

Pollen-induced chemiluminescence: inhibition by serum from allergic individuals

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The interaction between pollen grains, polymorphonuclear leukocytes (PMNs) and serum results in the formation of "pollen rosettes" and the production of chemiluminescence (CL). Significantly more CL is produced by cells exposed to pollen and sera from nonallergic individuals compared with sera from allergic individuals. The source of the PMNs, however, has no effect on the CL produced. When IgG is isolated from the sera of allergic individuals and added to the test system, a large CL response is seen that is inhibited by the IgG-depleted fraction. No similar inhibition is produced by the IgG-depleted fraction of sera from nonallergic individuals. The inhibitor of CL in sera from allergic individuals appears to be IgE, since its removal leads to an enhanced CL response. These results suggest that competition for a limited number of binding sites on pollen grains exists between pollen specific IgG and IgE antibodies in serum from allergic patients. (J ALLERGY CLIN IMMUNOL 69:388, 1982.)

The interaction between a potential allergen and the host determines whether the host will experience allergic rhinitis and asthma or no clinical response. Since the initial interaction often occurs in the nose and nasopharynx, we examined the interaction between pollen, serum, and one of the cells capable of interacting with particulate antigens, the PMN,¹ to determine whether there are differences in the responses of these components between allergic and nonallergic individuals. Microscopic observation showed no internalization of pollen but close contact was established and maintained leading to the formation of PRs. This close contact suggested membrane activation, which we indirectly measured by a previously

described CL technique.²⁻⁴ The difference between sera from allergic and nonallergic individuals led to subsequent serum fractionation studies.

MATERIALS AND METHODS

Subjects

Normal and allergic subjects were selected after informed consent was obtained. Patients were screened by history and skin testing (prick test) to determine pollen reactivity. Nonatopic subjects were identified by history and prick and intradermal skin testing. PRIST and RAST reactivity were also studied in serum specimens as described below.

Serum collection

Blood was obtained by venipuncture and allowed to clot at room temperature for 60 min. Serum was collected and frozen in aliquots at -70°C . Portions of each sample were heat inactivated at 56°C for 3 hr, centrifuged at 10,000 rpm for 30 min, and filtered ($0.2\ \mu\text{m}$ pore size) to remove aggregated proteins.

PMN collection

PMNs were isolated from peripheral venous blood as described by Ferrante and Thong.⁵ Briefly, heparinized human blood was layered on a Hypaque-Ficoll gradient composed of two parts 85% Hypaque and nine parts 9% Ficoll (density = $1.095\ \text{gm/ml}$). The tube was then centrifuged at $200 \times g$ for 30 min at room temperature. The middle band, containing PMNs, was washed twice with RPMI 1640 and kept in ice until used (approximately 1 hr).

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Abbreviations used

PMN:	Polymorphonuclear leukocyte
CL:	Chemiluminescence
PR:	Pollen rosette
CPM:	Counts per minute
ELISA:	Enzyme-linked immunosorbent assay
PRIST:	Paper radioimmunosorbent test
RAST:	Radioallergosorbent test
FCS:	Fetal calf serum
PBS:	Phosphate-buffered saline

The PMN preparation contained at least 95% PMNs, which were 98% viable as determined by trypan-blue dye exclusion.

Pollen

Pollen grains were obtained from Mr. Harry Hayes of the Tucson Clinic Laboratory and stored in dry form until used. Unless otherwise noted, 2.5 mg of whole pollen was suspended in 1 ml of RPMI 1640 and 0.1 ml of the mixture added to a plastic tube containing 0.2 ml serum. After 30 min of incubation at 37° C, the serum-pollen mixture was added to a suspension of PMNs.

Pollen used in these experiments include the following: (1) Bermuda grass (*Cynodon dactylon*), found from Maryland to Oklahoma to California and southward; (2) short ragweed (*Ambrosia elatior*), found nearly throughout the United States; (3) rabbit bush (*Franseria deltoidea*), found in south-central Arizona; (4) careless weed (*Amaranthus palmerii*), found from Kansas to Texas, Arizona, and California; and (5) olive tree (*Olea europaea*), cultivated in California, Arizona, and southern Nevada.⁶

IgE and RAST measurements

Total serum IgE was measured by the PRIST assay.⁷ Serum IgE antibodies directed against allergens were measured by RAST.^{8, 9}

CL

CL was enhanced with FCS saturated with luminol (approximately 0.2 mg/ml) according to a method described previously.¹⁰ To ensure that PMNs were capable of phagocytosis, in each experiment a control vial containing 1.5×10^8 *Staphylococcus aureus* (heat killed at 56° C for 30 min) was included. When pollen was used, each reaction mixture included 0.2 ml of serum and 0.1 ml of a pollen suspension (2.5 mg/ml RPMI 1640) preincubated together for 30 min at 37° C before being added to 5×10^5 PMNs in 3.8 ml RPMI 1640 and FCS-luminol in a dark-adapted scintillation vial. Studies using several media with and without phenol red showed small differences with regard to peak CPM but no interference with regard to comparisons between sera from allergic vs nonallergic individuals. Vials containing serum, FCS-luminol, PMNs, and buffered media

were placed in the scintillation counter (Beckman LS-230) for 30 min for final dark adaptation. The reaction was initiated by the addition of pollen and serum. Each vial was counted for 1 min and the counting cycle was repeated for 3 hr at room temperature without further mixing. The results were recorded in CPM. The CL response induced by pollen-serum mixtures was inhibited by superoxide dismutase, sodium benzoate, and sodium azide in a dose-dependent fashion.

Interaction between pollen and PMNs.

The interaction between PMNs, pollen, and serum was viewed microscopically and photographed on Ektachrome 160 film.

Isolation of IgG

The pH of the serum was brought to 5.5 with 1M acetic acid. Undiluted octanoic (caprylic) acid was added dropwise at room temperature with stirring (7 ml octanoic acid/dl serum) to yield a white precipitate. Stirring was continued for 1 hr and the tube was centrifuged at 10,000 rpm for 30 min at 5° C. The liquid containing IgG was filtered (Whatman No. 1 filter paper) and its pH was raised to 6.5 to 7.0 with 0.1M phosphate buffer. The filtrate was then dialyzed extensively against PBS (0.0175M PO₄, 0.015M NaCl, pH 6.8) and poured over a diethylaminoethyl column (equilibrated with 0.0175M phosphate buffer, pH 6.8) to yield IgG. The precipitate (referred to as serum-minus-IgG or serum-G) was resuspended in 0.015M NaCl, pH 10.0, and dialyzed extensively against PBS (0.01M PO₄, 0.015M NaCl, pH 7.2). Immunoelectrophoresis showed the IgG fraction to be free of contaminating proteins. The serum-G fraction contained less than 18 mg/dl of IgG plus a mixture of other proteins, including IgE at levels greater than 65% of those seen prior to precipitation.

Measurement of pollen-specific IgG

The analysis of pollen-specific IgG was performed via an ELISA as previously described,¹¹ except that glutaraldehyde-fixed pollen acted as the solid-phase antigen. Pollen was incubated with 3% glutaraldehyde in 0.1M PBS (pH 7.4) for 1 hr and washed four times with PBS. Sera from recent immigrants (individuals who had resided in Tucson less than a month) and a long-term resident (one who had lived in Tucson for more than 5 yr) were added to the fixed pollen for 1 hr, followed by three washes in PBS-Tween 20. Alkaline phosphatase-coupled goat anti-human IgG (Tago Chemical Co.) was added to the mixture, incubated for 1 hr, and washed three times with PBS-Tween 20, and substrate was added (1 mg *p*-nitrophenyl-phosphate/ml 0.1M glycine buffer at pH 10.4). After 4 hr of incubation, the reaction was stopped with 50 μ l of 3N NaOH, the pollen was centrifuged, the supernatant was placed in cuvettes, and the optical density was read in a Gilford cuvette reader at 400 nm. Estimates of specific antibody levels were calculated from a standard curve that was established by adhering known concentrations of human IgG (Sigma Chemical Co.,

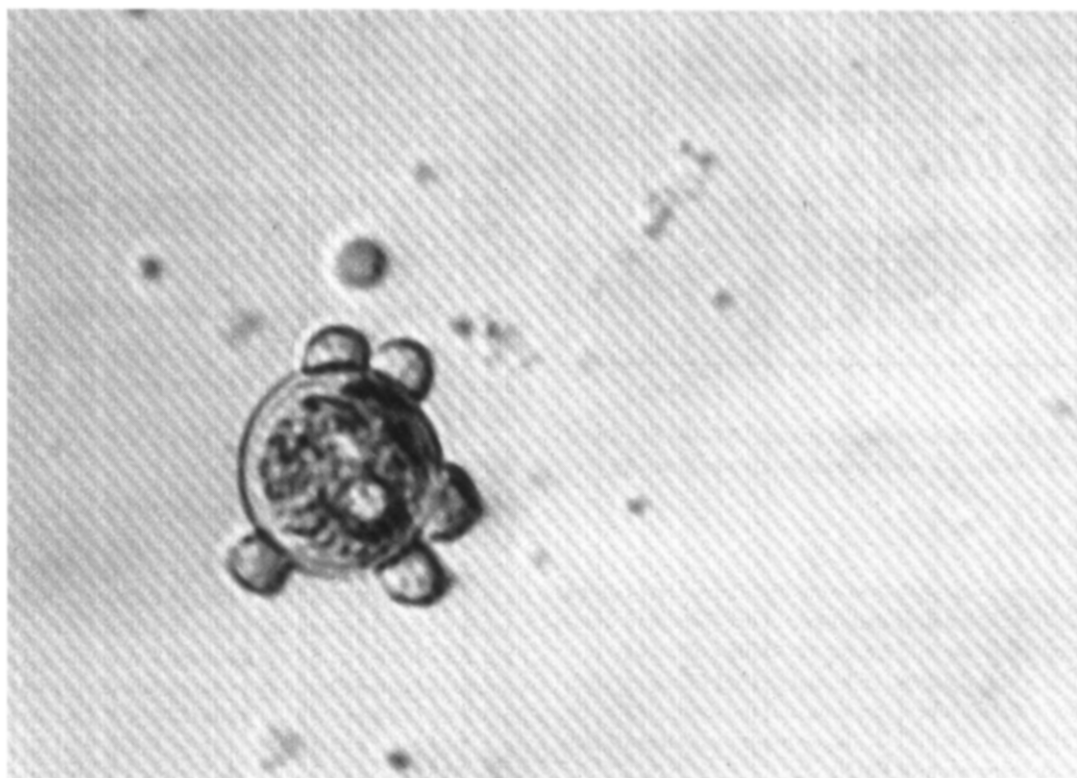


FIG. 1. Photomicrograph of a PR. Bermuda grass pollen and serum from a nonallergic individual were incubated together at 37° C for 30 min before being added to PMNs.

St. Louis, Mo.) to cuvettes, followed by the addition of alkaline phosphatase-coupled goat anti-human IgG and the addition of substrate.

Removal of IgE

IgE was removed from serum by a 1-hr incubation of serum with anti-human IgE-linked agarose beads. Each gram of swollen agarose gel contains 0.72 mg of affinity isolated anti-human IgE (E-chain specific) (Sigma). Analysis of serum after incubation with anti-IgE beads by PRIST assay showed IgE levels had decreased by 60% to 90%.

Statistics

The data were analyzed by the Student's *t* test.

RESULTS

Microscopy

The interaction between PMNs and Bermuda grass pollen that had been incubated with serum from a nonallergic individual is shown in Fig. 1. Bermuda pollen is approximately twice the size of PMNs (PMNs are 12 to 15 μ m in diameter,¹² and most pollen grains range from 20 to 60 μ m in diameter¹³). Therefore there is no internalization of pollen, but deformation of PMNs takes place around pollen grains, producing PRs. The interaction between pollen and PMNs is not static, but rather the PMN mi-

grates across the surface of the pollen, maintaining close contact so that its membrane conforms to the contour of the pollen. PRs consist of three to 15 PMNs around each pollen grain. When serum from allergic individuals was used, fewer PMNs were seen in contact with pollen and the PMN membrane did not conform to the shape of the pollen grain.

CL

Phagocytic interaction between PMNs, serum, and heat-killed staphylococci was the same for allergic or nonallergic serum donors. The peak CL from 5×10^5 cells averaged 2×10^5 CPM after 90 min of incubation with 0.1 ml of the suspension of staphylococci and 0.2 ml of serum. The CL induced by staphylococci returned to baseline after 4 hr.

When Bermuda grass pollen is incubated with heat-inactivated sera from nonallergic donors prior to being added to the PMNs, CL is produced (Fig. 2). No CL is seen when PMNs are incubated with soluble allergen free of pollen grains, pollen alone, or heat-inactivated serum alone. The CL response is dependent on the number of pollen grains and the serum concentration; an increase in the concentration of pollen leads to an increase in the CL produced (Table I). Increasing the concentration of serum also increases

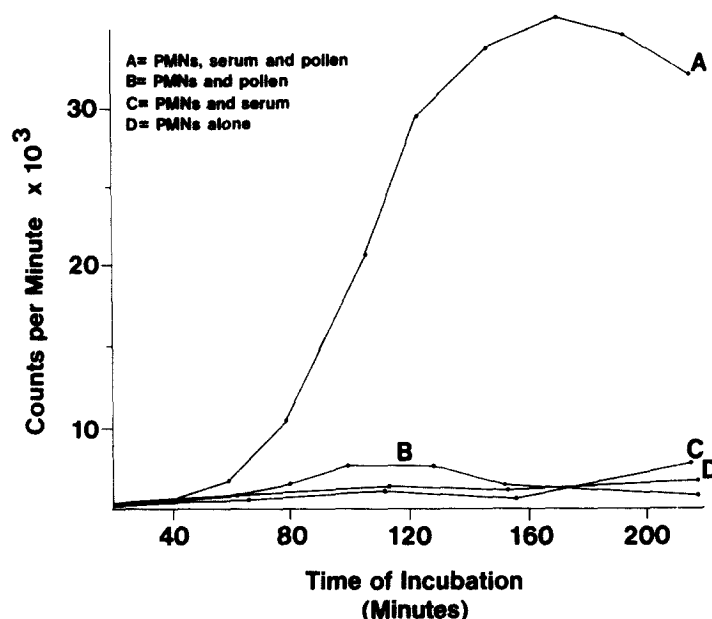


FIG. 2. CL produced by PMNs incubated with and without pollen and serum from a nonallergic individual.

CL production but to a lesser extent than increasing the pollen concentration. The serum donor had no history of allergic symptoms or skin-test reactivity to Bermuda antigen despite exposure to this pollen for many years.

The results of CL responses of PMNs from atopic and nonatopic individuals and the effects of serum on CL are shown in Fig. 3. The sera from allergic individuals produced a lower CL response compared with sera from nonallergic individuals, while the source of the PMN had no effect on the CL response.

The next series of experiments were designed to determine whether sera from atopic individuals produces a diminished response to all pollen or only those to which they are allergic.

As shown in Table II, sera from atopic subjects who reacted by skin testing to some pollens but not to others, produced less CL in response to pollen to which they were sensitive. For example, serum from subject R. B., who is sensitive to Bermuda grass pollen but not to olive tree pollen, produced much less CL with Bermuda grass pollen in the mixture than with olive tree pollen. Therefore the CL response observed in the presence of allergic sera is antigen-specific and always reduced in the presence of pollen to which the individual is sensitive. Skin-test reactivity and the CL response were correlated in all individuals tested thus far ($n = 16$), that is, sera from individuals who were skin-test negative to a particular pollen (nonallergic) always produced more CL than sera from skin test-positive (allergic) individuals.

TABLE I. Dose response of PMNs*

Serum (ml)	Antigen (mg)	CPM (peak)
0.2	0	230
0.2	0.025	6,800
0.2	0.25	29,500
0.2	5.0	420,000
0	0.25	690
0.1	0.25	10,200
0.2	0.25	29,500
2.0	0.25	76,400
4.0	0.25	92,800

*Results are given as the mean of the peak CPM from triplicate experiments. The serum came from a nonatopic donor.

The reproducibility of this phenomenon is shown in Fig. 4. When peak CPM from 13 subjects were compared, subjects who were sensitive to a particular pollen showed significantly less CL compared with nonallergic individuals ($p < 0.01$). The exception to this was seen in sera from nonallergic individuals who had never been exposed to a particular pollen (those living in Tucson for less than a month—"newcomers"). Sera from these individuals (who were skin-test negative to Bermuda grass and careless weed pollen) produced substantially less CL to "new" pollen than to pollen to which they had been exposed (Fig. 5, top). For example, Bermuda grass is generally not found north of the thirty-fifth parallel, and since the three newcomers to Tucson, who did not respond to

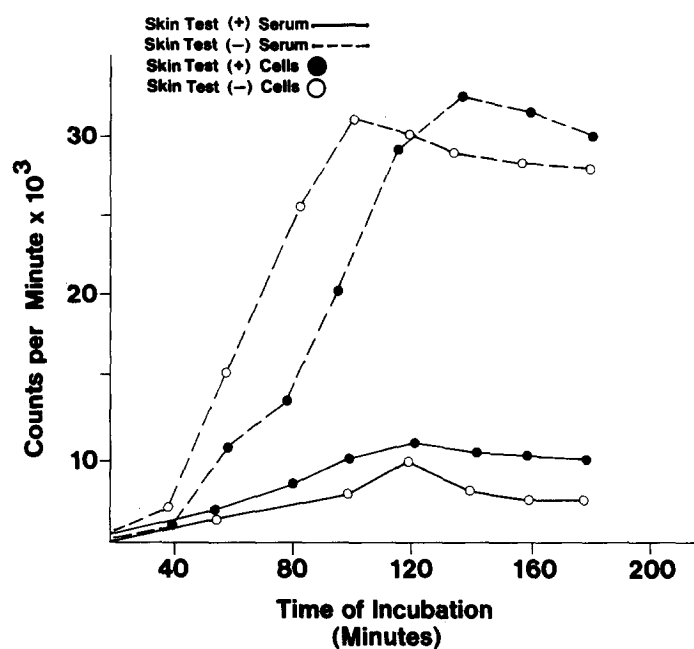


FIG. 3. Comparison of the CL response produced by cells and sera from allergic and nonallergic individuals.

TABLE II. Comparison of skin test, RAST, PRIST, and CL test results

Subject	Pollen	Skin-test reactivity*	RAST result†	CPM × 10 ³ in CL test		Total IgE (IU/ml)
				Serum HI	Serum not HI	
J. L.	Mulberry	0	0	42.6	35.4	515
	Olive	0	1+	31.5	29.7	
	Careless weed	2+	2+	12.5	6.4	
	Bermuda	3+	2+	6.4	5.2	
G. B.	Bermuda	0	2+	31.5	27.6	
	Olive	2+	0	11.5	9.0	
R. B.	Bermuda	2+	3+	10.0	7.5	101
	Olive	0	0	30.0	25.0	
H. W.	Bermuda	0	1+	31.4	29.0	
	Olive	3+	1+	9.6	8.9	
	Mulberry	0	0	38.7	36.0	
J. J.	Bermuda	2+	2+	7.5	6.8	47
	Olive	0	0	24.2	25.2	
R. L.	Bermuda	0	0	32.5	28.1	172
	Olive	0	0	31.0	29.5	
	Mulberry	0	0	29.2	27.3	
	Careless weed	0	0	27.3	24.8	

HI = heat inactivated.

*Wheal diameter measured 15 min after a prick with a 1:20 dilution of allergen: 0 mm = 0; 1-2 mm = ±; 3-6 mm = 1+; 7-10 mm = 2+; over 10 mm = 3+; pseudopods = 4+.

†CPM of ¹²⁵I radioactivity: less than 1500 CPM = 0; 1500-2100 CPM = 1+; 2100-7400 CPM = 2+; 7400-20,100 CPM = 3+.

Bermuda in the CL test, had not lived south of the thirty-fifth parallel, they probably had not come in contact with Bermuda pollen. However, careless weed or pollen that cross-reacts with careless weed (e.g., Lamb's quarters) is found throughout the U.S.,

and the responses of newcomers to this pollen was similar to those of other nonallergic subjects. Analysis of their sera by ELISA showed little IgG antibody to Bermuda grass pollen but high levels against careless weed pollen (Fig. 5, bottom).

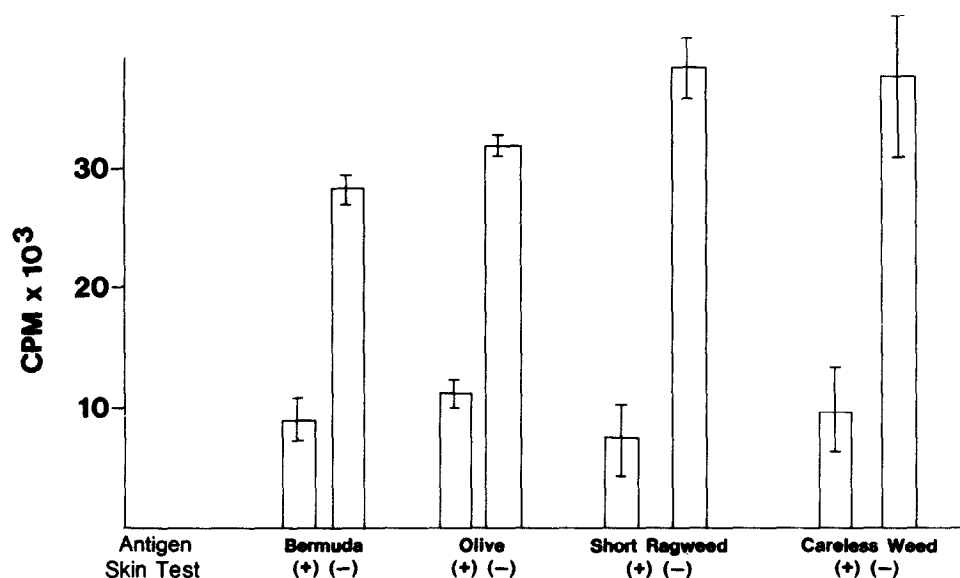


FIG. 4. CL response produced by sera from individuals who are skin-test positive (+) or skin-test negative (-) to a particular type of pollen. Results are expressed as the mean + the 95% confidence interval.

Serum factors

To exclude complement as the serum factor that is activating PMN membranes and stimulating CL,¹⁴ sera were heat inactivated at 56° C for 120 min before being added to the reaction mixture. Since the CL produced by heat-inactivated serum was no less than that produced by serum which was not heat inactivated, it is not likely that complement activation is responsible for the differences in CL production between allergic and nonallergic serum.

To determine whether the lower CL response is specifically stimulated by a particular pollen but expressed nonspecifically to a number of different species of pollen, serum from an allergic individual was incubated simultaneously with two different pollens—one to which he was sensitive ("S-pollen") and one to which he was not ("NS-pollen"). If the interaction between serum and S-pollen leads to the formation of a substance that inhibits CL, we would expect the S-pollen to inhibit the response to the NS-pollen. Results in Table III indicate that the simultaneous addition of S- and NS-pollens leads to a response typical of the addition of NS-pollen alone. Therefore the inhibition of CL is associated with pollen to which an individual is allergic; the inhibition has no effect on the response to pollen to which the individual is not allergic.

The specificity of the response suggests that immunoglobulins may be involved. Therefore we measured total and specific IgE levels by PRIST and RAST and compared these with skin-test reactivity and CL. Table II shows the total IgE level in the sera of 4/6 of

TABLE III. Specificity of inhibition of CL

Subject	Antigen	Skin-test reactivity	CPM
J. J.	Bermuda	+	452
	Olive	-	20,000
	Bermuda + olive		21,000
J. L.	Bermuda	+	4,000
	Olive	-	33,000
	Bermuda + olive		36,700
J. P.	Bermuda	-	31,000
	SR	+	3,900
	Bermuda + SR		33,000
M. M.	Bermuda	+	1,500
	SR	+	5,200
	Bermuda + SR		5,100

SR = short ragweed.

the patients as well as the RAST result for each pollen tested. Neither the total IgE level nor RAST result could consistently predict CL results, whereas enhanced CL was always associated with negative skin-test reactivity.

Since IgG is an important opsonin for PMN phagocytosis, we tested the capacity of IgG-rich and IgG-depleted fractions from both allergic and nonallergic individuals to stimulate CL. Results from these studies showed that the IgG-rich fraction from both allergic and nonallergic individuals induced CL (Table IV). However, the IgG-depleted fraction from allergic individuals (1) produced little or no CL itself and (2) inhibited CL produced by the IgG-rich fraction

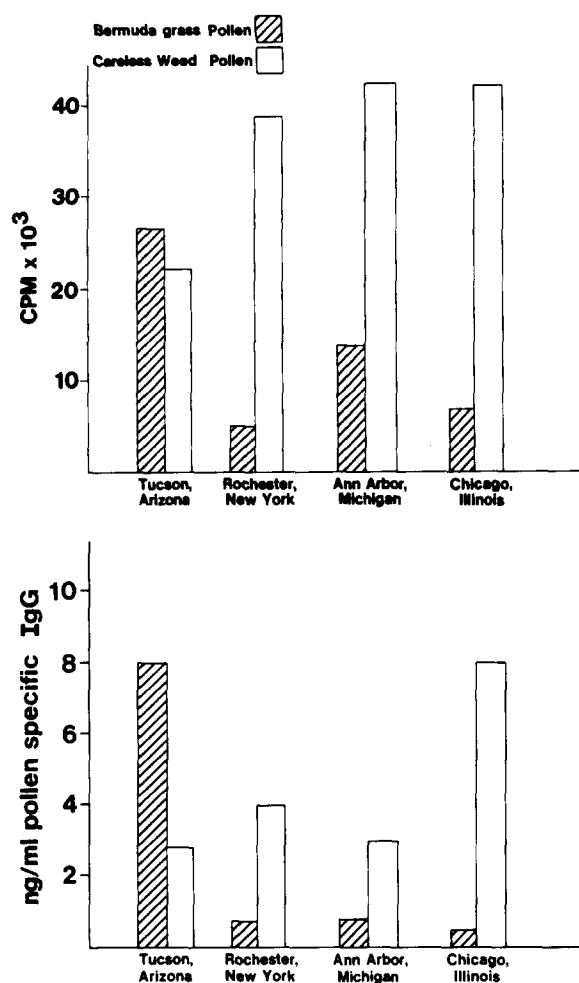


Fig. 5 Top, CL response produced by serum from a long-term resident of Tucson who is skin-test negative to both Bermuda grass and careless weed pollen compared with that produced by sera from recent immigrants who are also skin-test negative to both pollens. **Bottom,** Levels of pollen-specific IgG in the serum of new and long-term residents of Tucson are compared.

from both allergic and nonallergic individuals. Therefore it appears that an inhibitor of CL production is present in allergic serum. The IgG-depleted fraction from nonallergic individuals did not inhibit CL. Confirmation that specific antipollen IgG stimulates high CL response is provided by studies with new and long-term residents of Tucson, neither of whom are allergic to Bermuda grass pollen. Long-term residents had higher levels of anti-Bermuda grass pollen IgG than new residents (Fig. 5, bottom). Each bar of the histogram represents the mean of four determinations of IgG directed against Bermuda grass pollen or careless weed pollen. Pooling these values from newcomers and comparing them with the levels determined for long-term residents showed significantly

TABLE IV. CL response stimulated by fractions of serum from allergic and nonallergic individuals*

Serum fraction	Allergic serum (CPM ± SEM)	Nonallergic serum (CPM ± SEM)
Whole serum	6,400 ± 250‡	46,000 ± 4200
IgG	51,000 ± 2600	74,000 ± 2500
Serum-G†	250 ± 30‡	13,700 ± 900
IgG + serum-G	1,490 ± 200‡	43,000 ± 3500
Nonallergic IgG + allergic serum-G	3,800 ± 200	

*Whole serum, the IgG fraction, or the fraction of serum lacking IgG was incubated with Bermuda grass pollen prior to addition to the PMN suspension. Results show the mean of the peak CPM ± SEM of triplicate assays.

†Serum components after IgG has been removed.

‡Indicates a significant difference in the CL produced by serum or fractions thereof between allergic and nonallergic individuals ($p < 0.001$).

TABLE V. Effects of the removal of IgE on CL to Bermuda grass pollen

Subject	Skin-test reactivity to Bermuda grass	CL (CPM)	
		Whole serum	Serum-IgE
R. L.	0	35,000	49,000
R. A.	1+	9,200	25,000
A. G.	4+	11,000	27,000
D. P.	4+	9,000	24,000
Mean ± SEM*		9,700 ± 640	25,000 ± 900

*CPM produced in the CL assay by unfractionated serum from three allergic individuals were pooled and the mean compared to that produced after IgE was removed from the serum. A significant increase in CL resulted from the removal of IgE ($p < 0.01$).

less IgG against Bermuda grass pollen in sera from newcomers compared with sera from long-term residents (mean ± 95% confidence interval = 0.6 ± 0.2 ng/ml for newcomers, 8.3 ± 6.1 ng/ml for long-term residents). There was no difference in IgG levels against careless weed pollen between newcomers and long-term residents. Therefore exposure to pollen leads to production of pollen-specific IgG in nonallergic individuals, which correlates with high CL.

The specificity of the inhibitor suggested that it may be an immunoglobulin. Therefore we removed IgE with agarose beads to which anti-human IgE was covalently bound and tested the serum for its capacity to stimulate CL. Addition of sera from allergic individuals treated in this fashion showed enhanced CL production (Table V).

DISCUSSION

This study was designed to probe the interaction between pollen, PMNs, and serum by means of an *in vitro* test. Because pollen grains are larger than PMNs, internalization does not occur in this test system; rather, their interaction leads to "frustrated phagocytosis"¹² and the formation of PRs. This association leads to membrane activation, which we measured by CL. The essential variables in this interaction are serum and pollen. PMNs from allergic or nonallergic individuals respond similarly; however, PMNs together with serum from nonallergic individuals who had been exposed to the test pollen produced highly specific and reproducible CL. PMNs with sera from allergic patients, on the other hand, produced less CL when incubated with pollen for which skin tests were positive than when incubated with pollen for which skin tests were negative.

Serum from newcomers who were nonallergic and skin-test negative did not produce the expected response to pollen found in the Tucson area, e.g., Bermuda grass pollen. This is not surprising if it is assumed that specific antibody is required to prepare the pollen for adherence and CL and the subjects have not had the opportunity to develop an antibody response against it. The apparent lack of anti-Bermuda pollen IgG in their sera, as measured by ELISA, supports this contention (Fig. 5, *bottom*). Even though careless weed, like Bermuda grass, is not found north of the thirty-fifth parallel,⁶ it has cross-reacting antigens with other widely distributed pollen such as Lamb's quarters.¹⁵⁻¹⁷ This cross-reactivity may account for the enhanced CL to careless weed pollen that we observed. This is supported by measurements of pollen-specific IgG by ELISA, which showed that little IgG against Bermuda grass pollen was present in their sera but appreciably more IgG was detected against careless weed.

The serum fractionation experiments support the contention that specific IgG is responsible for mediating CL production. IgG isolated from the serum of either allergic or nonallergic individuals led to a large CL response. This suggests the presence of pollen-specific IgG in both allergic and nonallergic individuals (Table V) only after they have come in contact with the pollen.

It is possible that CL could be accentuated by the activation of complement. The resulting C3 fragments could then bind to and activate PMN membranes and thereby contribute to the observed response.¹⁴ The activation of human complement by the addition of atopic sera to allergens to which subjects are allergic has been reported, but the results did not correlate well with clinical symptoms.^{18, 19} Our results show

that the removal of complement activity by heat inactivation has no effect on the CL produced, indicating that complement is not responsible for mediating the CL response.

Doll et al.²⁰ have recently reported that immune complexes will inhibit CL by PMNs. The inhibition shown by these authors is nonspecific in that aggregated human globulin inhibits the response to opsonized zymosan. The inhibition shown in our study is specific; if immune complexes were responsible we would expect to see inhibition of CL when sera from allergic individuals and cells are incubated with a mixture of both "sensitive" and "nonsensitive" pollen. Since the CL response to "nonsensitive" pollen is not depressed by "sensitive" pollen, a mechanism other than suppression of CL by immune complexes is suggested.

A likely candidate for the inhibitor of CL production by PMNs is IgE. The presence of specific anti-pollen IgG in nonallergic serum presumably enhances CL via the Fc receptors on PMNs. Pollen-specific IgG antibodies are also present in allergic individuals, but IgE may cover antigenic sites and effectively inhibit IgG binding. Since PMNs lack receptors for the Fc portion of IgE,²¹ this interference could result in lower CL. Further support for IgE acting as an inhibitor of CL is provided by experiments that showed a significant increase in CL after IgE was removed (Table V).

Thus it appears that competition for binding sites on pollen grains between specific anti-pollen IgG and IgE may be responsible for these observations. Quantitation of specific IgE and IgG in allergic and nonallergic individuals and CL response changes over time in allergic individuals during immunotherapy are the subjects of future studies.

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