

Allergen Drives Class Switching to IgE in the Nasal Mucosa in Allergic Rhinitis¹

Pooja Takhar,* Lyn Smurthwaite,* Heather A. Coker,* David J. Fear,* Graham K. Banfield,† Victoria A. Carr,† Stephen R. Durham,† and Hannah J. Gould^{2*}

IgE-expressing B cells are over 1000 times more frequent in the nasal B cell than the peripheral blood B cell population. We have investigated the provenance of these B cells in the nasal mucosa in allergic rhinitis. It is generally accepted that expression of activation-induced cytidine deaminase and class switch recombination (CSR) occur in lymphoid tissue, implying that IgE-committed B cells must migrate through the circulation to the nasal mucosa. Our detection of mRNA for activation-induced cytidine, multiple germline gene transcripts, and ϵ circle transcripts in the nasal mucosa of allergic, in contrast to nonallergic control subjects, however, indicates that local CSR occurs in allergic rhinitis. The germline gene transcripts and ϵ circle transcripts in grass pollen-allergic subjects are up-regulated during the season and also when biopsies from allergic subjects are incubated with the allergen ex vivo. These results demonstrate that allergen stimulates local CSR to IgE, revealing a potential target for topical therapies in allergic rhinitis. *The Journal of Immunology*, 2005, 174: 5024–5032.

Immunoglobulin E Abs are synthesized by plasma cells in the nasal mucosa in allergic rhinitis, providing a local source for the sensitization of tissue mast cells against common inhaled aeroallergens (1). This IgE synthesis reflects the large proportion of B cells committed to IgE synthesis in the nasal mucosa compared with the circulation. It is estimated that 1 in 25 B cells and 1 in 5 plasma cells in the nasal mucosa in allergic rhinitis, compared with 1 in 10,000 in peripheral blood B cells, express IgE (2, 3). The bias toward IgE is also revealed by the high frequency of B cells containing ϵ -chain mRNA in the nasal mucosa in allergic rhinitis (4, 5). This bias in the nasal mucosa is not widely recognized, and the mechanism by which it occurs is not understood.

B cells must undergo class switch recombination (CSR),³ changing the C region of the H chain (C_H) to express IgE. It is generally accepted that CSR occurs in lymphoid tissues (6). However, if CSR to IgE occurs only in lymphoid tissues, the IgE-committed cells would have to migrate through the circulation into the nasal mucosa to produce IgE locally. It is unclear how selective recruitment or expansion might occur to account for the difference between the circulating and local B cell populations, and local CSR to IgE may represent an alternative explanation. It is not generally acknowledged that CSR can occur outside lymphoid tissue,

but there are reasons to predict that the allergic nasal mucosa may be an exception.

There are nine different Ab classes ($C\mu$, $C\delta$, $C\gamma 3$, $C\gamma 1$, $C\alpha 1$, $C\gamma 2$, $C\gamma 4$, $C\epsilon$, and $C\alpha 2$) encoded in a tandem array on human chromosome 14 (Fig. 1). The 5' flanking sequence of each C_H (except $C\delta$) contains a germline (I) exon and a switch (S) region containing GC-rich tandem repeats that contain the potential sites of nonhomologous recombination in CSR (7). Germline gene transcription from both donor and acceptor C_H , initiated from the I exon promoters and continuing through the S regions and C_H exons, is required for class switching (8). The enzyme, activation-induced cytidine deaminase (AID), normally expressed only in germinal center B cells in lymphoid tissues, is also required for CSR (9, 10). Exogenous AID expression induces CSR in a fibroblast cell line (11), indicating that there are conditions in which AID can be the limiting factor for CSR.

IL-4 stimulates the transcription of all the germline genes and is necessary for stimulation of the ϵ germline gene (12–15). IL-4 also stimulates the expression of AID (16). A further requirement for CSR in primary B cells is CD40 signaling (12, 17). Allergen stimulates the expression of IL-4 and CD40 ligand by Th2 cells and mast cells in the nasal mucosa in allergic rhinitis (18, 19). Thus, we have reason to suggest that aeroallergens might stimulate the expression of AID and CSR to IgE in the nasal mucosa in allergic rhinitis. In the present work, we sought to test this hypothesis.

During recombination between the expressed/donor and targeted/acceptor S regions, the intervening DNA is looped out and deleted. The ends of the deleted fragments are linked to form extrachromosomal circular DNA templates containing the I exon promoter upstream of the targeted S region, now linked to the previously expressed S region and C_H gene (20). This switch circle DNA is transiently transcribed into specific circle transcripts (CT), which provide an ideal marker for ongoing CSR (21). The appearance of CT has been shown to depend on the presence of AID (22). Fig. 1 illustrates the process of direct and sequential CSR from IgM (via IgG) to IgE.

To test our hypothesis of local CSR in the nasal mucosa, we have taken biopsies from grass pollen (GP)-allergic donors in and

*The Randall Division, King's College London, London, United Kingdom; and †Upper Respiratory Medicine, Imperial College School of Medicine, National Heart and Lung Institute, London, United Kingdom

Received for publication November 10, 2004. Accepted for publication January 31, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by National Asthma Campaign Grant S99/068 and 03/055, British Lung Foundation Grant P00/7, and the Clinical Research Committee Royal Brompton and Harefield Hospitals National Health Service Trust.

² Address correspondence and reprint requests to Prof. Hannah J. Gould, Randall Division, New Hunt's House, King's College London, Guy's Campus, London SE1 1UL, U.K. E-mail address: hannah.gould@kcl.ac.uk

³ Abbreviations used in this paper: CSR, class switch recombination; AID, activation-induced cytidine deaminase; CT, circle transcript; GLT, germline gene transcript; GP, grass pollen; I exon, germline exon; S region, switch region.

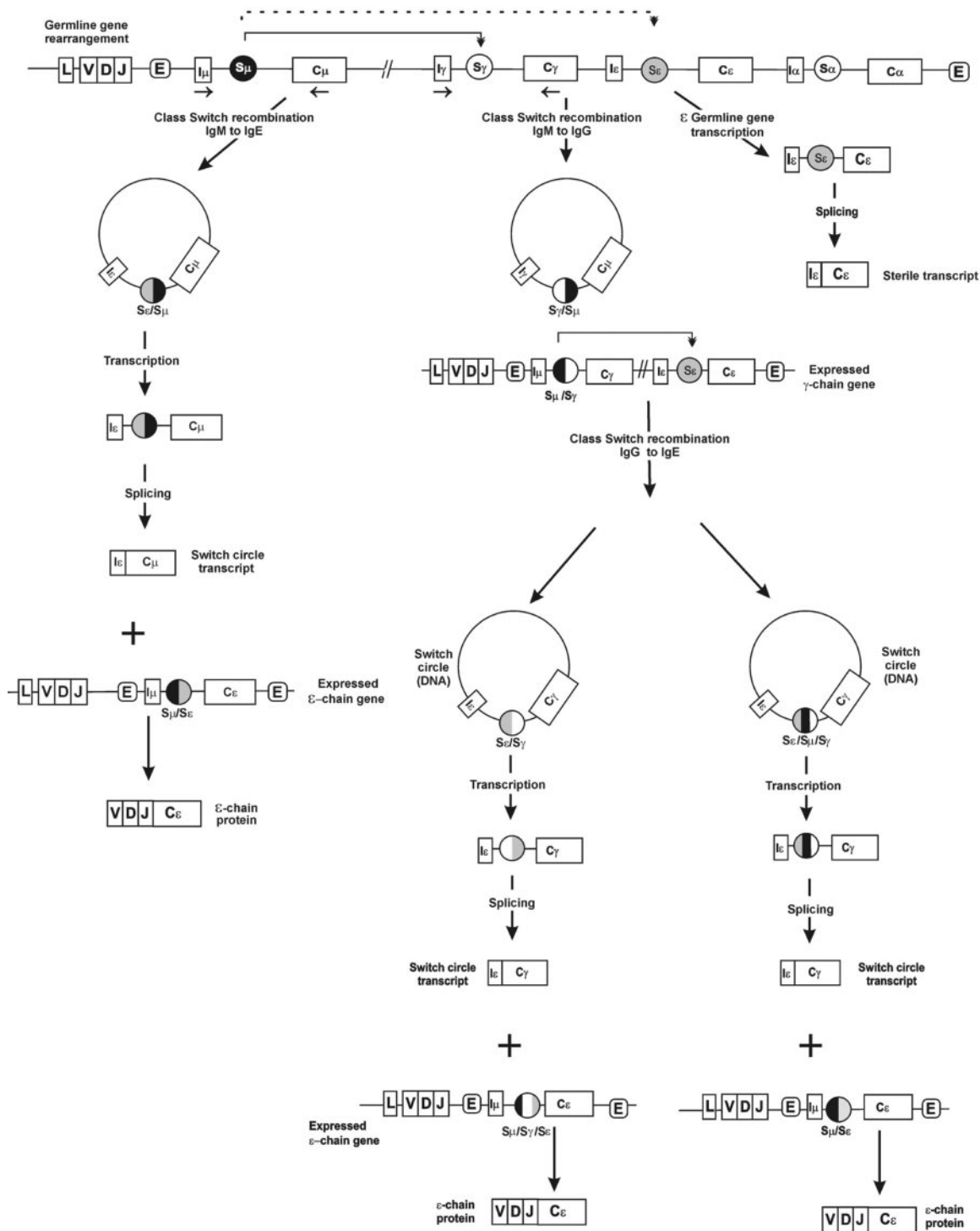


FIGURE 1. Mechanism of direct and sequential CSR to IgE. The expressed H chain gene loci and the molecular events involved in class switching to IgE are shown schematically. S, switch region; I, I exon of the germline gene; C, C region gene.

out of season, and have incubated a cohort of out-of-season biopsies with allergen *ex vivo*. We have extracted RNA from biopsies and conducted RT-PCR to detect AID mRNA, germline gene transcripts (GLT), and CT corresponding to CSR from IgM, IgG1, IgG3, and IgG4 to IgE. We have compared the results with those from nonallergic control subjects. Our results provide direct evidence of local CSR in allergic disease or indeed in any human disease.

Materials and Methods

Participants and biopsy procedure

Nasal biopsies were taken from the inferior turbinate using 2.5-mm-diameter cup and ring forceps (Gerritsma forceps) under local anesthetic, as described previously (23). All allergic volunteers had a clinical history of at least 2 years of seasonal hay fever and/or perennial allergic rhinitis and a positive skin prick test to the relevant allergen extract (Soluprick; ALK) (Table I). Total and specific serum IgE concentrations were measured by

Table I. Comparison of skin prick test, serum total, and serum-specific IgE data for cohort of study subjects^a

Patient	Status at Biopsy	Age	Gender	Allergen (SPT mm)	Serum Total IgE (ng/ml)	Serum-Specific IgE (ng/ml)
SM	I	24	F	G (5), T (3), B (3), H (2), PC (2)	116	G (31.3)
DT	I	24	M	G (7), H (4), A (3), T (4), B (5), C (4), D (3), PC (5)	319	G (101), H (2.12) C (0.80)
AA	I	20	M	G (15), H (15), PC (8)	ND	ND
NR	I	28	F	G (6), T (6), C (6), PC (6)	112	G (34)
KC	I	57	M	G (6), PC (12)	22	G (8.5)
TK	I	21	M	G (9), PC (5)	1053	G (101)
MK	O	26	F	G (3), H (5), A (3), D (2), PC (6)	46	G (0.86), H (7.04)
ST	O	35	F	G (9), H (5), C (5), PC (4)	543	G (0.34), H (101) C (28.5)
JA	O	34	F	G (5), T (4), B (6), C (4), D (2), PC (6)	58	G (7.97), H (0.34) C (0.34)
KB	O	23	F	G (5), A (3), B (6), D (4), PC (5)	101	G (19.7), H (0.34) C (0.34)
TH	O	33	M	G (10), D (4), PC (5)	100	G (22.8), H (0.34) C (0.34)
NW	O	24	M	G (7), B (5), PC (6)	224	G (79.5)
AT	O	44	M	G (10), H (3), PC (6)	62	G (22.5)
CM	O	27	F	G (5), H (5), C (4), D (4), PC (5)	120	G (35.9)
KG	O	24	F	G (8), PC (7)	172	G (65.7)
SS	O	23	F	G (8), T (3), B (3), D (4), PC (5)	425	G (87.9)
JF	N	31	M			
JM	N	27	M			
VC	N	28	F			
SY	N	32	M			

^a I, In season; O, out of season; N, normal; G, grass pollen; T, tree pollen; B, birch; H, house dust mite; C, cat; D, dog; A, *Artemisia*; PC, histamine-positive control; SPT, skin prick test.

radioallergosorbent test. All volunteers stopped all medication for at least 2 days before the study. The biopsies from 12 seasonal GP-allergic hay fever patients were taken, 6 during the GP season (June–August) and 6 out of season (November–March), for the in vivo study. The biopsies from a further 4 seasonal GP-allergic subjects were taken out of season for ex vivo allergen challenge. Control nasal biopsies were taken from 4 healthy subjects with no clinical history of allergy and negative skin prick test with no nasal symptoms. All the biopsies were washed in HBSS medium to remove any traces of blood. The study was performed with the approval of the Royal Brompton Hospital Ethics Committee and the patients' written informed consent.

Ex vivo allergen challenge

Nasal biopsies were obtained from four GP-allergic patients out of the GP season. Due to ethical constraints, we could not collect more than one biopsy per patient. Each biopsy was divided into two equal-sized explants of ~1 mm³. Explants were cultured for 24 h in 200 µl of Ysells medium (24) with or without 10 µg/ml GP allergen Phlp 5 (Soluprick) in a 96-well plate under standard conditions (37°C and 5% CO₂). After culture, tissue was removed and immediately frozen at –80°C until further analysis.

RNA isolation and RT-PCR

Total mRNA was isolated from biopsies using the Promega RNA isolation kit (Promega Life Sciences), following the manufacturer's instructions. RNA was suspended in nuclease-free water and quantified by spectrophotometry at 260 nm.

For RT-PCR, 5 µg of total RNA was denatured at 100°C for 2 min and then placed on ice for 2 min before adding to a 20-µl reaction mix containing 5× first strand buffer, 0.1 M DTT, 10 mM dNTP mix, oligo(dT) primer (0.5 µg/µl), 40 U/µl RNase OUT, random d(N)₁₀ primers (2 µg/µl), and 200 U/µl Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). The reaction was first incubated at 37°C for 10 min, followed by 40-min incubation at 42°C and a final incubation of 10 min at 50°C. Finally, the cDNA reaction was diluted with 80 µl of nuclease-free water and incubated at 100°C for 2 min.

AID expression was analyzed by PCR and confirmed by nucleotide sequencing and Southern blot hybridization. AID transcripts were amplified by primer sets aidF1/aidR1 and aidF2/aidR2 in a nested PCR amplification protocol (Table II). For amplification of AID mRNA, 5 µl of cDNA was added to a 25 µl PCR comprising 0.2 mM dNTPs, 1 mM

Table II. Sequences of oligonucleotide primers used to amplify AID mRNA, I_H-C_H GLT, and Iε-C_H CT^a

Primer Name	Sequence	GenBank Accession No.
IµF	5'-GTGATTAAGGAGAAACACTTTTGAT-3'	X58529
IγF	5'-GATGCCAGGATGGGCACGAC-3'	S79588
IεF1	5'-GGGAGCTGTCCAGGAACCCGACAGAGC-3'	X56797
IεF2	5'-GGCCACACATCCACAGGC-3'	X56797
CµR	5'-GTTGCCGTTGGGGTGCTGG-3'	X17115
CµR2	5'-AGACGAGGGGAAAAGGGTT-3'	X17115
CγR	5'-CCAACTCTCTTGTCACCTTGG-3'	X04646
γ1R	5'-GGCATGTGTGAGTTTGTGTCACAA-3'	J00228
γ3R	5'-GGCACGGGGGAGG-3'	X99549
γ4R	5'-GGGCATGGGGGACCATA-3'	KO1316
CεR	5'-GGGGTGAAGTCCCTGGAGC-3'	X95746
aidF1	5'-GAGGCAAGAAGACACTCTGG-3'	AB040431
aidR1	5'-GTGACATTCCTGGAAGTTGC-3'	AB040431
aidF2	5'-TAGACCTGGCCGCTGTACC-3'	AB040431
aidR2	5'-CAAAAGGATGCGCCGAGCTGTCTGGAG-3'	AB040431
GAPDHF	5'-ATTTGGTCGTATTGGGCGCCTGGTC-3'	AF261085
GAPDHR	5'-TCATACCTCTCATTGTTTCACACCCATG-3'	AF261085

^a GAPDHF, GAPDH forward primer; GAPDHR, GAPDH reverse primer.

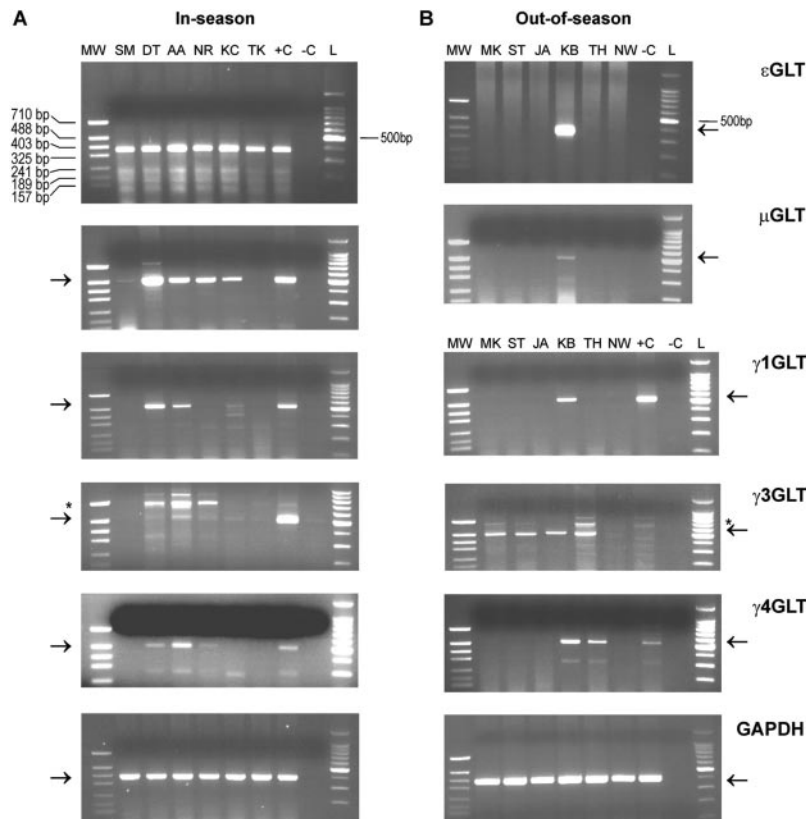


FIGURE 2. Analysis of GLT in the nasal mucosa. ϵ GLT, 379 bp; μ GLT, 529 bp; γ 1 GLT, 510 bp; γ 3 GLT, 489 bp; γ 4 GLT, 505 bp were PCR amplified from the nasal mucosa of 12 hay fever patients. **A**, GP-allergic in-season patients. **B**, GP-allergic out-of-season patients. Lanes: MW, m.w. marker; L, 100-bp DNA ladder. Lanes +C and -C represent PCR-positive control and the PCR-negative control, respectively. GAPDH (393 bp) was amplified to control cDNA loading. Gels are representative of one of three independent PCR amplifications yielding similar results. Arrows indicate the expected PCR product. Asterisk indicates the PCR by-products formed due to mispriming to the similar repeats of the S region.

MgCl₂, 0.2 μ M each primer, and 1.25 U of platinum *Taq* polymerase (Invitrogen Life Technologies). The first cycle was conducted at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 5 min. A second round of PCR was performed on a 5- μ l aliquot of the first round PCR product with essentially the same conditions as described above, except that an inner set of primers and annealing temperature of 60°C were used. Amplification of AID mRNA from the RAMOS cell line was used as a positive control.

GLT (I_H-C_H) were detected using primers homologous to sequences in I exon and C_H regions of specific GLT. μ GLT were PCR amplified with the I μ F forward and C μ R2 reverse primer pair; ϵ GLT were PCR amplified by primer pair I ϵ F2 and C ϵ R (Table II). Similarly, γ 1 GLT, γ 3 GLT, and γ 4 GLT were amplified by the same forward primer I γ F, which lies in the highly homologous I γ regions, but reverse primers γ 1R, γ 3R, and γ 4R, respectively (Table II).

CT corresponding to CSR to IgE from different C_H were detected by primers homologous to sequences in I ϵ and the donor C_H (I ϵ -C_H; Table II). Earlier work has suggested switching from γ 1, γ 3, and γ 4, but not γ 2 to IgE (13). We therefore analyzed CSR from IgM, total IgG, and specifically from IgG1, IgG3, and IgG4 to IgE.

Direct μ to ϵ switching was investigated with primer sets I ϵ F1/C μ R and I ϵ F2/C μ R in a seminested PCR to amplify the resultant I ϵ -C μ CT. Primer set I ϵ F1/C γ R and I ϵ F2/C γ R were designed to detect I ϵ -C γ CT resulting from sequential switching to IgE via any IgG using a seminested PCR approach. Primer C γ R is derived from the published sequence of the first C γ exon that is identical between subclasses (25, 26). Such I ϵ -C γ CT could be generated either by an earlier direct or sequential switch from μ to one or more γ genes (Fig. 1).

To amplify γ -specific subtype γ to ϵ CT (I ϵ -C γ 1,3,4), we used forward primers I ϵ F1 or I ϵ F2 with reverse primers γ 1R, γ 3R, or γ 4R in seminested PCR amplification protocol. γ 1R, γ 3R, and γ 4R were designed from the unique C γ 1, C γ 3, and C γ 4 hinge regions (26) (exon II) (Table II). Different γ genes share <60% homology in the hinge region (26). The orientation of the primers ensures that the amplified CT are from the excised switch circle and not from the rearranged genomic DNA.

PCR for GLT and CT were performed in a 30- μ l reaction containing 2.5 mM MgCl₂, 0.3 mM dNTP mix, 0.3 μ M each primer, and 2.5 U of *Taq* polymerase (Promega Life Sciences). One-twentieth of the cDNA preparation was used as the template. For ϵ GLT and γ GLT amplification, after an initial denaturation at 94°C for 5 min, 40 cycles of PCR were performed at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for

1 min, with a final extension step of 10 min at 72°C. The μ GLT PCR was conducted under conditions similar to those described above, except for annealing temperature at 68°C. I ϵ -C μ and I ϵ -C γ CT were amplified by seminested PCR (35 cycles each). The first cycle was conducted at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 66°C for 1 min, and 72°C for 1 min. A second round of PCR was performed on a 5- μ l aliquot of the first round PCR product with essentially the same conditions as described above, except for the annealing temperature of 60°C. Amplification of mRNA isolated from tonsil B cells stimulated with IL-4 and anti-CD40 was used for positive controls.

The expected product sizes are as follows: ϵ GLT, 379 bp; μ GLT, 529 bp; γ 1 GLT, 510 bp; γ 3 GLT, 489 bp; γ 4 GLT, 505 bp; I ϵ -C μ CT, 408 bp; I ϵ -C γ CT, 339 bp; AID, 335 bp; GAPDH, 393 bp. PCR products were size separated on a 1.5% agarose Tris-borate-EDTA electrophoresis buffer gel, stained with ethidium bromide, and visualized under UV. GAPDH, a housekeeping gene, was amplified from all samples to check the integrity of cDNA and to control the loading of cDNA.

Southern blot hybridization

PCR products were fractionated by electrophoresis in a 1.5% agarose Tris-borate-EDTA electrophoresis buffer gel, denatured, and transferred onto nylon membrane using standard procedures (27). Blots were then hybridized with specific probes labeled with [³²P] α -cytidine triphosphate by random priming. I ϵ -CH CT were hybridized with a PCR-generated 350-bp probe specific for the I ϵ exon.

Cloning and sequencing

PCR products were cloned using the GEM-T Easy System cloning method following the manufacturer's instructions (Promega Life Sciences). Positive clones containing the insert were purified by miniprep and subsequently sequenced using an automated 310 sequencer (ABI PRISM; PerkinElmer). A blast search was performed on each sequence, providing alignments with sequences of Ig H chain and AID sequences present in GenBank/European Molecular Biology Laboratory databases.

Immunohistochemistry

The surface B cell marker CD19 was visualized in 6- μ m frozen sections from a nasal biopsy taken out of season. Images were visualized with an Axioscop microscope (Zeiss) and processed using an AxioCam color charge-coupled device camera and Axiovision software. The protocol was

Table III. Distribution of GLT, CT, and AID mRNA in GP-allergic patients

	In-Season Patients						Out-of-Season Patients					
	SM	DT	AA	NR	KC	TK	MK	ST	JA	KB	TH	NW
ϵ GLT	+	+	+	+	+	+				+		
μ GLT	+	+	+	+	+					+		
$\gamma 1$ GLT		+	+		+					+		
$\gamma 3$ GLT		+	+	+	+		+	+	+	+		
$\gamma 4$ GLT		+	+	+						+	+	
I ϵ -C μ CT												
I ϵ -C γ CT		+	+	+						+		
I ϵ -C $\gamma 1$ CT		+	+									
I ϵ -C $\gamma 3$ CT			+		+					+		
I ϵ -C $\gamma 4$ CT												
AID	+	+	+	+			+	+	+	+	+	

based on that previously published (2). Briefly, nasal biopsy sections were acetone fixed, washed in TBS, and blocked for 15 min with Iscove's medium (Invitrogen Life Technologies) that additionally contained 10% FCS (Invitrogen Life Technologies), 10% normal goat serum (Vector Laboratories), 10% normal horse serum (Vector Laboratories), 10 U of penicillin/streptomycin (Invitrogen Life Technologies), and 2 mM glutamine (Invitrogen Life Technologies). Abs were also diluted in this blocking medium. An avidin/biotin block (Vector Laboratories) was then applied, and the sections were washed in TBS, followed by incubation with mouse anti-human CD19 Ab (25 μ g/ml) (Beckman Coulter) or the control mouse MOPC21 Ab (5 μ g/ml) (Sigma-Aldrich) for 60 min. The sections were washed and incubated with biotinylated goat anti-mouse IgG (10 μ g/ml) (Vector Laboratories) for 30 min. After washing, the sections were incubated with alkaline phosphatase-conjugated streptavidin (5 μ g/ml) (Vector Laboratories) for 30 min, washed, and then incubated for 10 min with fuchsin chromogenic substrate containing levamisole (DakoCytomation) for 10 min. Finally, the sections were washed in TBS before being mounted with glycerol (DakoCytomation).

Results

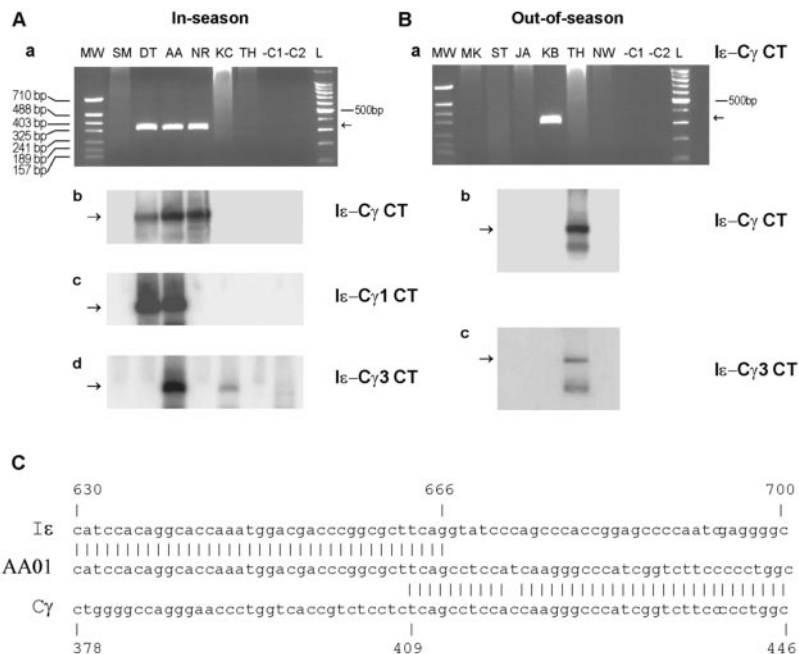
The nasal mucosa of seasonally allergic subjects contains multiple GLT in season

We first used RT-PCR to detect GLT in biopsies from 12 GP-allergic subjects, comprising 6 biopsies at the peak of the GP season (June-August; SM, DT, AA, NR, KC, TK), 6 out of season (November-March; MK, ST, JA, KB, TH, NW), and 4 nonallergic

controls (JF, JC, VC, SY). The subjects were selected on the basis of skin prick tests and assays of IgE against specific allergens in serum (Table I). RNA was isolated from the biopsies and used as templates for RT-PCR. The RT-PCR products analyzed by agarose gel electrophoresis are shown in Fig. 2, and the results are summarized in Table III.

ϵ GLT (379 bp) was observed in all 6 (6 of 6) of the GP-allergic subjects examined during the season. μ GLT (529 bp) was observed in 5 of 6, $\gamma 3$ GLT (489 bp) in 4 of 6, $\gamma 1$ GLT (510 bp) in 3 of 6, and $\gamma 4$ GLT (505 bp) in 3 of 6 biopsies (Fig. 2A). These GLT were observed much less frequently in the GP-allergic group examined out of season, μ GLT in 1 of 6, ϵ GLT in 1 of 6, $\gamma 3$ GLT in 4 of 6, $\gamma 1$ GLT in 1 of 6, and $\gamma 4$ GLT in 2 of 6 biopsies (Fig. 2B). In the latter group, all 5 different GLT, comprising 5 of the 6 total signals, were observed in the biopsy from a single subject (KB). KB was a multiallergic subject (Table I) and may have been exposed to a non-GP allergen shortly before donating the biopsy. Thus, GLT were expressed predominantly in the GP season. IgM and all 3 IgG subclasses were thus primed to switch to IgE in the nasal mucosa, and indeed, ϵ GLT was the most frequently transcribed germline gene, showing the potential for IgE switching. No GLT were observed in the nasal biopsies from the 4 nonallergic control subjects (result not shown).

FIGURE 3. Analysis of CT in the nasal mucosa. Gel electrophoresis of PCR-amplified fragments for I ϵ -C γ CT obtained from nasal mucosa of hay fever patients. **A**, Cohort of six GP-allergic in-season patients. **a**, Lanes: MW, m.w. marker; L, 100-bp DNA ladder. Lanes -C1 and -C2 are the first and second round PCR-negative control, respectively. **b**, The RT-PCR products detected by agarose gel electrophoresis in **a** were radioactively probed by Southern blotting. **c** and **d**, I ϵ -C $\gamma 1$ and I ϵ -C $\gamma 3$ CT were visualized by Southern blot hybridization with the I ϵ probe. **B**, Cohort of GP-allergic patients out of season. **a**, Lanes -C1 and -C2 represent first and second round PCR-negative control, respectively. **b**, The RT-PCR products detected by agarose gel electrophoresis were radioactively probed for I ϵ by Southern blotting. **c**, I ϵ -C $\gamma 3$ CT were visualized by Southern blot hybridization with the I ϵ probe. **C**, Nucleotide sequence surrounding the breakpoint of I ϵ -C γ CT cloned from an in-season patient, AA. The sequence data for clone AA01 are available from European Molecular Biology Laboratory under accession no. AJ567669. Vertical lines indicate the identical nucleotide bases. Gels are representative of one of three independent PCRs yielding similar results. Arrows indicate the expected size of the PCR product.



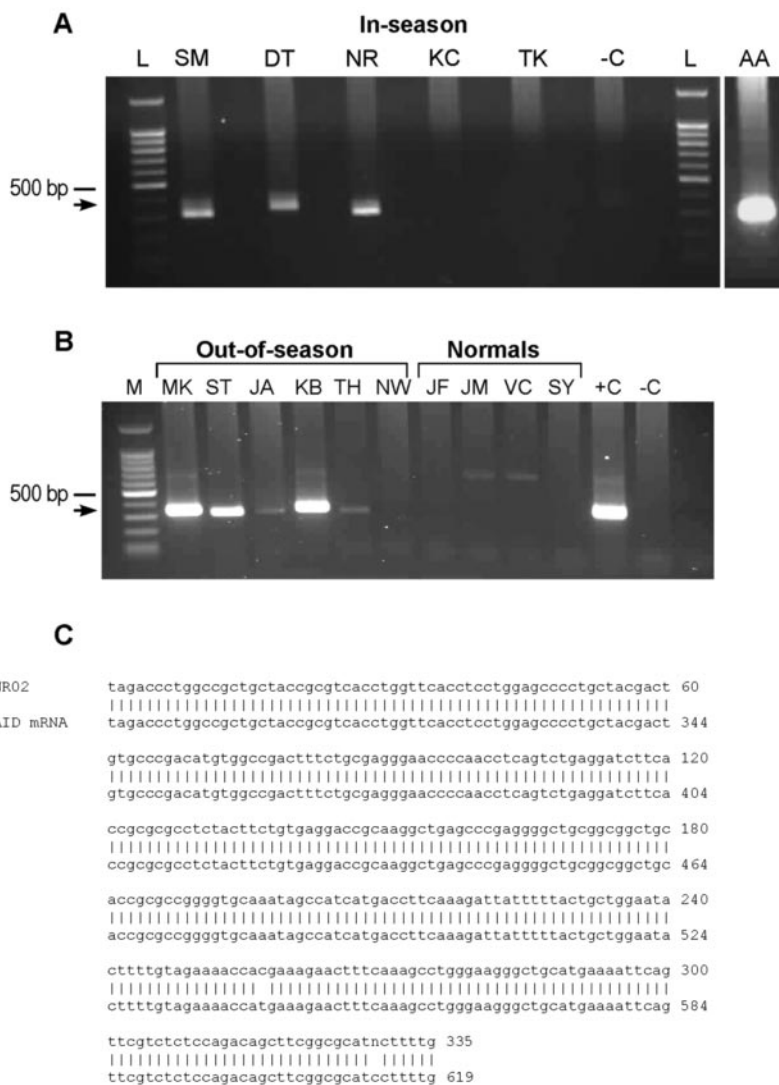


FIGURE 4. Expression of AID mRNA in the nasal mucosa. The 335-bp AID mRNA band was PCR amplified from the nasal mucosa of hay fever patients and nonallergic controls. *A*, GP-allergic patients in season. *B*, GP-allergic patients out of season and nonallergic normal controls. Lanes +C and -C are the PCR-positive and -negative controls, respectively. Lane L represents the 100-bp DNA molecular marker ladder. Gels are representative of one of three independent PCRs (except AA) yielding similar results. Arrows indicate the expected PCR product. *C*, Nucleotide sequence of AID transcripts cloned from in-season subject NR. These sequence data for clone NR02 are available from European Molecular Biology Laboratory under accession no. AJ577811. Vertical lines indicate the identical nucleotide bases.

The nasal mucosa of allergic subjects contains multiple CT

We first looked for Iε-Cμ CT, the product resulting from direct CSR from IgM to IgE in the same biopsies. We used a seminested PCR method, followed by Southern blot hybridization with the Iε probe. No Iε-Cμ CT were detected in any of the 16 biopsies. We, therefore, looked for Iε-Cγ CT, the product resulting from CSR from any IgG (IgG1, IgG2, IgG3, or IgG4) to IgE. Iε-Cγ CT (339 bp) were detected in 3 of 6 in-season biopsies and 1 of 6 out-of-season biopsy (Fig. 3, *Aa* and *Ba*). The out-of-season biopsy that gave a positive result was from the same multiallergic subject (KB) who expressed all 5 GLT. No Iε-Cγ CT could be detected from the nasal biopsies of the nonallergic control subjects (results not shown). To confirm their identity, PCR products from the positive biopsies were cloned and sequenced. The expected sequences were obtained in all cases; one such example (Iε-Cγ CT) is shown in Fig. 3C.

To determine which of the IgG subclasses switched to IgE in these subjects, subclass-specific primers were used in the RT-PCR. Fig. 3, *A*, *b-d*, and *B*, *b-d* shows Southern blots of the amplified products hybridized with the Iε probe to detect CT. Iε-Cγ1 CT and Iε-Cγ3 CT were each observed in 2 of 6 in-season biopsies (both in 1 of the biopsies), and Iε-Cγ3 CT were observed in 1 of 6 out-of-season biopsy (KB). No Iε-Cγ4 CT were observed in the biopsies from the allergic subjects. KB was again the exception,

giving the only CT signal (Iε-Cγ3 CT) in the out-of-season group. No CT were observed in the biopsies from the nonallergic control subjects (results not shown). The predominance of CT in the GP-allergic subjects indicates that CSR from at least 2 of the 3 IgG subclasses (IgG3 and IgG1) to IgE occurred in the nasal mucosa during the GP season.

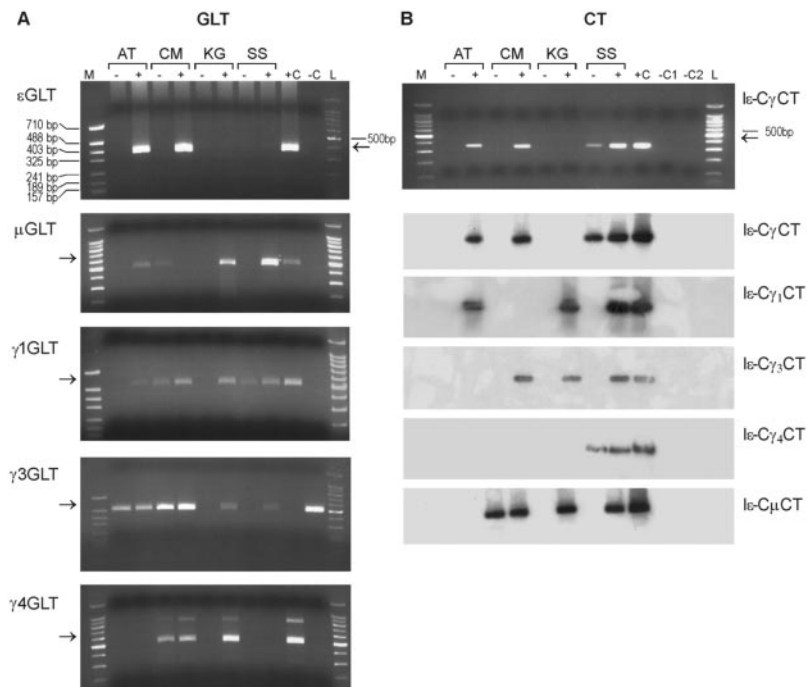
Nasal biopsies from allergic subjects contain AID mRNA

As shown in Fig. 4, *A* and *B*, we obtained a 335-bp band corresponding to the expected size of AID mRNA in 9 of 12 GP-allergic subjects (4 of 6 in season and 5 of 6 out of season). The PCR-amplified products were confirmed by Southern blot hybridization with a labeled probe spanning exons III and IV of AID mRNA (results not shown). DNA sequencing of the amplified product further confirmed its identity, as shown in Fig. 4C. AID mRNA was not detected in any of the biopsies from the 4 nonallergic control subjects (Fig. 4B). Surprisingly, local AID expression appears to correlate with allergic rhinitis, but does not require stimulation by allergen.

Expression of GLT and CT after ex vivo allergen challenge

The increased frequency of GLT and CT in the in-season compared with the out-of-season biopsies from the GP-allergic subjects (Figs. 2 and 3 and Table III) suggests that allergen stimulates

FIGURE 5. Analysis of GLT and CT in the nasal mucosa following ex vivo allergen challenge. **A**, GLT expression in GP-allergic patients challenged with GP allergen ex vivo. GLT were PCR amplified from the nasal mucosa of four hay fever patients cultured for 24 h either in medium alone (–) or with 10 μ g/ml GP allergen, Phlp 5 (+). Lanes: M and L represent the m.w. markers. Lanes +C and –C are the PCR-positive control and –negative control, respectively (lanes 10 and 9 in γ 3 GLT, respectively). **B**, CT analysis in GP-allergic patients challenged with GP allergen ex vivo. Lanes marked – correspond to AT, CM, KG, and SS cultured in medium alone; lanes marked + represent AT, CM, KG, and SS incubated with allergen; lanes +C, –C1, and –C2 correspond to the PCR-positive control and first and second round PCR-negative control, respectively. PCR-amplified I ϵ -CH CT were probed with radioactively labeled I ϵ probe by Southern blot hybridization. Arrows indicate the expected size of the PCR product.



CSR to IgE in the nasal mucosa in allergic rhinitis. This is consistent with our observation of AID mRNA in the tissue (Fig. 4 and Table III). However, these products do not provide definitive evidence for local CSR to IgE within the tissue, because it is conceivable that the B cells may have been recruited to the nasal mucosa after switching to IgE in lymphoid tissue while still containing the mRNA transcripts.

To circumvent this problem, we divided out-of-season biopsies from 4 allergic subjects (AT, CM, KG, and SS; Table I) into two halves, incubated one-half with GP allergen and the other half with medium alone for 24 h, and assayed GLT and CT (Fig. 5 and Table IV). As shown in Fig. 5A, we observed μ GLT in 3 of 4 allergen-stimulated and 1 of 4 nonstimulated half biopsies, ϵ GLT in 2 of 4 stimulated and 0 of 4 nonstimulated half biopsies, γ 1 GLT in 4 of 4 stimulated and 2 of 4 nonstimulated half biopsies, γ 3 GLT in 4 of 4 stimulated and 2 of 4 nonstimulated half biopsies, and γ 4 GLT in 2 of 4 stimulated and 1 of 4 nonstimulated half biopsies. Across the board (Table IV), all 5 GLT were expressed more frequently after allergen challenge, consistent with the seasonal results shown in Figs. 2 and 3 and Table III.

CT in the same cDNA was analyzed (Fig. 5B and Table IV). In this study (in contrast with the results obtained with untreated bi-

opsies; Table III), we observed the product of IgM switching to IgE (I ϵ -C μ CT) in 3 of 4 stimulated and 1 of 4 nonstimulated, and IgG4 switching to IgE (I ϵ -C γ 4 CT) in 1 of 4 stimulated half biopsies and 1 of 4 nonstimulated half biopsies. We also observed the products of IgG1 switching to IgE (I ϵ -C γ 1 CT) in 3 of 4 stimulated vs 0 of 4 nonstimulated half biopsies, and IgG3 switching to IgE (I ϵ -C γ 3 CT) in 3 of 4 stimulated vs 0 of 4 nonstimulated half biopsies. Across the board, CT, like GLT, predominated, 13 of 20, in the half biopsies stimulated with allergen vs 3 of 20 nonstimulated half biopsies (Table IV). Allergen induced CSR to IgE ex vivo from all the isotypes examined in this study, IgM, IgG1, IgG3, and IgG4, and therefore consistent with earlier studies on the stimulation of isolated B cells with IL-4 and anti-CD40 in vitro (12, 13, 17).

Immunohistochemical detection of B cell clusters in the nasal mucosa

Germinal center-like structures have been observed in the target organs of autoimmune diseases, notably rheumatoid arthritis (28). The appearance of these structures has been correlated with the evidence of local somatic hypermutation and, to a limited extent, CSR (29, 30). The structures appear to evolve from loose B cell clusters into well-defined organelles, with the appearance and composition of classical germinal centers in lymphoid follicles during the progression of the disease. To examine the distribution of B cells in the nasal mucosa, we stained frozen sections of a biopsy taken from a subject out of season with an Ab against the B cell-specific protein, CD19. We observed both isolated B cells and loosely organized B cell clusters in the lamina propria of the nasal mucosa (Fig. 6), which resembled those seen in sinovial tissues in the early stages of rheumatoid arthritis (28).

Discussion

AID, an enzyme required for CSR, has not been observed outside lymphoid tissue, and the evidence for local CSR itself is mostly indirect. An exception is one study of CSR from IgM to IgA in murine B cells from the gut mucosa, induced by TGF- β or contact

Table IV. Distribution of GLT and CT in out-of-season GP-allergic patients following ex vivo allergen challenge

	Culture Medium				Allergen Challenge			
	AT	CM	KG	SS	AT	CM	KG	SS
ϵ GLT					+	+		
μ GLT		+			+		+	+
γ 1 GLT		+		+	+	+	+	+
γ 3 GLT	+	+			+	+	+	+
γ 4 GLT		+				+	+	
I ϵ -C μ CT		+				+	+	+
I ϵ -C γ CT				+	+	+		+
I ϵ -C γ 1 CT					+		+	+
I ϵ -C γ 3 CT						+	+	+
I ϵ -C γ 4 CT				+				+

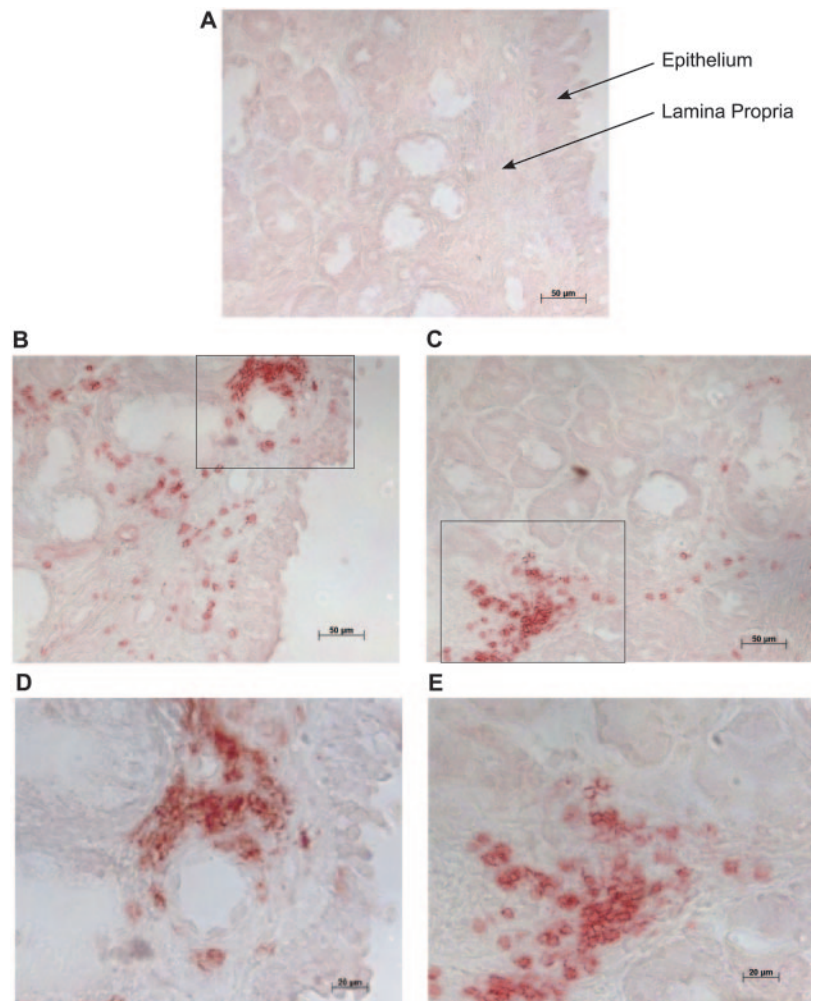


FIGURE 6. B cell clusters in the nasal mucosa. The surface B cell marker, CD19, was immunohistochemically stained with fuschin red chromogen. Each image is orientated such that the epithelium is on the *right*. *A*, No staining was observed when the sections were stained with a control Ab. The epithelium and lamina propria are detailed here. *B* and *C*, Both individual and clusters of CD19⁺ B cells in the same section. *D* and *E*, B cell clusters from *B* and *C*, respectively, at greater magnification. *A*–*C*, The scale bar represents 50 μ m. *D* and *E*, The scale bar represents 20 μ m.

with stromal cells *ex vivo* (22). Several studies have provided indirect evidence for CSR in the target organs of allergic rhinitis and asthma, in particular the appearance of IL-4 mRNA and ϵ GLT after local allergen challenge (4, 19) or seasonal exposure to allergen in the nasal mucosa in allergic rhinitis (5) and in the bronchial mucosa (31, 32) and bronchoalveolar lavage (33) in asthma. The demonstration that IL-4 and ϵ GLT are induced by incubation of the nasal biopsies from allergic subjects with allergen *ex vivo* (19) is a step closer to proof of our hypothesis. However, these markers indicate only that the B cells are primed to undergo CSR (because germline gene transcription is required for CSR), but not that CSR actually occurs.

H chain class switching is generally accompanied by somatic hypermutation, and both processes depend on AID (10). Allergen leads to the up-regulation of IL-4 in the nasal mucosa in allergic rhinitis (5), and IL-4 induces AID expression (16). We therefore predicted that AID would be expressed in the nasal mucosa of hay fever subjects who are exposed to allergens. However, we found that while AID was expressed in season, it was also expressed in the nasal mucosa of GP-allergic subjects out of season, although not in the nasal mucosa of the nonallergic control subjects. The expression of AID in out-of-season patients may reflect the ongoing secretion of IL-4 by mast cells, which may be activated by local IgE (1). Recent studies have shown that IgE itself may cross-link Fc ϵ RI on mast cells and stimulate the release of cytokines, including IL-4 (34).

In earlier work, we demonstrated that B cells in the nasal mucosa of GP-allergic subjects contain ϵ GLT during the GP season

(4) and after local allergen challenge between seasons (19), demonstrating the potential of the resident B cells to undergo H chain switching to IgE. In the present work, we demonstrate the GP-allergic subjects contain μ and γ GLT (γ 1, γ 3, and γ 4 GLT), in addition to ϵ GLT, in the GP season, providing evidence that all four isotypes have the potential to switch to IgE.

Cameron et al. (35) have presented evidence that ragweed allergen stimulates the formation of DNA circles corresponding to CSR from IgM and IgG4 to IgE in the nasal mucosa of ragweed-allergic subjects *ex vivo*, and the dependence of this process on IL-4. The present work demonstrates that GP allergen stimulates ϵ CT and CSR from IgM, IgG1, IgG3, and IgG4 *ex vivo*, and also upon seasonal exposure to the allergen *in vivo*. These studies provide the first direct evidence of local CSR from multiple isotypes to IgE in the nasal mucosa in allergic rhinitis. The role of allergen in inducing this switch must be important in the pathogenesis of allergic rhinitis, because allergen induces the degranulation of mast cells, sensitized by the IgE Abs, and this IgE must be replaced to maintain the sensitization of the mast cells. We have shown that IgE synthesis occurs in the nasal mucosa in allergic rhinitis (1), and thus, presumably, the class-switched B cells also differentiate into the plasma cells that secrete the IgE.

The direct evidence of local CSR in the nasal mucosa in allergic rhinitis may be extrapolated to other diseases in which there is indirect evidence of its occurrence. As mentioned above, such evidence has been presented in asthma (31, 32). Another type of indirect evidence comes from the observations of clonal families of V_H regions comprising IgE together with other isotypes in the

nasal mucosa in allergic rhinitis (36) and asthma (37). We may extrapolate further to autoimmune diseases in which similar indirect evidence of CSR has been reported in rheumatoid and reactive arthritis (28, 38), Sjögren's syndrome (39), ankylosing spondylitis (40), and diabetes (41). This evidence is further substantiated in autoimmune diseases by the evidence for the formation of germinal center-like structures viewed by immunohistochemistry. The observation of B cell clusters in the nasal mucosa in allergic rhinitis (Fig. 6), resembling the clusters that develop in synovial tissues in the early stages of rheumatoid arthritis (28, 29), suggests a similar development in rhinitis.

We suggest therefore that inflammation can give rise to processes, including AID expression, class switch recombination, and the generation of structures promoting these processes in the target organs of allergic and autoimmune diseases that occur normally only in lymphoid tissue. Because of the accessibility of the respiratory tract, CSR in allergic rhinitis and asthma may be a suitable target for topical therapies. Inhibition of CSR to IgE may bring about the long-term suppression of allergic disease.

Acknowledgments

We thank ALK for providing us with GP allergen, Phlp 5. We thank Kate Kirwan for assistance with the figures.

Disclosures

The authors have no financial conflict of interest.

References

- Smurthwaite, L., S. N. Walker, D. R. Wilson, D. S. Birch, T. G. Merrett, S. R. Durham, and H. J. Gould. 2001. Persistent IgE synthesis in the nasal mucosa of hay fever patients. *Eur. J. Immunol.* 31:3422.
- KleinJan, A., J. G. Vinke, L. W. F. M. Severijnen, and W. J. Fokkens. 2000. Local production and detection of (specific) IgE in nasal B cells and plasma cells of allergic rhinitis patients. *Eur. Respir. J.* 15:491.
- King, C. L., G. Thyphronitis, and T. B. Nutman. 1990. Enumeration of IgE secreting B cells: a filter spot-ELISA. *J. Immunol. Methods* 132:37.
- Durham, S. R., H. J. Gould, C. P. Thiesnes, M. R. Jacobson, K. Masuyama, S. Rak, O. Lowhagen, E. Schotman, L. Cameron, and Q. A. Hamid. 1997. Expression of ϵ germ-line gene transcripts and mRNA for the ϵ heavy chain of IgE in nasal B cells and the effects of topical corticosteroid. *Eur. J. Immunol.* 27:2899.
- Cameron, L., S. R. Durham, M. R. Jacobson, K. Masuyama, S. Juliusson, H. J. Gould, O. Lowhagen, E. M. Minshall, and Q. A. Hamid. 1998. Expression of IL-4, C ϵ RNA, and I ϵ RNA in the nasal mucosa of patients with seasonal rhinitis: effect of topical corticosteroids. *J. Allergy Clin. Immunol.* 101:330.
- Gray, D. 1993. Immunological memory. *Annu. Rev. Immunol.* 11:49.
- Snapper, C. H., and F. D. Finkelman. 1999. Immunoglobulin class switching. In *Fundamentals of Immunology*, 4th Ed. W. E. Paul, ed. Lippincott, Philadelphia, p. 831.
- Stavnezer, J. 2000. Molecular processes that regulate class switching. *Curr. Top. Microbiol. Immunol.* 245:27.
- Muramatsu, M., V. S. Sankaranand, S. Anant, M. Sugai, K. Kinoshita, N. O. Davidson, and T. Honjo. 1999. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J. Biol. Chem.* 274:18470.
- Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102:553.
- Okazaki, I. M., K. Kinoshita, M. Muramatsu, K. Yoshikawa, and T. Honjo. 2002. The AID enzyme induces class switch recombination in fibroblasts. *Nature* 416:340.
- Jabara, H. H., S. M. Fu, R. S. Geha, and D. Vercelli. 1990. Synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med.* 172:1861.
- Fujieda, S., K. Zhang, and A. Saxon. 1995. IL-4 plus CD40 monoclonal antibody induces human B cells γ subclass-specific isotype switch: switching to γ 1, γ 3, and γ 4, but not γ 2. *J. Immunol.* 155:2318.
- Cerutti, A., H. Zan, E. C. Kim, S. Shah, E. J. Schattner, A. Schaffer, and P. Casali. 2002. Ongoing in vivo immunoglobulin class switch DNA recombination in chronic lymphocytic leukemia B cells. *J. Immunol.* 169:6594.
- Fear, D. J., N. McCloskey, B. O'Connor, G. Felsenfeld, and H. J. Gould. 2004. Transcription of Ig germline genes in single human B cells and the role of cytokines in isotype determination. *J. Immunol.* 173:4529.
- Zhou, C., A. Saxon, and K. Zhang. 2003. Human activation-induced cytidine deaminase is induced by IL-4 and negatively regulated by CD45: implications of CD45 as a Janus kinase phosphatase in antibody diversification. *J. Immunol.* 170:1887.
- Zhang, K., E. A. Clark, and A. Saxon. 1991. CD40 stimulation provides an IFN- γ -independent and IL-4-dependent differentiation signal directly to human B cells for IgE production. *J. Immunol.* 146:1836.
- Pawankar, R., M. Okuda, H. Yssel, K. Okumura, and C. Ra. 1997. Nasal mast cells in perennial allergic rhinitis exhibit increased expression of the Fc ϵ RI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells. *J. Clin. Invest.* 99:1492.
- Cameron, L., Q. A. Hamid, E. Wright, Y. Nakamura, P. Christodoulopoulos, S. Muro, S. Frenkiel, F. Lavigne, S. R. Durham, and H. J. Gould. 2000. Local synthesis of ϵ germline gene transcripts, IL-4, and IL-13 in allergic nasal mucosa after ex vivo allergen exposure. *J. Allergy Clin. Immunol.* 106:46.
- Zhang, K., F. C. Mills, and A. Saxon. 1994. Switch circles from IL-4-directed ϵ class switching from human B lymphocytes: evidence for direct, sequential, and multiple step sequential switch from μ to ϵ heavy chain gene. *J. Immunol.* 152:3427.
- Kinoshita, K., M. Harigai, S. Fagarasan, M. Muramatsu, and T. Honjo. 2001. A hallmark of active class switch recombination: transcripts directed by I promoters on looped-out circular DNAs. *Proc. Natl. Acad. Sci. USA* 98:12620.
- Fagarasan, S., K. Kinoshita, M. Muramatsu, K. Ikuta, and T. Honjo. 2001. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* 413:639.
- Fokkens, W. J., T. M. Vroom, V. Gerritsma, and E. Rijntjes. 1998. A biopsy method to obtain high quality specimens of nasal mucosa. *Rhinology* 26:293.
- Yssel, H., J. E. De Vries, M. Koken, W. Van Blitterswijk, and H. Spits. 1984. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods* 72:219.
- Mills, F. C., M. P. Mitchell, N. Harindranath, and E. E. Max. 1995. Human Ig S γ regions and their participation in sequential switching to IgE. *J. Immunol.* 155:3021.
- Takahashi, N., U. Shintaro, M. Obata, T. Nikaide, S. Nakai, and T. Honjo. 1982. Structure of human immunoglobulin γ genes: implications for evolution of a gene family. *Cell* 29:671.
- Sambrook, J., T. Maniatis, E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- Kim, H. J., V. Krenn, G. Steinhauser, and C. Berek. 1999. Plasma cell development in synovial germinal centers in patients with rheumatoid and reactive arthritis. *J. Immunol.* 162:3053.
- Williams, D. G., S. P. Moyes, and R. A. Mageed. 1999. Rheumatoid factor isotype switch and somatic mutation variants within rheumatoid arthritis synovium. *Immunology* 98:123.
- Randen, I., D. Brown, K. M. Thompson, N. Hughes-Jones, V. Pascual, K. Victor, J. D. Capra, O. Forre, and J. B. Natvig. 1992. Clonally related IgM rheumatoid factors undergo affinity maturation in the rheumatoid synovial tissue. *J. Immunol.* 148:3296.
- Ying, S., M. Humbert, Q. Meng, R. Pfister, G. Menz, H. J. Gould, A. B. Kay, and S. R. Durham. 2001. Local expression of ϵ heavy chain of IgE in the bronchial mucosa in atopic and nonatopic asthma. *J. Allergy Clin. Immunol.* 107:686.
- Humbert, M., G. Menz, S. Ying, C. J. Corrigan, D. S. Robinson, S. R. Durham, and A. B. Kay. 1999. The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences. *Immunol. Today* 20:528.
- Fujieda, S., D. Diaz-Sanchez, and A. Saxon. 1998. Combined nasal challenge with diesel exhaust particles and allergen induces in vivo IgE isotype switching. *Am. J. Respir. Cell Mol. Biol.* 19:507.
- Kitaura, J., J. Song, M. Tsai, K. Asai, M. Maeda-Yamamoto, A. Mocsai, Y. Kawakami, F. T. Liu, C. A. Lowell, B. G. Barisac, et al. 2003. Evidence that IgE molecules mediate a spectrum of effects on mast cell survival and activation via aggregation of the Fc ϵ RI. *Proc. Natl. Acad. Sci. USA* 100:12911.
- Cameron, L., A. S. Gounni, S. Frenkiel, F. Lavigne, D. Vercelli, and Q. A. Hamid. 2003. SeS μ and SeS γ switch circles in human nasal mucosa following ex vivo allergen challenge: evidence for direct as well as sequential class switch recombination. *J. Immunol.* 171:3816.
- Coker, H. A., S. R. Durham, and H. J. Gould. 2003. Local somatic hypermutation and class switch recombination in the nasal mucosa of allergic rhinitis patients. *J. Immunol.* 171:5602.
- Snow, R. E., R. Djukanovic, and F. K. Stevenson. 1999. Analysis of immunoglobulin E V H transcripts in a bronchial biopsy of an asthmatic patient confirms bias towards V H 5, and indicates local clonal expansion, somatic mutation and isotype switch events. *Immunology* 98:646.
- Krenn, V., A. Konig, F. Hensel, C. Berek, M. M. Souto Carneiro, W. Haedicke, Y. Wang, H. Vollmers, and H. K. Muller-Hermelink. 1999. Molecular analysis of rheumatoid factor (RF)-negative B cell hybridomas from rheumatoid synovial tissue: evidence for an antigen-induced stimulation with selection of high mutated IgV H and low mutated IgV L / λ genes. *Clin. Exp. Immunol.* 115:168.
- Stott, D. I., F. Hiepa, M. Hummel, G. Steinhauser, and C. Berek. 1998. Antigen-driven clonal proliferation of B cells within the target tissue of an autoimmune disease: the salivary glands of patients with Sjögren's syndrome. *J. Clin. Invest.* 102:938.
- Voswinkel, J., K. Weisgerber, M. Pfreundschuh, and A. Gause. 2001. B lymphocyte involvement in ankylosing spondylitis: the heavy chain variable segment gene repertoire of B lymphocytes from germinal center-like foci in the synovial membrane indicates antigen selection. *Arthritis Res.* 3:189.
- Ludewig, B., B. Odermatt, S. Landmann, H. Hengartner, and R. M. Zinkernagel. 1998. Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue. *J. Exp. Med.* 188:1493.