm$ 50.4 pg/mL vs 11.5 $\pm$ 7.1 pg/mL, *P* = .005) (Fig. 1). There is a trend towards increased IL-4 levels among patients with asthma compared with control subjects that does not reach statistical significance (25.0 $\pm$ 7.7 pg/mL vs 11.5 $\pm$ 7.1 pg/mL, *P* = not significant [NS]). Among all patients, the elevated IL-4 levels correlate positively with increased IgE levels (*r* = 0.69). We conclude that sera IL-4 levels are markedly elevated in patients with HIE and that in both diseases these elevations correlate significantly with increased IgE levels.

IL-13, another T_{H2} cytokine, shares the function of regulating IgE class switching in B cells in vitro with IL-4.⁵ Because IgE-mediated mechanisms are known to be involved in extrinsic asthma and HIE, then IL-13, like IL-4, may contribute to their pathogenesis. However, fewer data exist on the expression of IL-13 in peripheral

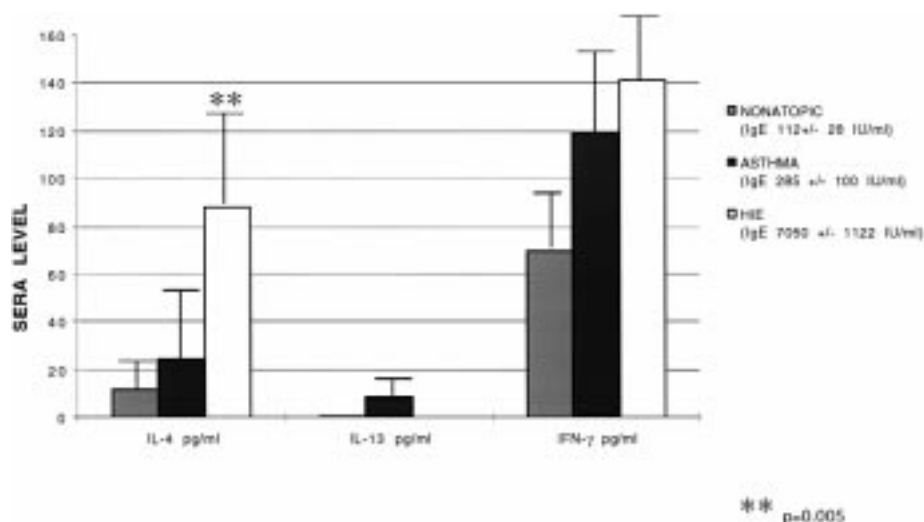


FIG 1 IL-4, IL-13, and IFN- γ expression during IgE-mediated disease. Sera cytokine levels were measured by ELISA. Data are shown as mean $\pm$ SE. IL-4 levels were significantly greater in patients with HIE than nonatopic control subjects, whereas IL-13 and IFN- γ levels did not differ among patients with HIE, asthma, or nonatopic control subjects. ** $P = .005$.

blood during allergic inflammation, although one group has reported that sera levels were undetectable in patients with perennial allergic rhinitis.⁴³ To provide data on the systemic expression of IL-13 in other IgE-mediated diseases and to determine whether IL-13 levels correlate with IL-4 or IgE levels, sera IL-13 levels were measured in all patients and control subjects. The mean IL-13 level is 0.6 ± 0.6 pg/mL among nonatopic control subjects, 12.2 ± 12.2 pg/mL among patients with asthma, and 0.4 ± 0.4 pg/mL among patients with HIE and are not statistically different (Fig. 1). Only 1 subject with asthma had a markedly increased level of serum IL-13 (97.9 pg/mL vs 0.1 ± 0.1 pg/mL for the remaining patients). This patient has no detectable IL-4, a markedly elevated IFN- γ level of 530.5 pg/mL, and a minimally increased IgE level of 164 IU/mL. These data suggest that sera levels of IL-13, unlike IL-4, usually are not elevated in association with moderately increased IgE levels in extrinsic asthma or markedly increased IgE levels in HIE.

Most allergen-specific CD4⁺ T-cell clones derived from atopic patients' peripheral blood lymphocytes secrete no detectable or low amounts of IFN- γ .^{37,38} In comparison, sera IFN- γ has been reported to be decreased,⁴⁴ nonelevated,^{45,46} and paradoxically increased⁴⁵ in individuals with asthma exacerbations. In comparison, some investigators report that the production of IFN- γ by both T-cell clones and PBMCs from adults with HIES in response to phytohemagglutinin stimulation or galactose-oxidase induction is decreased.^{41,47} In addition, IFN- γ inhibits IgE production by PBMCs both in vitro and in vivo in this disease,⁴⁸ and it has been proposed that defective production of IFN- γ is responsible for the overproduction of IgE in HIES.⁴¹ However, the data are mixed as another group was unable to reproduce such findings or show

that the secretion of IFN- γ decreases after induction with PMA and ionomycin.⁴² To characterize further the expression of systemic IFN- γ during asthma first seen at the emergency department and in cases of HIE and to determine the relation between IFN- γ levels and IL-4 and IgE levels, sera from all patients and control subjects were analyzed for IFN- γ . The mean IFN- γ levels were not significantly decreased in subjects with asthma (119 ± 65 pg/mL vs 70 ± 30 pg/mL among control subjects, $P = \text{NS}$) or in the 3 subjects with HIE (140 ± 25 pg/mL vs 70 ± 30 pg/mL among control subjects, $P = \text{NS}$). The IFN- γ levels do not correlate with the IL-4 levels or the IgE levels ($r = -0.1$ and 0.16 , respectively). We conclude that in these 2 patient populations, despite the elevated IL-4 and IgE levels, there is no consistent pattern of expression of IFN- γ in the sera.

Stat6 protein levels

Stat6 has been shown to be essential for the induction of genes by IL-4 and IL-13. Furthermore, in transfection experiments the overexpression of Stat6 in certain cell lines leads to increased sensitivity to IL-4 and increased IL-4-mediated transcription.⁴⁹ These data suggest that the level of Stat6 protein may be the limiting factor for IL-4-induced transcription. Therefore one model for diseases associated with high IgE levels is that cells from these individuals express increased levels of Stat6, which would lead to a heightened response to IL-4 and increased class switching to IgE. To determine whether the level of expression of this protein is increased in PBMCs from subjects with IgE-mediated disease, levels of Stat6 were measured from whole cell extracts and compared among patients and control subjects. B-cell line Ramos and PBMCs stimulated with recombinant IL-4 in vitro served as positive controls for Stat6 expression, and protein levels from these whole

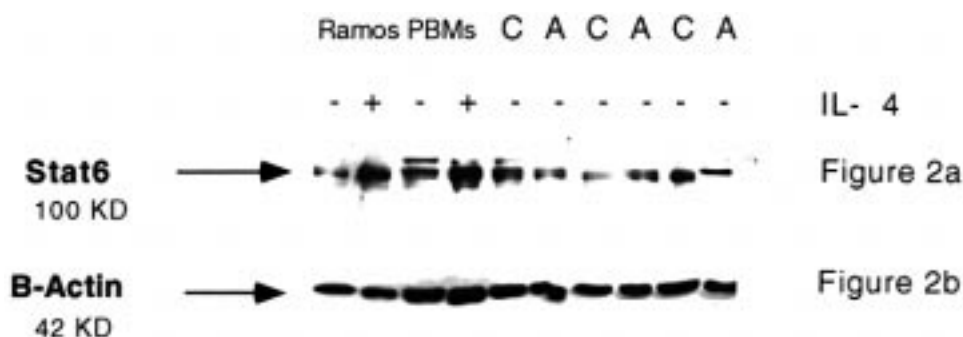


FIG 2. Western blot of whole cell extracts derived from PBMCs of 3 different representative subjects with asthma and simultaneously recruited nonatopic control subjects probed with α -Stat6 (**A**) and α - β -actin (**B**). Ramos cells and PBMCs (nonatopic control subjects) were stimulated with recombinant human IL-4 (100 U/mL) for 15 minutes before generation of whole cell extracts. Although nonactivated Stat6 is present in all asthmatic subjects (**A**) and nonatopic control subjects (**C**) represented here and in all subjects not shown, levels are not increased in patients (**A**). β -Actin levels are equivalent in all specimens (**B**).

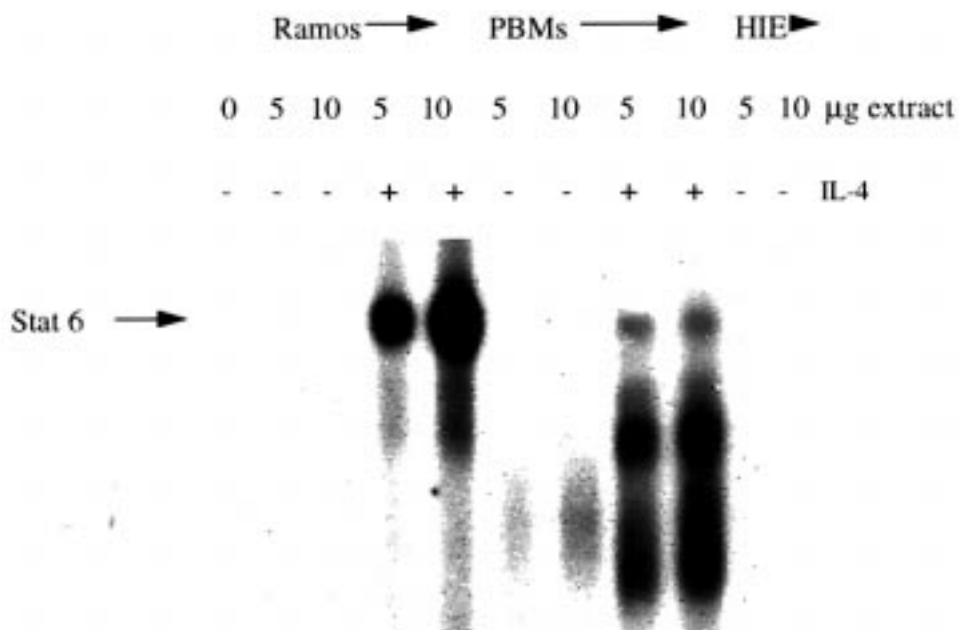


FIG 3. EMSA of representative patient with HIE. Whole cell extracts of PBMCs were probed with GAS element from IRF-1 gene. Ramos cells and PBMCs stimulated in vitro for 15 minutes with recombinant IL-4 (100 U/mL) served as positive controls for Stat6. Stat6 activation is not detected in this patient with HIE and markedly elevated IgE (9268 IU/mL) and IL-4 level (174.7 pg/mL) or in any other patients or nonatopic control subjects tested.

cell extracts were compared with those derived from patients with extrinsic asthma or HIE incubated with an antibody to Stat6. Stat6 was widely expressed in PBMCs derived from all tested individuals with extrinsic asthma and HIE and nonatopic control subjects (Fig. 2, A). The level of expression in patients with extrinsic asthma or HIE is not increased over that of control subjects. Probing of this Western blot with β -actin, a protein expressed in equal amounts in all cells and used as a control for total protein levels, confirms that the small variation in Stat levels are not associated with variations in the total protein incubated with the antibody (Fig. 2, B). These results imply that heightened IL-

4-induced IgE production is not related to increased systemic expression of Stat6.

Stat6 activation

The essential role of Stat6 activation in IL-4 signaling and IgE production in vivo has been demonstrated in murine models.²¹⁻²³ We hypothesized that some individuals with increased sera IgE may demonstrate evidence of increased systemic Stat6 activation in the setting of elevated sera IL-4 and IgE levels. To examine for Stat6 activation, EMSA was used, and the induction of DNA binding activity was assessed by probing with the GAS element from the IRF-1 gene. As positive controls we

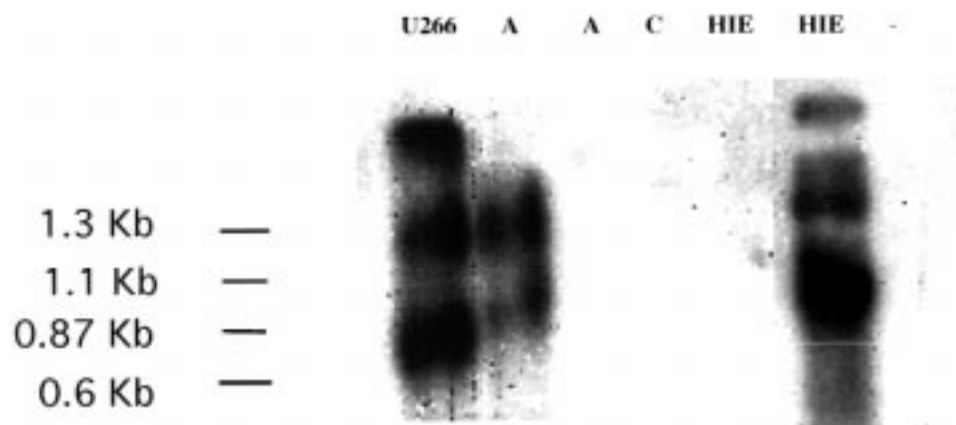


FIG 4. Southern blot of DNA extracted from PBMCs isolated from 2 patients with asthma (A), 2 patients with HIE, and 1 nonatopic control subject (C). U266 (IgE-secreting myeloma cells) served as positive controls. DNA was amplified by using nested primer PCR of switch fragments (S μ /S ϵ), and specific products were determined by Southern transfer, hybridization, and incubation with a probe against S ϵ . Switch fragments are detected in U266 cells, as well as in 2 of 8 patients with asthma (1 case shown in second lane) and 2 of 3 patients with HIE (1 case shown in sixth lane) but not in any of the nonatopic control subjects. The last lane represents no DNA (negative control).

used both Ramos cells and PBMCs isolated from a nonatopic control subject and treated half of these cells with recombinant hIL-4 *in vitro*. Stat6 was not detected on EMSA in the patient with HIE and the highest measured IL-4 and IgE level (Fig. 3), the other 2 tested patients with HIE, any tested patients with extrinsic asthma and moderately elevated IL-4 and IgE levels, or any of the nonatopic control subjects. These results demonstrate that despite the presence of elevated circulating IL-4 levels, subjects with elevated IgE do not exhibit evidence of systemic Stat6 activation. We also assayed for the presence of germline- ϵ transcripts as another marker for cells undergoing active class switching to C ϵ after Stat6 activation^{16,50} and found these were absent in all patients and nonatopic control subjects tested (data not shown). These data imply that in these 2 IgE-mediated diseases, either the peripheral B cells have already isotype-switched to IgE or that intracellular signaling is not induced systemically by the increased IL-4 in the circulating environment.

Switch recombination

The absence of detectable systemic Stat6 activation and production of germline- ϵ transcripts despite elevated sera IL-4 and IgE levels may imply that B cells in the blood of patients have already undergone class switching. If this were the case, switch junction fragments (S μ /S ϵ) representing DNA rearrangements and deletional recombination would be present. Such fragments have been found in human B cells in the presence of IL-4 infected with the Epstein-Barr Virus⁵¹ or stimulated with anti-CD40 antibodies.³⁵ To assess for the presence of switch-junction fragments, DNA was derived from the IgE-secreting myeloma cell line U266 and used as a positive control, and it was extracted from the PBMCs of the patients and nonatopic control

subjects. Nested PCR and Southern blot analysis of all specimens was performed with primers derived from S μ /S ϵ fragments and incubated with a P³²-labeled probe to S ϵ . Switch fragments are amplified from DNA isolated from PBMCs derived from 2 of 8 patients with asthma, 2 of 3 patients with HIE, and 0 of 14 nonatopic control subjects (Fig. 4). Switch fragments are not amplified in the patient with HIE and the greatest measured IL-4 and IgE level. Therefore cells are present in the blood of these patients that have undergone class switching to IgE.

DISCUSSION

The characterization of cytokine signaling and the JAK-STAT pathway that are critical for IgE class switching in mice has furthered our understanding of the mechanisms of IgE regulation. Our aim was to study mechanisms of cytokine-mediated intracellular signal transduction and IgE class switching in human diseases characterized by elevated levels of IgE. We hypothesized that evidence of systemic cytokine-mediated intracellular signal transduction and IgE class switching could be detected in some individuals with extrinsic asthma and HIE. In this study sera IL-4 levels are significantly elevated in subjects with HIE. The increased level of IL-4 measured in the sera in these patients contrasts with other studies that have examined IL-4 production from CD4⁺ T-cell clones derived from patients with HIES⁴¹ or assessed IL-4 production after *in vitro* stimulation with mitogens.^{41,42} The data imply that the cellular source of IL-4 is unclear. One possibility is that *in vivo* stimuli exist that induce secretion of this cytokine. An alternative possibility is that the source of IL-4 in these patients are either T cells present in other organs or other cells, such as basophils or mast cells. A third interpretation is that IL-4 production differs between HIES and HIE.

IL-13, another T_{H2} cytokine, has been shown to induce IgE class switching *in vitro*,⁵⁻⁷ but it has not been well studied during human IgE-mediated disease. In both asthma and HIE, the systemic expression is not increased, suggesting that IL-13 plays no significant role in inducing heightened IgE production in these diseases. These results also suggest that therapy for HIES or its variants could be targeted towards inhibiting the T_{H2} cytokine IL-4 alone. Furthermore, even though one may predict that increased IL-4 and IgE levels are related to downregulated IFN- γ levels, no consistent pattern of IFN- γ expression is detected among both groups of patients. This finding challenges the model that T_{H1} - and T_{H2} -type cytokines regulate class switching to IgE *in vivo* by antagonistically regulating transcriptional activity at the germline- ϵ promoter. However, it is compatible with the mixed cytokine data reported in previous studies on patients with asthma,^{37-39,45,46} suggesting either that these cytokines do not necessarily act in a strictly polarized manner *in vivo* or that variations may be attributed to other factors such as severity or chronicity of disease. Also, the absence of decreased IFN- γ in HIE provides new data on cytokine expression in HIE and indicates that downregulation of T_{H1} cytokines and IFN- γ signaling is not prominent in these cases.

In addition to examining cytokine expression during IgE-mediated diseases, our aim was to conduct the first study of intracellular cytokine signaling and the role of STAT transcription factors in IgE class switching in humans. The genetics of HIES and its variants are not well known, and therefore we hypothesized that some individuals with markedly increased IgE levels would exhibit increased Stat6 protein expression. The presence of unaltered protein levels here implies that this is not the important genetic defect in HIE or HIES or that this population is heterogeneous.

Another question addressed in this study is whether T_{H2} cytokines in the circulating blood are able to activate Stat6 systemically in IgE-mediated disease. These data demonstrate that despite the presence of increased systemic expression of IL-4 and IgE, Stat6 is not activated in PBMCs. Because IL-4 alone can induce Stat6 activation and the production of germline- ϵ transcripts in human B cells *in vitro*,⁵² it is unclear why Stat6 activation was not detected in the patients examined. One possibility is that synergistic costimulatory signals provided by CD4⁺ T cells, such as CD40 ligand, are required for the induction of Stat6 activation to detectable levels.²⁵ A second explanation is that a subset of B cells present in the blood are induced by IL-4, but our assays are not sensitive enough to detect this activity.

Because increased sera IgE levels do not appear to be the result of active class switching in the blood, one could postulate that other sources or mechanisms may be involved. One explanation is that IgE is being produced by cells that have already undergone class switching (ie, "memory" B cells). The detection of switch junction fragments in several subjects, a requisite precursor to IgE heavy-chain polypeptide production,

implies that cells are present in the blood that have already class switched to IgE.

An additional explanation for the absence of active class switching to IgE in the blood is that the cellular source may be the spleen or lymph nodes in the case of HIE or the airways where antigen sensitization occurs in the case of asthma. Indeed, it is clear that T_{H2} cytokines are elevated in the bronchoalveolar lavage fluid or transbronchial biopsy specimens of patients with allergic asthma⁵³⁻⁵⁷ and in the nasal mucosa of patients with symptomatic allergic rhinitis.^{43,58,59} Furthermore, germinal centers within the parenchyma of inflamed and hyperresponsive lungs after airway antigenic challenge have been identified in mice (ie, bronchus-associated lymphoid tissue) and shown to produce IgE.⁶⁰ Only a few studies have compared the systemic versus local T_{H2} immune response either temporally or quantitatively. In a murine model of allergen-induced asthma, IL-4 levels after antigenic challenge peaked in bronchoalveolar lavage fluid later, although to a similar level to that of the sera.⁶¹ After experimental allergen bronchoprovocation, another group demonstrated that peripheral blood T_H cells decreased in association with their selective increase in the lung.⁶² A plausible corollary is that the expression of T_{H2} -type cytokines in the airways during the inflammatory response of late-phase asthma or allergic rhinitis may be later or greater than the systemic expression. This pattern has been described for IL-13.⁴³ Therefore it is likely that the activation of the JAK-STAT pathway is occurring locally in patients with allergic airway disease but not systemically.

In summary, even though increased IgE production correlates with elevated sera IL-4 levels, it is not associated with increases in the protein expression of Stat6 or the prevalence of Stat6 activation. These results suggest that active intracellular cytokine signaling is not occurring systemically in either extrinsic asthma or HIE. In comparison, the presence of switch fragments in several patients suggests that PBMCs in some individuals may have already class switched to IgE, even if the process is not active. These results imply that increased IgE antibodies may reflect B cells that have already undergone class switching or that cytokine-mediated Stat activation and IgE induction may occur predominantly in target organs in IgE-mediated disease.

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Correction

The following correction applies to the article by Busse et al entitled "Budesonide delivered by Turbuhaler is effective in a dose-dependent fashion when used in the treatment of adult patients with chronic asthma," which appeared in volume 101, pp 457-463, 1998, of *The Journal*.

An incorrect version of Fig 2 appeared in the article. The correct version, accompanied by its legend, appears below.

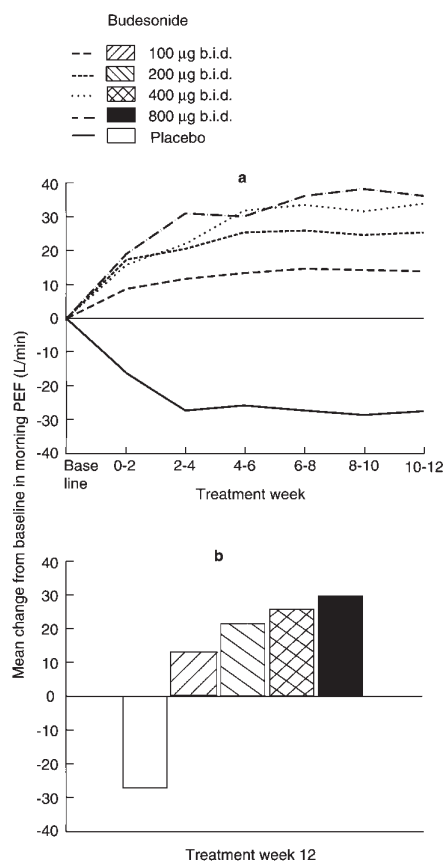


FIG 2. Mean change from baseline in morning PEF (L/min) for placebo- and budesonide-treated groups. a, 2-week intervals and b, from baseline to week 12.