

Isotypic and antigenic restriction of the blocking antibody response to ryegrass pollen: Correlation of rye group I antigen-specific IgG1 with clinical response

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To investigate the role of blocking antibodies in allergen immunotherapy (IT), we analyzed IgE, IgG, and IgG subclass 1 to 4 antibody responses to ryegrass group I antigen (RGGI) in a prospective double-blind, heterologous allergen, allergen-controlled trial of grass-pollen IT in 18 adults with seasonal rhinitis and asthma. Serum was assayed preseasonally before starting IT and again in midseason at time of documented highest natural exposure. Antibodies were measured by ELISA, and immunogenic specificities of ryegrass extract were examined by Western immunoblots. Nine subjects receiving grass-pollen IT and nine control subjects had similar clinical and immunologic status before IT. RGGI-specific IgE antibodies (sIgE) did not change from pretreatment levels in actively treated subjects but increased in control subjects ($p < 0.002$). RGGI sIgG increased approximately thirteenfold with active IT versus threefold during natural seasonal exposure ($p < 0.0005$). The IgG-blocking response to RGGI was restricted to IgG1 and IgG4. Ten nonatopic subjects had similar RGGI sIgG1 but lower or undetectable sIgE and sIgG4 than the 18 atopic study subjects. Active IT dramatically increased RGGI sIgG4 ($p < 0.001$) and to a lesser extent RGGI sIgG1 ($p < 0.01$). Immunoblots demonstrated eight IgE-binding ryegrass-polypeptide allergens, with RGGI ubiquitous, and 11 IgG-binding polypeptides, including all eight allergens. A negative correlation between seasonal rhinitis symptom-medication scores and RGGI sIgG1 levels was found ($r_s = -0.62$, $p < 0.01$), but no other immunologic parameters assayed were related to clinical improvement. Although RGGI sIgG4 predominates in the blocking response and is a useful marker of effective IT, early beneficial biologic effects may involve IgG1 antibodies. (J ALLERGY CLIN IMMUNOL 1987;79:387-98.)

It has been known since the 1930s that IT induces heat-stable serum antibodies that block the ability of homologous reaginic antibodies to produce an immediate cutaneous reaction.^{1,2} These "blocking" antibodies were demonstrated by Lichtenstein et al.³ to be of IgG isotype in 1968. Many studies of the efficacy and mechanism of action of IT have been conducted.⁴ Of the many immunologic changes induced by IT, increases in serum-blocking antibody levels correlate

Abbreviations used

RGGI:	Ryegrass group I antigen
sIgG:	Specific immunoglobulin G antibody
IT:	Allergen immunotherapy
SMS:	Symptom-medication scores
PBS:	Phosphate-buffered saline
RIA:	Radioimmunoassay
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
CBB:	Coomassie brilliant blue

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best with clinical response.⁵ However, clinicians aspiring to monitor IT by in vitro testing and researchers aspiring to understand the scientific basis of IT are frustrated by an inability to predict individual response by serum-blocking antibody level.

There are several possible reasons for this limitation. One is that blocking antibodies are epiphe-

nomenologic markers that do not play a role in the actual amelioration of symptoms. Another is that assays used to measure blocking antibodies imprecisely define isotypic and antigenic specificities that might mediate observed clinical changes. Finally, the complexities of natural exposure to allergens, biologic reactions involved, and subjective perceptions induced in different individuals, even when they are well matched for sensitivity, diminishes the efficacy of any *in vitro* assay.⁴

In an attempt to address these issues, we developed assays to examine the blocking antibody response to ryegrass pollen, the major pollen aeroallergen of northern California, which causes widespread seasonal allergic rhinitis and asthma in sensitive individuals.⁶ These assays enabled us to dissect the blocking response into its isotypic and antigen-specific components.

In a randomized, double-blind, heterologous, allergen-controlled clinical trial of grass-pollen IT in adults with seasonal rhinitis and asthma, we found evidence that the natural and IT-induced blocking antibody responses to RGGI demonstrate restriction to IgG1 and IgG4 isotypes and concurrence of IgE and IgG antibody specificities for discrete ryegrass antigens. We also found a previously unreported strong inverse correlation between serum RGGI IgG1 antibody levels and individual clinical responses, as measured by seasonal rhinitis SMS.

MATERIAL AND METHODS

Study subjects and serum samples

Patient selection, clinical and immunologic characteristics, quantification of natural and therapeutic RGGI exposure, and clinical efficacy data are presented in detail elsewhere.⁶ Briefly, 18 adults with a history of seasonal rhinitis and asthma occurring only during the grass-pollen season (April through July), 4+ positive skin prick test to ryegrass-pollen extract (erythema >20 mm with wheal and pseudopods), and no prior IT were randomly assigned to receive aqueous allergen extracts containing a seven grass-pollen mix standardized by RGGI content (active IT) or extracts containing allergens to which the subjects were also sensitive on skin testing but without grass pollen ("heterologous allergen control") in a double-blind protocol. Heterologous allergen was used instead of a true placebo such as carmelized histamine because prior experience demonstrated that the latter could be distinguished by physicians and subjects because of differences in reactions that could compromise the blinding of the study.⁷ With this methodology, eight of nine subjects receiving active grass IT and seven of nine receiving nongrass extracts believed they were receiving active IT at the end of the study. Blood was drawn for serologic studies at enrollment (winter 1984 to 1985) and again between May 15-22, 1985, near the peak of nat-

ural grass-pollen exposure. The nine subjects receiving active IT received cumulative RGGI doses of 7.2 to 99 μ g (mean = 34.8 μ g) at the time of seasonal sampling. Additional serum samples from 10 individuals with no history of clinical allergy, low serum IgE levels (<10 IU/ml), and no detectable allergen-specific IgE serum antibodies to 13 common allergens (RAST class 0) were also included as nonatopic control subjects.

Antigens

Aqueous grass-pollen allergen extracts (1:20 w/v in 50% glycerin-0.4% phenol) were obtained from Hollister-Stier, Spokane, Wash. The seven grass mix used for active IT comprised equal parts of Kentucky blue grass, orchard, timothy, sweet vernal, meadow fescue, and perennial ryegrass pollen containing 18.6 μ g/ml of RGGI at maintenance dosage (1:100 w/v) by ELISA inhibition.⁶ RGGI reference standard was obtained from the National Institute of Allergy and Infectious Diseases (catalog No. A611-901-185).

Purified immunoglobulin G (n = 8) and A (n = 1) myelomas were generously provided by the Stanford University Medical Center Blood Bank, courtesy of Dr. Carl Grumet. The IgG myelomas used had the following isoallotypes: IgG1m(f)_L-SC, IgG1m(f)_L-HE, IgG2m(n)_K-KO, IgG2m(n)_K-WO, IgG3m(g)_K-CR, IgG3m(b)_K-AR, IgG4_L-HA, and IgG4_K-TE. IgE_K-PS myeloma was kindly provided by Dr. Lewis Perelmutter, Division of Immunology, Bureau of Microbiology, Ottawa, Canada.

ELISA for RGGI sIgG

All ELISAs were performed in 96-well "U" microtiter plates (Dynatech Laboratories, Alexandria, Va.). Wells were coated with 10 μ g/ml of RGGI, solubilized in normal saline by adsorptive incubation of 100 μ l per well at 37° C for 1 hour. These concentrations were chosen on the basis of previous immunosorbent antigen-binding studies with trace radiolabeled antigen and high-titer sera. For each pair of antigen-coated test wells, a pair of control wells received saline without antigen. After aspiration, 200 μ l per well of blocking solution (0.1% human serum albumin-PBS-0.01% thimerosal) was added for 1 hour at 37° C. After aspiration, three washes with cold-wash buffer (PBS-0.05% Tween 20-thimerosal), and air drying, 100 μ l per well of test sera diluted 1:100, 1:500, and 1:1000 in dilution buffer (10% normal goat serum-PBS-thimerosal) was added to wells for 2 hours at 22° C. After aspiration, washing, and drying, all wells were exposed to affinity-purified goat antihuman IgG-peroxidase conjugate (Sigma Chemical Co., St. Louis, Mo.), 1:1000 in dilution buffer, for 1 hour at 37° C. After aspiration and washing, the plates were developed with 0.5 mg/ml of o-phenylenediamine in phosphate-citrate buffer, pH 6.0, at 22° C for 10 minutes. The reaction was stopped with 5 N sulfuric acid, and absorbance was immediately read in a microtiter plate spectrophotometer (Bio-Tek, Burlington, Vt.) at 490 nm. Net absorbances were generated by subtracting the average absorbance of paired control wells from the corresponding paired antigen-coated wells.

Standardization of IgG ELISA

A single reference serum with the highest titer of RGGI sIgG was used to standardize results. Serial dilutions of the reference standard were prepared for each microtiter plate and developed simultaneously with test sera. RGGI sIgG content of test sera was derived by interpolation to the standard curve at dilutions exhibiting linear parallelism. The RGGI sIgG content of this reference serum was investigated by saturation analysis in double antibody RIAs as described by Adkinson et al.⁸ When 10 ng of ¹²⁵I-RGGI (see below) was added to appropriate dilutions of reference serum, calculations of antibody content yielded values of ~175 µg/ml of RGGI sIgG. However, saturation curves never reached a flat plateau, a problem previously reported in attempts to standardize reference sera by this method.^{9, 10} Because this raised doubts regarding the accuracy of mass unit calculations, results are therefore reported in arbitrary ELISA units per milliliter where 1 unit is congruent to 1 µg/ml in RIA as described above.

Serial transfer ELISA for RGGI sIgE and blocking antibody

Quantitative ELISA for determination of sIgE and calculation of competitive antigen-binding antibodies of other isotypes were performed as previously described.¹¹

Double antibody RIA for RGGI sIgG

¹²⁵I-RGGI was prepared by a modification of the chloramine-T method.¹² RGGI (40 µl) was mixed for 10 minutes at 22° C with 40 µl of 0.3 mol/L of phosphate buffer, pH 7.5, 15 µl of ¹²⁵I (2 mCi/20 µl, specific activity, 17.4 Ci/mg, New England Nuclear, Boston, Mass.), and 40 µl of chloramine-T (30 mg/10 ml of phosphate buffer). The reaction was stopped with 40 µl of sodium metabisulfite (60 mg/10 ml of phosphate buffer) at 22° C for 5 minutes, and then 80 µl of 1% potassium iodide, and 220 µl of bovine serum albumin (1 mg/ml in PBS) was added. The mixtures were then chromatographed on Sephadex G-25 columns (Pharmacia Fine Chemicals, Piscataway, N. J.), packed in 10 ml plastic pipettes, and equilibrated with 1 mg/ml of bovine serum albumin (Sigma Chemical Co.) in PBS-0.1% sodium azide. The columns were eluted with equilibration buffer with collection of 10 drops per fraction. Each fraction was counted, and the four highest fractions from the first elution peak were pooled. Two 10 µl aliquots were withdrawn and counted for total radioactivity. Each aliquot then received 100 µl of pooled human serum and 1 ml of 20% trichloroacetic acid, followed by incubation for 1 hour at 22° C and centrifugation at 3000 rpm for 20 minutes. The pellets were then recounted to determine total precipitable protein.

Serum samples (100 µl) were diluted 1:40 in sodium borate buffer, pH 8.0, and incubated with an equal volume of ¹²⁵I-RGGI (5 ng) for 4 hours at 22° C. Then, 100 µl of affinity-purified rabbit antihuman IgG_R (Dako Corp., Santa Barbara, Calif.) 1:2 in test buffer was added and incubated overnight at 4° C, and the mixture was centrifuged

3000 rpm for 15 minutes at 4° C. The supernatants were decanted, and the pellets were washed four times with 2.5 ml of test buffer. Results were expressed as percent of total radioactivity added that was precipitated by the rabbit anti-serum.

ELISA for RGGI sIgG1 to 4 antibody

Determination of IgG subclass 1 to 4 antibodies to RGGI was performed as described above for IgG antibodies with the following exceptions: (1) Serum samples were diluted 1:10. (2) Murine monoclonal antihuman IgG1 to 4 subclass antibodies were used. These were all of IgG1 isotype and demonstrated to be human immunoglobulin subclass-specific in direct binding and competitive inhibition assays as described below. The antibodies used were as follows: anti-IgG1 was No. 912, Hybritech, La Jolla, Calif.; anti-IgG2 was clone GOM-1, Seward Laboratories, Birmingham, U. K.; anti-IgG3 was clone 211, Bethesda Research Laboratories, Gaithersburg, Md.; and anti-IgG4 was clone SK-44, Bio-Yeda, Rehovot, Israel. Monoclonal antihuman IgG subclass antibodies were incubated after aspiration of serum samples from wells for 2 hours at 22° C at equivalent antigen-binding titers determined as described below. For anti-IgG1, the dilution used was 1:2500; for anti-IgG2, 1:8000; for anti-IgG3, 1:15,000; and for anti-IgG4, 1:8000. (3) After washing and drying, 100 µl per well of goat antimouse IgG-peroxidase conjugate (Boehringer Mannheim, Indianapolis, Ind.) was incubated for 1 hour at 37° C. After aspiration and washing, substrate was added, and plates were developed as described above for IgG antibodies.

Standardization of IgG1 to 4 ELISA

Although IgG subclass assay standardization could be achieved semiquantitatively by use of myelomas and subclass antibodies in isotypic dilution curves, such an assay could not be considered accurate because of variable binding of myelomas to immunosorbent and variable primary antibody affinities for homologous myeloma antigens.¹³ Results are expressed as net absorbances, yielding semiquantitative comparative data.

Immunoblot analysis of ryegrass pollen protein antigens and antibodies

Polypeptides of perennial ryegrass pollen were separated by SDS-PAGE under dissociating conditions with discontinuous buffer by use of 12.5% SDS-polyacrylamide gels (16 by 16.8 by 0.15 cm) loaded with 10 µl per channel of dialyzed, lyophilized rye extract (1 mg/ml) and run in 0.1% SDS-Tris buffer.¹⁴ Calibration curves for determination of molecular weight of rye polypeptides were constructed by plotting relative migration values (Rf) for low molecular weight (Calibration kit proteins, Pharmacia) versus the logarithms of their corresponding molecular weight.

After SDS-PAGE was completed and marginal strips were removed for staining, the remaining middle portion of the gel was brought into apposition with nitrocellulose sheeting

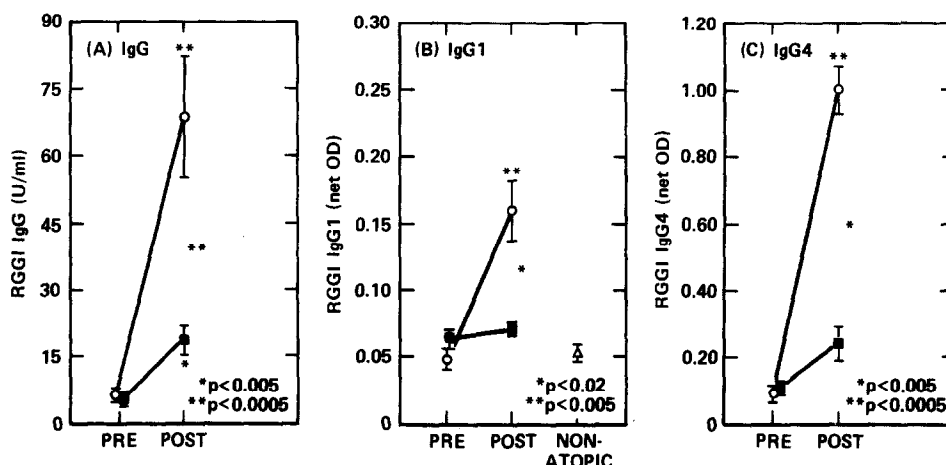


FIG. 1. A, RGGI-specific sIgG levels in nine actively treated subjects (open circles) and nine control subjects treated with heterologous allergens (closed squares) measured preseasonally before institution of IT (PRE) and seasonally while they were receiving maintenance IT (POST). B, RGGI sIgG1 levels in these two groups. Note similar sIgG1 levels in 10 nonatopic control subjects (open triangle) compared with the atopic groups. C, RGGI sIgG4 levels in the two IT groups. Note difference in subclass response scale (y axis) from sIgG1.

(Bio-Rad Laboratories, Richmond, Calif.) and transferred electrophoretically in a Hoefer scientific transfer unit at 100 V (initially 650 mA) and run for 45 minutes.¹⁵ After transfer, a marginal strip containing rye polypeptides and molecular weight standards was cut and stained with 0.25% CBB R-250. The remainder of the sheet was then prepared for immunoblotting.

The sheet was first blocked by incubation with 0.1% human serum albumin-PBS-thimerosal for 1 hour at room temperature with continuous gentle agitation. After three washes with 0.9% NaCl-0.5% Tween 20 wash buffer, the membrane was cut into 1 cm strips and incubated with serum diluted in wash buffer (for IgE, IgG1, and IgG4, 1:10 and for IgG, 1:100) for 3 hours at room temperature. After three washes, strips were then incubated with monoclonal anti-human IgE, IgG1, or IgG4 1:5000 overnight at room temperature with agitation, followed by a final incubation with goat antimouse IgG-peroxidase conjugate 1:1000 in wash buffer. (For IgG, strips were incubated with goat antihuman IgG-peroxidase 1:1000 and developed.) After three washes, the strips were transferred into freshly prepared substrate buffer (5 mg of 3,3'-diaminobenzidine, 10 ml of PBS, 4 μ l of 30% H₂O₂, and 25 μ l of 10% NiCl₂) and developed at room temperature. The reaction was stopped by washing the strips twice in distilled water.

Statistical analysis

Mean antibody levels between groups were compared by the nonparametric Mann-Whitney statistic, proportions were tested by chi-square analysis with Yates correction, and correlations between sample data were tested by nonparametric Spearman rank coefficients. Statistics were computed by use of Statview software programs (BrainPower, Inc., Calabasas, Calif.) on a Macintosh 512K microprocessor (Apple Computer, Cupertino, Calif.). Variances are expressed as standard error of the mean throughout the text.

RESULTS

RGGI sIgG antibody responses

RGGI sIgG ELISA. The ELISA for RGGI sIgG was sensitive, specific, and reproducible. The interassay coefficient of variation was approximately 11% for test sera ($n = 5$). Sensitivity, determined with doubling dilutions of the reference serum, was 2 ELISA units (≈ 2 ng/ml) by use of +2 SD above the mean absorbance generated by filler-coated control wells as the cutoff. The assay was antigen-specific; heterologous antigens did not appreciably affect absorbances at concentrations up to 1 mg/ml.

IgG antibody levels to ryegrass extract determined by quantitative ELISA were compared to those obtained by double-antibody RIA and inhibition of IgE binding in transfer plate ELISA.^{9, 10} ELISA results correlated well with those obtained by isotype-specific double-antibody RIA ($y = 50.7 \times + 33.1$, $r_s = +0.88$, $p < 0.001$) and also with inhibition in the plate-transfer method for quantitation of IgE antibodies, which detects competitive antigen-binding antibodies of non-IgE isotypes ($y = 2.4 \times + 22$, $r_s = +0.71$, $p < 0.001$).

RGGI sIgG levels in relation to natural exposure and grass-pollen IT (Fig. 1, A). Preseasonal pretreatment RGGI sIgG levels were similar in the actively treated and control IT groups (5.8 ± 1.4 and 5.0 ± 1.3 U/ml, respectively). Seasonal levels in the control group increased a mean of 3.4-fold to 18.4 ± 3.2 U/ml ($p < 0.005$), whereas those in the actively treated group increased a mean 12.7-fold to 68.6 ± 13.8 U/ml ($p < 0.0005$). The difference in posttreatment seasonal levels between the actively treated

and control IT groups was highly significant ($p < 0.0005$). RGGI sIgG closely paralleled IgG antibody levels to crude rye-grass-pollen extract (data not presented). Of note, no subject receiving heterologous allergen IT had serum seasonal RGGI sIgG levels >32 U/ml, whereas no subject receiving grass-pollen IT had seasonal RGGI sIgG levels <44 U/ml.

RGGI sIgG antibodies in nonatopic sera were investigated by saturation analysis of ^{125}I -RGGI precipitated in double antibody RIA with pooled sera from 10 nonatopic subjects; this experiment yielded an RGGI sIgG content of 0.2 U/ml. Four of these 10 individuals' serum samples had low but detectable RGGI sIgG in ELISA.

RGGI sIgE responses

RGGI sIgE levels in relation to natural exposure and grass-pollen IT. RGGI sIgE antibody levels were measured by transfer ELISA. Mean RGGI sIgE rose from 90.5 IU/ml preseasonally to 103 IU/ml seasonally in the actively treated group; this increase was not statistically significant. In contrast, mean RGGI sIgE rose from 28 to 64 U/ml in the control group ($p < 0.001$).

RGGI sIgG subclass 1 to 4 antibody responses: Subclass ELISA

Binding of myeloma proteins to immunosorbent. To investigate the use of human IgG subclass-specific murine monoclonal antibodies for use in ELISA, the binding of myeloma antigens involved in preliminary sensitivity and specificity assays first required verification. This was done by coating microtiter wells with identical concentrations of the various IgG myelomas and developing the plates uniformly with goat anti-human IgG_{FC}-peroxidase. When binding of myeloma IgG1-SC was arbitrarily established as 100% binding (net absorbance = 1.669 under test conditions), binding of other myelomas was found to be sufficiently comparable to allow comparative analysis (range of myeloma binding = 83.7% for IgG4-HA to 101.4% for IgG2-KO).

Assessment of nonspecific binding by antimouse IgG-peroxidase conjugate to human myeloma proteins. In order to achieve sufficiently low background absorbances to allow quantitative assays, it was necessary to use a developing antimouse antibody-enzyme conjugate that had little or no cross-reactivity with human IgG antigenic determinants. To test a number of potential conjugates, plate wells were coated with the various myelomas, exposed to the candidate conjugates, and directly developed with substrate. With the absorbance generated by the goat antihuman IgG-peroxidase conjugate as 100% reactivity, a wide variety of cross-reactivity was observed

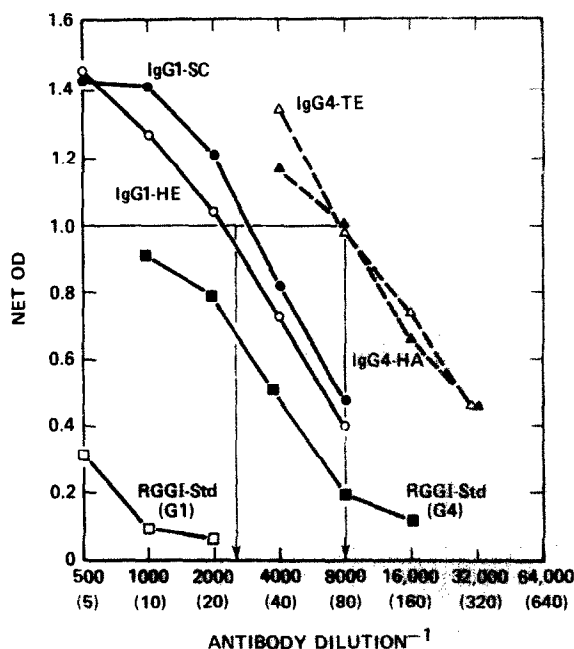


FIG. 2. Titration curves for determination of equivalent antigen-binding dilutions of monoclonal antihuman IgG1 and antihuman IgG4. Dilutions of monoclonal antibody yielding net absorbance of 1.0 in primary reactivity with homologous human myelomas were selected for use in IgG subclass ELISA. For anti-IgG1, this was 1:2500; for anti-IgG4, this was 1:8000. Titration curves for reference serum RGGI sIgG1 and sIgG4 antibodies are also presented with corresponding dilutions indicated in parentheses along the x axis, illustrating parallelism of isotype and antibody dilution curves.

with different conjugates tested (maximum cross-reactivity, 76.7% with one commercial antibody tested). One antibody conjugate (Hybritech) that exhibited $<5\%$ cross-reactivity with human myelomas was selected for use in the ELISA assay (cross-reactivity range = 2.5% with IgG2-WO to 4.8% with IgG4-TE).

Monoclonal antibody-equivalent, antigen-binding titer. To allow comparison of subclass antibody responses, it was necessary to correct for the differing affinities, which the various murine monoclonal antibodies used, exhibited for their respective antigens.¹⁶ This was accomplished by constructing titration curves of each antibody against homologous myeloma proteins and examining the results for (1) linear parallelism with each other, (2) the dilution of each monoclonal antibody that yielded the same net absorbance with its homologous myelomas as the other subclasses, and (3) linear parallelism between anti-isotype curves and RGGI-antibody titration curves. Fig. 2 illustrates the titration curves for antihuman IgG1 and IgG4 against homologous myelomas done just before analysis of sera. Here, the equivalent antigen-binding absorbance chosen was 1.0, which oc-

TABLE I. Direct binding assay for cross-reactivity of anti-isotype monoclonal antibodies

Myeloma	Percent binding by			
	Anti-IgG1	Anti-IgG2	Anti-IgG3	Anti-IgG4
IgG1-SC	100	0.1	0.1	1.9
IgG1-HE	100	0	0	0.5
IgG2-KO	5.9	100	1.2	2.5
IgG2-WO	3.1	100	5.7	2.1
IgG3-CR	2.9	0.8	100	3.4
IgG3-AR	0.8	0	100	0
IgG4-TE	0	0	0	100
IgG4-HA	0	0	0	100

curred at a dilution of 1:2500 for anti-IgG1 and 1:8000 for anti-IgG4. Fig. 2 also illustrates that isotype-anti-isotype (IgG subclass myeloma-murine monoclonal antihuman IgG subclass antibody) titration curves exhibited linear parallelism with antigen-antibody (RGGI-human anti-RGGI sIgG) titration curves at the appropriate dilutions.

Equivalent myeloma-binding titers of anti-IgG2 and anti-IgG3 were 1:8000 and 1:15,000, respectively. However, RGGI sIgG2 or IgG3 antibodies were not detectable in screening of pretreatment or posttreatment sera from the 18 study subjects as well as a further 23 subjects receiving grass-pollen IT.

Specificity of murine monoclonal antibodies for human IgG subclasses. Specificity of a number of commercially available IgG subclass antibodies was assessed by two methods, a direct binding assay and an inhibition assay, as follows.

DIRECT BINDING ASSAY. Microtiter plate wells were coated with the various myelomas, exposed to homologous and heterologous monoclonal antibodies at equivalent antigen-binding titers, and identically developed with goat antimouse-peroxidase conjugate. Binding of a monoclonal antibody to its homologous subclass myelomas was taken as 100% for calculation of percent binding to heterologous myelomas. As presented in Table I, the four monoclonal antibodies selected for ELISA demonstrated <6% cross-reactivity with heterologous IgG subclass myelomas tested; 5.9% cross-reactivity of antihuman IgG1 with IgG2-KO and 5.7% cross-reactivity of antihuman IgG3 with IgG2-WO were the two highest values; others were <3.4%. No cross-reactivity was observed with IgA or IgE myelomas.

INHIBITION ASSAY. This method, verifying the specificity of the antihuman monoclonal antibodies, used the principle of dose-dependent inhibition of murine monoclonal antibody binding to RGGI-specific human IgG subclass antibodies immobilized on rye allergosorbent after preincubation of monoclonal an-

tibody with homologous, but not heterologous, myeloma protein. This method was applied to IgG1 and IgG4 subclass-specific monoclonal antibodies, since only these two RGGI-specific antibody isotypes were present in serum in sufficient amounts for analysis (see above). After coating of plates with rye extract followed by known high-titer serum, equivalent antigen-binding titers of murine monoclonal antihuman IgG1 or IgG4 were added and developed with anti-mouse IgG-peroxidase conjugate. These two monoclonal antibodies were preincubated with tenfold incremental concentrations (0.1 to 100 μ g/ml) of polyclonal human IgG and IgA and with IgE, IgG1, IgG2, IgG3, IgG4, and Bence Jones κ myeloma proteins before addition to the ELISA. As presented in Fig. 3, only homologous IgG subclass myelomas and polyclonal IgG could inhibit binding of the monoclonal antibody to immobilized serum antibodies with one exception, a slight inhibition of antihuman IgG1 by high concentrations of IgG3-CR (15% inhibition at 10 μ g/ml and 27% inhibition at 100 μ g/ml).

RGGI sIgG1 levels in relation to natural exposure and grass-pollen IT (Fig. 1, B). Preseasonal RGGI sIgG1 levels were similar in the actively treated and control IT groups (mean net absorbance = 0.047 ± 0.008 versus 0.065 ± 0.010 , respectively). Active IT increased RGGI sIgG1 levels to 0.160 ± 0.031 ($p < 0.005$), but no change was found in the control group (0.071 ± 0.009). RGGI sIgG1 levels in nonatopic individuals were similar to those observed in untreated allergic individuals (0.053). IgG1 antibody levels to crude ryegrass-pollen extract were quite similar to levels reported for purified RGGI in the various groups (data not presented).

RGGI sIgG4 antibody levels in relation to natural exposure and grass-pollen IT (Fig. 1, C). Preseasonal levels of RGGI sIgG4 did not differ in the two allergic study groups (net absorbance = 0.093 ± 0.019 in the active group and 0.110 ± 0.029 in the control group) and were undetectable in nonatopic sub-

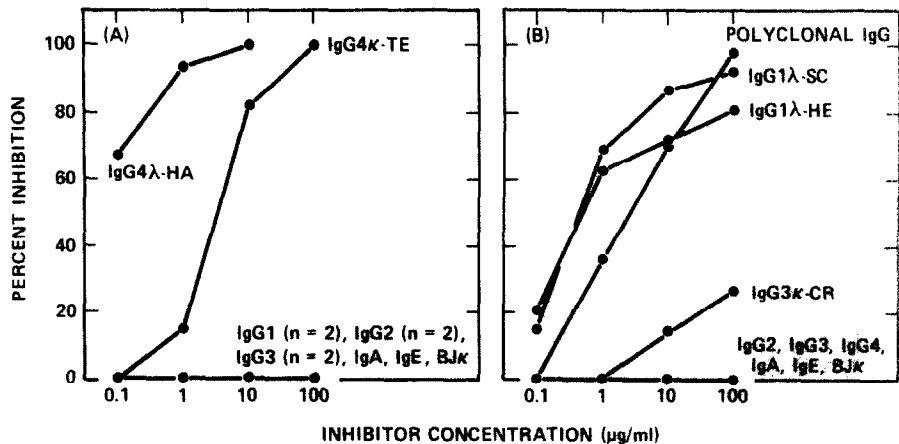


FIG. 3. Inhibition assays for determination of IgG subclass specificity of monoclonal antihuman IgG1 and antihuman IgG4. **A**, Inhibition of binding of anti-IgG4 to rye-immobilized high-titer serum antibody by preincubation with increasing concentrations of IgG4 myelomas HA and TE. Fifty percent inhibition was observed with <100 ng/ml of HA and 5 μg/ml of TE. In contrast, there was no inhibition by up to 100 μg/ml of IgG1 to 3, IgA, and IgE myelomas and Bence Jones κ protein. **B**, Inhibition assay for anti-IgG1 specificity. Binding was inhibited by homologous IgG1 myelomas SC and TE (50% inhibition at 500 ng/ml) and purified polyclonal human IgG (50% inhibition at 5 μg/ml) but not heterologous myelomas, except for IgG3-κ, which caused 27% inhibition at 100 μg/ml.

jects. Active grass-pollen IT dramatically increased RGGI sIgG4 levels (1.003 ± 0.099 , $p < 0.0005$), whereas the control group demonstrated a trend toward a small seasonal increase (0.242 ± 0.066 ; $p = 0.07$). The difference in the seasonal mean between the two groups was highly significant ($p = 0.0006$). As with IgG and IgG1, IgG4 antibodies to crude rye extract were similar in the various groups to antibodies reported above for purified RGGI (data not presented).

Immunoblot analysis of IgE, IgG, IgG1, and IgG4 antibody responses to rye antigens

After rye extract underwent SDS-PAGE, eight protein bands of molecular weight 12.2 to 86 kd were visible after CBB staining of the gel. After electrophoretic transfer to nitrocellulose, 11 bands were visible with CBB stain (Fig. 4, lane 4). With India ink staining, which was more sensitive than CBB, 18 bands could be visualized (Fig. 5, lane A). Examples of binding of IgG, IgG4, and IgE antibodies to rye-grass polypeptides are presented for two actively treated patients in Fig. 4, lanes 1 to 3 and 7 to 9. Of the proteins transferred from SDS-PAGE and stained on nitrocellulose with CBB, up to eight (molecular weight 13.2 to 52 kd) bound IgE antibodies in patient sera. RGGI (polypeptide No. 6 in Fig. 5, 33 kd) bound IgE and IgG (both IgG1 and IgG4) from all patients. IgG and IgG4 immunoblots before grass-pollen IT were few in number and weakly visualized. In con-

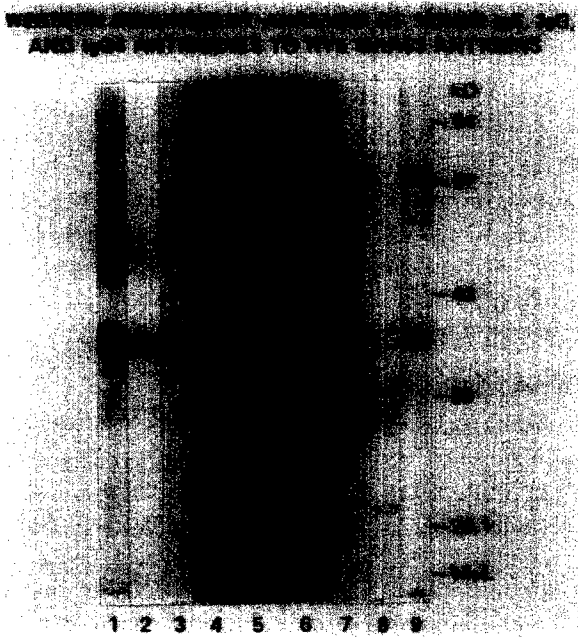


FIG. 4. Immunoblot analysis of rye-grass IgG-, IgG4-, and IgE-binding proteins. Rye polypeptides were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose, and stained with CBB (lane 4). Molecular weight standards are presented in lane 5 and purified RGGI (33 kd) in lane 6. Lanes 1 to 3 illustrate IgG-, IgG4-, and IgE-binding proteins, respectively, for one actively treated subject; lanes 7 to 9 illustrate the same for a second treated subject. Note concurrence of IgE- and IgG-binding proteins in individual subjects and heterogeneity of responses to non-RGGI antigens in different individuals.

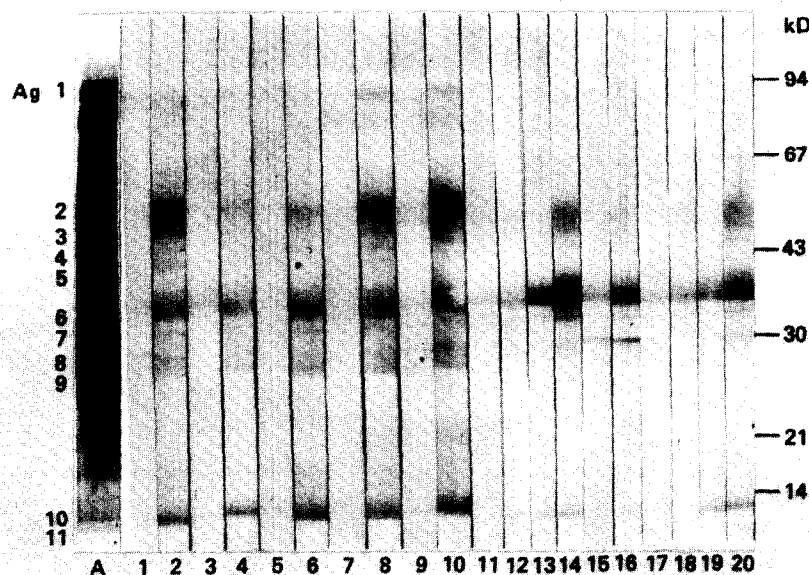


FIG. 5. Immunoblots of ryegrass proteins binding IgG (lanes 1 to 10) and IgE (lanes 11 to 20) before (odd numbered lanes) and after (even numbered lanes) institution of IT. Lane A illustrates rye proteins separated on SDS-PAGE, transferred to nitrocellulose, and stained with 0.5 μ l/ml of India ink for maximum sensitivity. IgG-binding proteins ($n = 11$) are indicated along left ordinate with corresponding molecular weight standard positions illustrated on right ordinate. Note immunogenic dominance of RGGI (33 kd, polypeptide No. 6), concurrence of IgG- and IgE-binding proteins in individuals, heterogeneity of IgG- and IgE-binding proteins between individuals, and effect of grass-pollen IT in increasing IgG (and, to a lesser extent, IgE) binding.

trast, after IT, up to 11 IgG-binding proteins (13.2 to 86 kd) were visualized (Fig. 5). Of note, all IgG-binding proteins also bound IgE (polypeptide Nos. 2, 3, 5 to 9, and 11 in Fig. 5). In addition to RGGI, three other antigens (polypeptide Nos. 2, 3, and 5) bound IgG1 in all subjects, including a nonatopic control subject (not presented).

Clinical immunologic correlations

Daily seasonal SMS were completed by each patient for both rhinitis and asthma, as previously described.⁶ There was a direct correlation between seasonal SMS for rhinitis and asthma ($r_s = +0.67$ and $p = 0.006$).

Correlation coefficients were calculated between asthma and rhinitis SMS and RGGI-specific antibody isotype levels as well as various antibody isotype ratios (e.g., IgG/IgE). RGGI-specific IgG1 levels correlated inversely with seasonal rhinitis SMS ($r_s = -0.62$, $p = 0.01$, and Fig. 6). However, there was no correlation between rhinitis SMS and other antibody isotypes or ratios. No correlations were found between asthma SMS and other antibody isotypes or ratios, with the exception of a suggestion of negative correlation between asthma SMS and RGGI sIgG ($r_s = -0.45$ and $p = 0.064$; Table II).

DISCUSSION

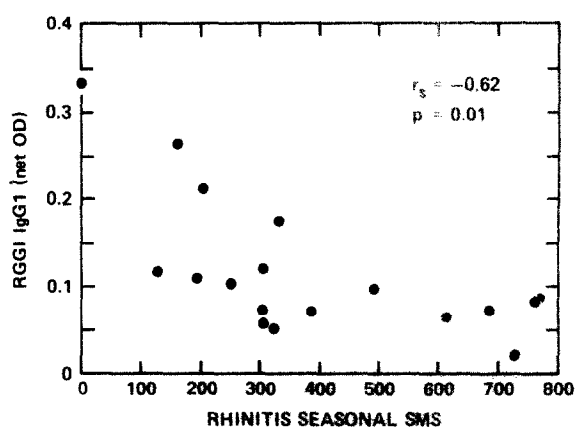
Although allergen-specific immunotherapy was introduced into clinical practice 75 years ago, treatment has remained essentially unchanged from its empirical origins, largely because of ignorance regarding mechanism of action and lack of reliable in vitro tests that correlate closely with the clinical response. A host of immunologic changes have been noted to occur with IT, including antigen-specific declines in immediate cutaneous reactivity, levels of serum IgE antibodies, basophil histamine release, lymphocyte proliferation, blunting of seasonal rises in specific IgE, and increases in suppressor T cell activity and specific IgG levels.¹⁷ However, only the rise in IgG antibodies (which block cutaneous reactivity in vivo and basophil histamine release in vitro) correlates statistically with clinical improvement in patients with allergic rhinitis caused by ragweed or grass pollen,^{18, 19} but the lack of predictive accuracy between blocking antibody level and symptomatic outcome in a given individual has led many to question a causal relationship between the two. This study represents an attempt to clarify this relationship by means of immunoassays that subdivide the blocking response into its isotypic components and allergen polypeptide specificities.

TABLE II. Correlation coefficients between seasonal rhinitis and asthma SMS and seasonal RGGI-specific serologic responses

Antibody	r_s	p (two-tailed)
For rhinitis		
IgG	-0.38	0.11
IgG1	-0.62	0.01
IgG4	-0.18	0.46
IgE	-0.40	0.10
IgG/IgE	-0.10	0.69
IgG1/IgE	-0.14	0.59
IgG4/IgE	+0.12	0.62
IgG1/IgG4	-0.30	0.21
For asthma		
IgG	-0.45	0.06
IgG1	-0.36	0.14
IgG4	-0.23	0.35
IgE	-0.25	0.30
IgG/IgE	-0.18	0.45
IgG1/IgE	-0.05	0.85
IgG4/IgE	+0.08	0.74
IgG1/IgG4	-0.12	0.62

One of our goals was to use a simple, reliable, and quantitative immunoassay for measuring IgG antibodies to allergens. Previously described methods include nonisotype-specific measurement of antibody by hemagglutination, ammonium sulfate-precipitable radiolabeled antigen-binding, inhibition of basophil histamine release, RAST interference, staphylococcal protein A, RIA, particle-counting immunoassay, and inhibition of specific IgE in the transfer ELISA.^{11, 20-25} In recent years, quantitative isotype-specific measurement of IgG allergen-binding antibodies by double-antibody RIA, quantitative ELISA, and microtiter solid-phase RIA have also been described.^{12, 26, 27} Quantitative ELISA has advantages of simplicity in performance and equipment required, reproducibility with high sensitivity, antigen- and antibody-isotype specificity, and no requirement for purified radiolabeled allergen or antibody. Our ELISA results correlated well with two other assays for specific IgG, double-antibody RIA, and IgE transfer plate-inhibition assay.^{11, 12}

We found that untreated individuals with grass pollen-induced rhinitis and asthma had substantial levels of RGGI sIgG serum antibodies compared to non-atopic individuals. Furthermore, seasonal natural exposure alone caused a threefold increase in sIgG levels. This is undoubtedly caused by the extremely high seasonal pollen counts encountered in northern California, which are five to 20 times higher than

**FIG. 6.** Correlation of rhinitis seasonal SMS (abscissa) with seasonal RGGI sIgG1 antibody levels in the 18 IT study subjects.

counts reported in studies from the Midwest, Eastern seaboard, or England.⁶ Despite this natural exposure, active grass-pollen IT cause a much greater ($p < 0.0005$), nearly thirteenfold increase, in RGGI sIgG levels (Fig. 1, A). We were disappointed but not surprised to find that specific IgG levels did not correlate with SMS for either rhinitis or asthma.

Our assay for RGGI sIgE, the transfer IgE ELISA, was designed to minimize the effect of competitive IgG antibodies.¹¹ As in many other studies, we found that IT blunted the seasonal rise in IgE antibody.

Although nonisotype-specific or IgG class-specific assays for blocking antibodies have not yielded predictive information, studies analyzing the IgG antibody response in terms of its subclass components suggest that IgG4 antibodies might be of special significance because this isotype demonstrated the most dramatic increases with IT.²⁸⁻³⁰ Aalberse et al.³¹ provided an attractive theoretical basis for these observations by demonstrating preferential induction of IgG4 isotype antibodies under conditions of large-dose, long-term parenteral immunization. The recent advent of monoclonal anti-isotype antibodies has greatly increased the accuracy of such studies by reducing the possibility of cross-reactivity. It is important to validate the sensitivity and specificity of such monoclonal antibodies for each antigen studied, since large variations have been documented in careful evaluations of anti-isotypic monoclonal antibodies.³² We established the use of the monoclonal antibodies used in this study by direct binding and inhibition experiments with a panel of purified myeloma proteins (Table I and Fig. 3).

The isotypic pattern of immune response to RGGI demonstrates that the higher levels of IgG antibodies

found in untreated allergic individuals preseasonally compared to nonatopic individuals (~ 5 versus ~ 0.2 U/ml, respectively) are due to IgG4 because RGGI sIgG1 levels are similar in atopic and nonatopic individuals (Fig. 1, B). This suggests that there are similar immunoregulatory controls operating for IgE and IgG4 production that may explain the prevalence of elevated IgG4 antibody levels to allergens in atopic individuals.³³⁻³⁶ Recent definition of the chromosomal arrangement of genes coding for immunoglobulin heavy chain constant region domains offers some support for such linkage.³⁷

Recently Djurup et al.,^{38, 39} studying the IgG subclass antibody response to timothy grass-pollen and yellow jacket venom in allergic patients receiving IT with monoclonal antibody-based immunoassays, noted the dramatic increases in IgG antibodies with IT previously reported with polyclonal reagents²⁸⁻³⁰ but, in addition, found isotypic restriction of the blocking response to IgG1 and IgG4. Of note, better clinical outcome was observed in both sets of patients if strong early and sustained IgG1 antibody responses and only late rises in IgG4 were induced by IT.

In this study, we report that the blocking antibody response to RGGI (and crude rye extract) is also restricted to IgG1 and IgG4. Although the IgG4 antibody response appeared to be dominant in the IgG antibody seasonal response in both untreated and treated patients, we found a surprisingly strong inverse correlation between IgG1 antibody levels to RGGI and SMS for rhinitis ($r_2 = -0.62$, $p = 0.01$, and Fig. 6). In contrast, no correlations were observed between specific IgG, IgG4, IgE, IgG/IgE, IgG1/IgE, IgG4/IgE, or IgG1/IgG4 ratios and clinical response. We could not confirm an earlier suggestion that specific IgG/IgE ratios are of greater value than single isotype antibody assays in evaluating IT.⁴⁰

It is thus possible that while measurements of specific IgG, and IgG4 in particular, reflect the immunologic stimulus of IT, IgG1 may be of greater importance biologically, at least early in IT. Possible mechanisms include competition with specific IgE for allergen in serum or secretions.^{4, 5, 17} Immunoregulation of IgE synthesis by feedback inhibition may account for late falls in IgE antibody levels but does not appear adequate to explain early clinical results in the face of absolute rises in IgE antibody. Provocative recent evidence from an animal model of allergic asthma and in IT of ragweed-induced allergic rhinitis suggests that blocking antibodies may mediate clinical improvement via suppressive effects on the late-phase allergic response.^{41, 42} Our results as well as those of Djurup et al.^{38, 39} suggest that attention should be paid

to the respective roles of specific IgG1 and IgG4 on changes in late-phase reactivity.

Besides the potential importance of antibody isotype in understanding the efficacy of IT, the role of the allergenic specificities involved must be considered. If we could measure antibodies of different isotypes directed at the same allergenic epitopes, would the quality of information obtained improve? We used the technique of immunoblotting to examine this question.^{15, 43, 44} Although it is possible that immunogenic polypeptides might be lost during electrophoretic transfer or immunostaining, our results confirm that immunoblotting can easily define allergens of major and minor importance and thus allow much greater precision in identifying and selecting allergens for analysis of immune response.

Immunoblotting revealed the complexity of the IgE and IgG responses to rye extract with at least eight and 11 immunogenic antigens discernable, respectively. It demonstrated the immunogenic dominance of RGGI as allergen and blocking antibody. The interindividual variations in the specificity of response to minor antigens were also demonstrated. Finally, of most interest and importance clinically, it indicated concurrence of IgE and IgG antibody responses to discrete antigens in individuals. This pattern of "parallel antigen recognition" by IgE and IgG antibodies has been described in immunoblot studies of the response to parasitic infestation in which blocking IgG antibodies appear to modulate immediate hypersensitivity reactions³⁶ and strongly suggest a protective role for blocking antibodies directed against identical or similar antigenic epitopes as those provoking an IgE response.

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The temporal relationship between increases in airway responsiveness to histamine and late asthmatic responses induced by occupational agents

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*The temporal relationship between increases in airway responsiveness and the late asthmatic response was assessed in nine patients challenged with occupational agents toluene diisocyanate (one patient), carmine (one patient), maleic anhydride (two patients), colophony (four patients), and trimellitic anhydride (one patient). The provocation concentration of histamine causing a 20% decrease in FEV₁ (PC₂₀) was measured before challenge and at approximately 3 hours and 24 hours on control and active-challenge days. Thirteen active challenges provoked eight definite late asthmatic responses (maximum fall in FEV₁ > 15% at 3 to 11 hours). At 3 hours after the challenges that provoked late responses, there was a significant ($p < 0.02$) decrease in PC₂₀ that was more ($p < 0.03$) than that observed for the five tests provoking early (late FEV₁ fall 0% to 5%) or equivocal late (FEV₁ fall 6% to 15%) responses. At 24 hours, PC₂₀ remained decreased ($p < 0.05$), although it was less so than at 3 hours ($p < 0.05$) and not significantly when compared with challenge tests causing single early or equivocal late responses. The 3-hour decreases in PC₂₀ were identified when FEV₁ (five of seven observations) was >90% of prechallenge values. For the nine independent tests, the 3-hour decreases in PC₂₀ correlated ($r = 0.72$; $p < 0.05$) with the magnitude of the late falls in FEV₁, whereas this was not observed at 24 hours ($r = 0.35$; p , not significant). These results demonstrate that in subjects challenged with occupational agents, increases in histamine airway responsiveness precede the late asthmatic response, occur independently of changes in airway caliber, and correlate with the magnitude of the subsequent late response. We suggest that in subjects with occupational asthma, the late asthmatic response depends on the tissue events, possibly airway inflammation, that underlie the preceding increase in airway responsiveness. (*J ALLERGY CLIN IMMUNOL* 1987;79:398-406.)*

Airway hyperresponsiveness, an increased bronchoconstrictor response to nonspecific provocative

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Abbreviations used

PC₂₀: Provocation concentration of histamine causing a 20% decrease in FEV₁
TDI: Toluene diisocyanate

stimuli, is now considered a central feature of bronchial asthma. The association between increases in airway responsiveness and late asthmatic responses has been observed after inhalation of both aller-