Original articles

Circulating immune complexes during immunotherapy

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Chronic immunization may lead to the production of circulating immune complexes (CICs). This study was undertaken to determine the presence of circulating IgG immune complexes in 95 subjects with allergic rhinitis/asthma receiving immunotherapy, 46 individuals with similar diagnosis but not on immunotherapy, and 64 healthy controls. Modified Raji cell and murine leukemia cell (L-1210) assays, selected for a high density of Fc receptors and devoid of Epstein-Barr virus membrane antigen, were used. Other immunological parameters such as immunologlobulins G, A, M, E, and rheumatoid factor activity were also studied. The CIC concentrations in the treated group did not differ significantly from the untreated group, although both groups did have a significantly higher concentration than the healthy controls. The presence of CICs has no relationship with age or sex of patients, dosage of allergen administered, number and nature of allergens received, period between the time of last injection and the blood sampling, and the duration of immunotherapy. Serum IgG, IgA, IgM, and rheumatoid factor activity did not differ between the treated and untreated groups. IgE was significantly higher in the treated group when compared with the untreated, and IgE levels in treated patients with elevated CICs were significantly increased compared with CIC-negative treated patients. These data suggest that CICs are present in serum of atopic diseases such as allergic rhinitis/asthma. Significantly, an association of elevation of CICs with immunotherapy could not be demonstrated.

Immunotherapy of atopic disorders results in the formation of IgG antibodies directed against the immunizing antigens.[1] This could conceivably lead to the production of circulating immune complexes (CICs) made up of IgG antibodies and antigenic components of the immunotherapy extracts used.[2] Immunotherapy can be considered as a form of chronic stimulation of the immune system, potentially associated with the production of CICs.[3] Some animal models suggest that "anomalous," irrelevant antibodies far exceed the concentrations of specific antibodies induced following prolonged alloimmunization.[4] One mechanism for the production of CICs during immunotherapy could be the generation of antiantibodies with serological specificity for the idiotypic determinants expressed on the "antiallergen" antibodies. We have examined allergic patients during prolonged immunotherapy to determine if such treatment is associated with increased amounts of CICs.

A preliminary study has indicated that immunotherapy was not uniformly associated with the development of elevated CIC levels, but that a subset of individuals may be predisposed to produce CICs.[2] We have now studied a larger population of individuals with allergic rhinitis and/or asthma in an attempt to detect any association between production of CICs

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TABLE I. Characteristics of the tested populations

	Allergic		
	Untreated	Treated	Control
Number of subjects	46	95	64
Sex: Male/female (% male)	14/32 (30%)	24/71 (25%)*	30/34 (47%)
Age: Years ± SD	32 ± 11	48 ± 16	45 ± 17
Diagnosis: Rhinitis/asthma†	42/4	63/2*	—
Duration of therapy: Years ± SD	—	4.1 ± 5.7	—

*Statistically not significant by ANOVA.
†Rhinitis: Allergic rhinitis without clinical evidence of asthma[22]; Asthma: Asthma as major complaint, with or without associated allergic rhinitis.

and different treatment modalities—i.e., single versus multiple antigen immunotherapy, nature of the extracts administered, and duration of the treatment. The results show that the nature of the allergic symptoms, asthma versus allergic rhinitis, the age and sex of the treated individuals, and the characteristics of their treatment did not play any role as far as development of CICs. Serum IgG, IgA, and IgM levels and rheumatoid factor activity were identical whether the treated patients had increased or normal CIC values. However, serum IgE was elevated in the CIC-positive and treated group.

MATERIAL AND METHOD

Clinical sampling

The study group consisted of 141 adults with either allergic rhinitis and/or asthma attending the Allergy Clinic of the Royal Victoria Hospital. Patients with other concomitant allergic disorders and those receiving systemic steroids were excluded, as well as allergic individuals with intercurrent acute illnesses such as infections, or with known collagen vascular diseases. However, 3 patients among the untreated allergic population were found retrospectively to have some rheumatic disorders: 2 had active rheumatoid arthritis, but in a very early, symptomatic phase, and had normal CICs; the other had quiescent systemic lupus erythematosus (SLE) as confirmed by a consultant physician blind to the results of CICs or diagnosis. These three patients were not excluded, although the patient with SLE had elevated CICs and low complement (C3 and total hemolytic complement).

Distribution of the tested populations

Among the 141 allergic individuals, 95 were actively undergoing maintenance immunotherapy, whereas 46 had never been treated with allergen extracts (Table I). Only patients whom the attending allergist felt might benefit from immunotherapy, if it were instituted, were included in the nonimmunotherapy group. The treated population had been receiving immunotherapy with aqueous extracts for an average period of 4.1 ± 5.7 yr (SD). The ratio of male to female patients was similar between both groups. The treated population tended to be older (mean age of 48 ± 16 yr, vs 32 ± 11 yr for the untreated) and was composed of a larger proportion of asthmatics. However, statistical analysis by Student's t test showed no difference. Sixty-four healthy, nonatopic individuals comprised the control group. This group did not differ from the allergic populations in age, but there was a slight increase in the male-to-female ratio.

Preparation and storage of sera

For all analyses in this study, 5 ml of blood was obtained. For the treated, blood was collected before injections and preseasonally. The 64 normal individuals used as controls were bled at the same time.

Blood was kept at 4° C overnight to allow good clot retraction and was then centrifuged at 200 × g. The resulting sera were decomplemented at 56° C for 30 min and kept at −20° C until used for the tests.

Assay for detection of CICs

An assay was developed based on the use of Fc receptor–bearing cells to specifically adsorb IgG immune complexes, and staphylococcal protein A (pA) was radiolabeled[5] to quantitate the cell-adsorbed immune complexes. Protein A binds with very high affinity to human IgG subclasses 1, 2, and 4.[6] Although one of its sites of fixation on IgG is the CH_2 domain of the IgG,[7] pA can still react with immune complexes adsorbed onto cells via the C-terminal-CH_3 domain,[8] because the CH_2 domain of cell-bound IgGs remains spatially available.[9] However, when pA is used as a probe for CH_2, sera have to be decomplemented prior to being assayed,[10] since the fixation of C1q could interfere with the pA binding site.[11, 12] Thus, with this approach, CICs did not fix onto C3b receptors which were expressed on the Raji cells.[13]

Of more than 20 different cell lines pretested to obtain the optimal Fc receptor "CIC developer" cells, 2 were retained. Murine leukemia cells (L-1210; obtained from Dr. Chitnis from National Institutes of Health, Bethesda, Md.), when grown in ascitic fluid, had greater than 95% to 98% of Fc receptor–bearing cells on the seventh day of in vivo passage, and had the highest density of surface Fc receptors

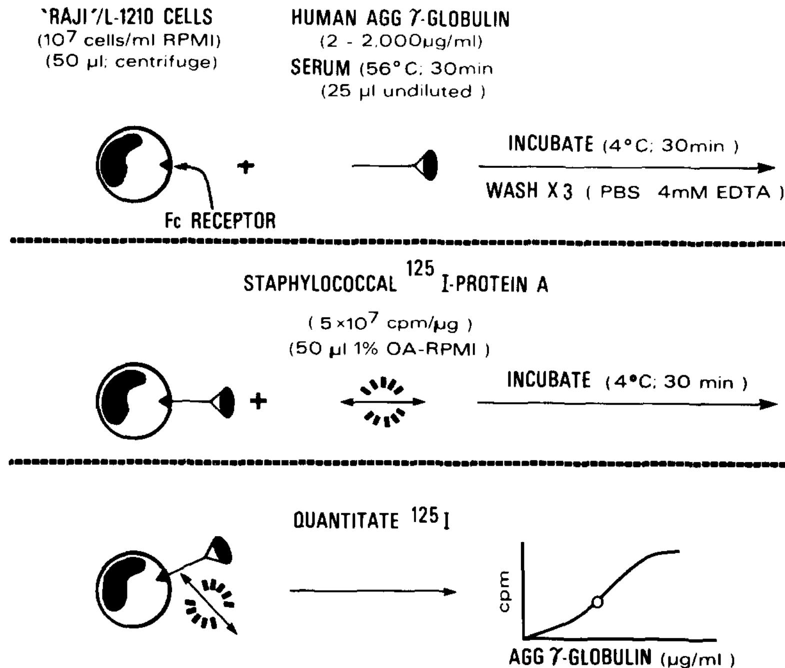

FIG. 1. Diagrammatic illustration of the methodology used in the modified Raji cell and murine leukemia cell (L-1210) assays.

among all cell lines examined. In addition, we used Raji cells (obtained from Dr. M. Jerry, Calgary, Alberta, Canada) after modification to increase their density in Fc receptors, as they do not express any surface-detectable HL-A–associated antigens, Ia antigens, or IgG determinants. Furthermore, there is no membrane antigen (MA)[14] from Epstein-Barr virus on Raji cells.

Selection for Raji cells that would bear a suitable amount of Fc receptors was achieved after repeated enrichment of cells forming rosettes with ox erythrocytes sensitized with a subagglutinating amount of IgG antibodies (so-called EA-RFC).[15] EA-RFC Raji cells were separated from non–EA-RFC by Ficoll gradient separation, as described previously.[16] The pellet of EA-RFC was recultured in tissue culture medium using RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin, 100 IU/ml; streptomycin, 50 μg/ml). After a period of 10 to 15 days, most of the bound erythrocytes had disappeared from the tissue culture. The enrichment procedure was repeated, giving a final yield of 50% to 60% EA-binding Raji cells. With time, this percentage tended to decrease, and the "selection procedure" had to be performed every three months.

Preparation of radiolabeled protein A

Purification of protein A and its radioiodination by the chloramine-T method has been extensively described elsewhere.[17] The specific activity was 400,000 cpm/μg protein A (I-pA).

Characteristics of the CIC "developer" cells

Variable amounts of L-1210 and/or "modified" Raji cells in 50 μl were introduced into triplicate wells of Microtiter "U" bottom plates (MRC-96; Linbro Scientific Co. Inc., New Haven, Conn., 10^4 to 10^6 cells/well). The plates were centrifuged at 100 × g for 5 min, and the supernatant was gently removed.[17] The pelleted cells were reacted in triplicate with 25 μl of different concentrations of aggregated IgG (1 pg to 2 mg/ml) prepared as follows: human IgG—"Connaught," 16.5%, was diluted 1:100 in phosphate-buffered saline (PBS) and preadsorbed on Raji cells; it was then treated at 60° C for 60 min, and aggregates were isolated by gel chromatography on Sephadex G-200.

Following incubation at 4° C for 30 min, the CIC "developer" cells were washed three times using PBS containing 4 mM ethylenediaminetetraacetic acid (EDTA). Thereafter, 50 μl of I-pA (50,000 cpm) diluted in 1% ovalbumin was added to each well, and a second incubation was performed as above. The cells were washed free from unbound I-pA using a MASH II apparatus. Trapping of non–cell-fixed I-pA was prevented by precoating the MASH tubings and the filter papers with 0.3% gelatin in PBS. Cell-bound radioactivity was then measured using a Beckman gamma counter. Although this test proved sensitive enough to detect as little as 2 ng aggregated IgG/ml, the assay was calibrated to provide linear curves with aggregated IgG concentrations from 2 to 2,000 μg/ml. Early screening experiments with sera from more than 100 healthy volunteers showed CIC values of 3.2 ± 4.1 μg/ml (SD). Serial experiments with sera from normal and active SLE revealed a high degree of reproducibility when compared to C1q binding assay.[18] Variation in results from consecutive experiments was always less than 10% but generally less than 5%.[19] The upper limit of values from normal individuals was found to be 20 μg/ml and will be used throughout to distinguish CIC positivity (CIC+) from normals (CIC−). In

TABLE II. Distribution of patients by diagnosis

	No.	CIC concentration: (μg/ml ± SD) With asthma*	With rhinitis*	p
Healthy	64	(2.8 ± 3.8)		NS
Untreated	46	4.8 ± 6.9	10.4 ± 20.8	
Treated	95	20.1 ± 42.2	25.3 ± 89.1	NS

NS, statistically not significant.
*Statistically not significant by ANOVA.

the present study, both kinds of "developer" cells gave identical results, although L-1210 exhibited a rather composite pattern of reactivity with aggregated IgG, presumably because of "crowding effect" from the very high density of Fc receptors on its surface.

For the "routine CIC assay," aliquots of 2×10^5 L-1210 or 5×10^5 Raji cells, in triplicates, were chosen as the optimal amount of CIC "developer" cells (Fig. 1). They were reacted with 25 μl of undiluted serum as mentioned above for aggregated IgG. Each 96-microwells plate contained coded sera from 20 "test-allergic" individuals (60 wells) and aggregated IgG at concentrations of 2 μg, 20 μg, 200 μg, and 2,000 μg/ml (12 wells). Three wells contained only buffer (25 μl of RPMI-1640), and the remaining 21 wells were used to include the same 7 "controls" sera throughout all CIC determinations: 1 from an agammaglobulinemia individual, 3 from different normal controls (the same throughout), and 3 from patients with bona fide connective tissue disorders exhibiting high levels of CIC as detected by pretests and by C1q binding assays.[18, 19]

The serum concentration of CIC was determined by converting bound radioactivity to μg/ml equivalent of aggregated IgG, after subtraction of the corresponding background agammaglobulinemia serum for test samples, and buffer alone for aggregated IgG. In some experiments, "competition-blocking studies" were performed to ensure that results with the Raji cells did not occur due to serologic fixation of antibodies: Fc receptors of Raji were saturated with aggregated IgG followed by coating with monovalent, unlabeled protein A[20] in excess. These cells were then used as "developer" cells for possible antibodies directed against Raji membrane antigens. In the present study, no false-positive CIC serum was encountered.

Clinical studies

A double-blind assessment of all clinical data/characteristics (age, sex, nature of symptoms, single versus multiple immunotherapy, nature of the extracts administered and duration of the treatment, total dose administered, number of allergens, and the time from last injection) was done by N. G., who was unaware of the CIC results at the time of assessment.

Quantitation of serum IgG, IgA, IgM, and IgE

Aliquots of sera obtained concomitantly with CIC sera samples were assayed nephelometrically for IgG, IgA, and IgM concentrations. Immunoglobulin standards and antisera were purchased from Hyland Laboratories (Div. Travenol Labs., Inc., Costa Mesa, Calif.). Quantitation of IgE was performed using IgE radioimmunoassay kits (RIST, Pharmacia Labs., Piscataway, N. J.).

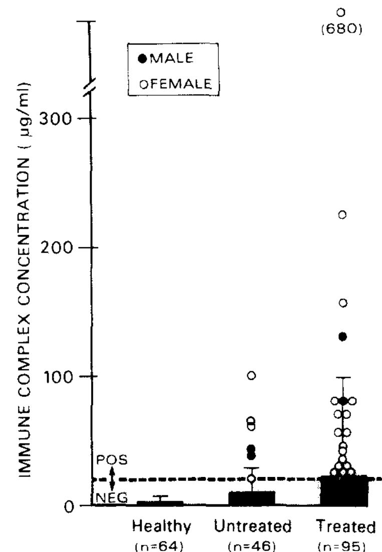

FIG. 2. Effect of immunotherapy on CIC concentration in 64 healthy controls, 46 subjects with allergic rhinitis/asthma not on immunotherapy, and 95 similar patients receiving immunotherapy. ● indicates male; ○ indicates female. Results are expressed as arithmetic means (μg/ml equivalent of aggregated IgG) with 1 SD values shown.

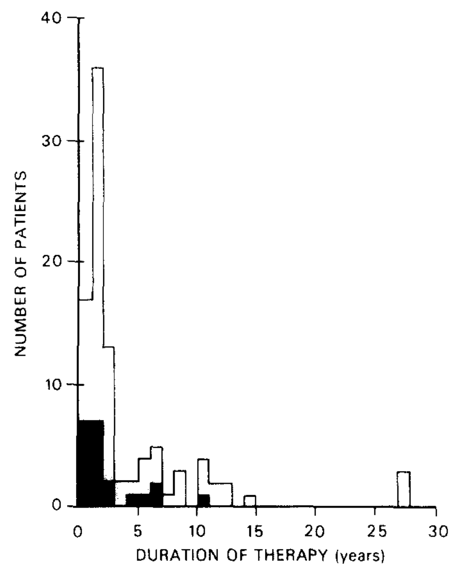

FIG. 3. Histogram illustrating the duration of therapy for treated patients. Open bars represent all patients; solid bars represent CIC-positive patients.

Rheumatoid factor detection

Rheumatoid factor (RF) activity in sera was determined by a nephelometric technique, in which the change in light scattering due to the reaction of RF with extrinsic aggre-

TABLE III. Characteristics of immunotherapy

	CIC-negative	CIC-positive	p
Total dose (PNU ± SD)	3,351 ± 2,016	3,900 ± 1,698	NS
No. of allergens (mean ± SD)	1.62 ± 0.77	1.48 ± 0.68	NS
Duration of immunotherapy (days ± SD)	4.5 ± 5.9	2.5 ± 2.7	0.02*
Time from last injection (days ± SD)	17.1 ± 10.9	17.2 ± 8.2	NS

NS, statistically not significant.
*p value < 0.02.

TABLE IV. Allergens used in immunotherapy

	All patients		Single		Multiple	
	Total	Positive	Total	Positive	Total	Positive
Dust	64	15	34	9	30	6
Ragweed	49	9	16	3	33	6
Grass	18	5	2	0	16	5
Tree	5	1	0	0	5	1
Bacteria	6	1	2	1	4	0
Miscellaneous*	9	0	0	0	9	0

*Includes: Mixed molds (5 patients); insects (1 patient); epidermals (dog, 1 patient; cat, 1 patient).

gated IgG is measured.[21] This technique quantitates IgM-RF, and light scattering values less than 2.6 ± 1.1% (SD) are negative for RF.[21] The relationship between light scattering (expressed as percent of relative light scattering, RLS) and latex titer (determined by the Hyland latex globulin RF slide agglutination technique) is expressed as $\log_{10}$ (% RLS) = 0.219 $\log_2$ (1/latex titer) + 0.037.

Calculations of the results and statistical analysis

Results are expressed as arithmetic means ± SD, with the exception of IgE values (geometric means ± SD). Statistical significance was calculated by Student's t test and by chi-square tests.

RESULTS

CIC versus treatment groups

The mean serum concentrations of CICs in healthy individuals, atopic individuals undergoing immunotherapy, and atopic individuals not undergoing immunotherapy are presented in Fig. 2. Untreated and immunotherapy-treated groups differed significantly from healthy individuals ($p < 0.05$ and $p < 0.01$, respectively), irrespective of deleting the 3 patients with rheumatic disorders ("selected untreated"). The 2 atopic groups did not differ significantly among themselves; however, the treated group had CIC levels exceeding those of the "selected untreated" group (CIC = 7.8 ± 17.5; $p < 0.05$). Six of the 46 atopic, nonimmunotherapy-treated patients had CIC concentrations exceeding 20 μg/ml, an incidence of 13% (compared with the "selected treated" group, with 5 positive, an 11% incidence), whereas 21/95 immunotherapy patients had CICs in excess of 20 μg/ml, an incidence of 22%. This difference was not statistically significant when analyzed by the chi-square test.

CIC versus sex and age of the patients, and nature of their allergic symptoms

The next questions concerned possible effects from clinical characteristics of individual groups on the CIC levels. As could be seen in Fig. 2, elevated serum CIC concentrations were not associated with sex among atopic individuals, whether they were receiving immunotherapy or not. Similarly, the age of individuals did not play any role: for the untreated group, the mean age of the CIC-negatives (31.9 ± 10 yr) did not differ from that of the CIC-positive patients (29.3 ± 18 yr); for the treated group, the mean age of the CIC-negative patients was 48.5 ± 17 yr, compared to 45.4 ± 13 yr for the CIC-positive individuals. When the nature of the allergic symptoms was analyzed, the rhinitis group had CIC levels comparable to those of asthmatics with or without rhinitis (Table II).

CIC versus characteristics of immunotherapy

Different characteristics of immunotherapy were analyzed to determine if any would be associated with the elevated CIC concentrations: (1) total dose admin-

TABLE V. Effect of immunotherapy on rheumatoid factor activity

	No.	Ne-RF activity* (%RLS ± SD)	No. RF-positive	Equivalent latex titer†
Untreated	46	4.5 ± 8.1	18	1:7
CIC-negative	40	4.0 ± 8.3	13	1:6
CIC-positive	6	7.8 ± 5.6	5	1:15
Treated	95	5.4 ± 9.8	42	1:9
CIC-negative	74	4.6 ± 7.6	32	1:7
CIC-positive	21	8.4 ± 14.9	10	1:17

*Rheumatoid factor–positive: Ne-RF activity > 2.6% RLS.
†Equivalent latex titer calculated by the equation: $\log_{10}$ (%RLS) = 0.219 $\log_2$ (1/latex titer) + 0.037.

TABLE VI. Effect of immunotherapy on serum immunoglobulin concentrations

		No.	Untreated (mean ± SD)	No.	Treated (mean ± SD)	p
IgG (mg/dl) (650-1,600)		46	1,163 ± 344	93	1,090 ± 283	NS
IgA (mg/dl) (65-305)		46	190 ± 95	93	206 ± 118	NS
IgM (mg/dl) (90-265)		46	115 ± 79	93	180 ± 104	NS
IgE (IU/ml) (25 ± 65)	Total	45	97 ± 14	95	116 ± 24	0.025*
	CIC (+)	6	103 ± 14	21	193 ± 35	NS
	CIC (−)	39	96 ± 14	74	100 ± 22	NS
	(p value CIC+ vs CIC−)		(NS)		(0.001†)	

NS, statistically not significant.
*p value < 0.025.
†p value < 0.001.

istered (calculated as protein nitrogen units [PNU]); (2) number of allergens administered; (3) duration of immunotherapy; (4) period between the time of the last injection and the blood sampling for the present study. As Table III indicates, the first two characteristics of immunotherapy were not associated with increased CICs. Similarly, the time interval between the last injection and the blood collection was identical among the CIC-positive and the CIC-negative patients.

Concerning the duration of immunotherapy, the CIC-positive group had been treated for a significantly shorter period than the CIC-negative group ($p < 0.02$). However, further analysis revealed that this difference was due to the absence of positive CIC individuals among those who had been submitted to prolonged immunotherapy, therefore creating a cluster of individuals which affected the overall distribution (Fig. 3). Thus, when considering only those patients who had undergone immunotherapy for less than 10 yr, the distribution of CIC-positive individuals was similar to that of CIC-negative patients.

CIC versus allergens used

The types of allergenic extracts used for immunotherapy were then assessed for possible increased incidence of development of CICs with a particular regimen, i.e., nature and number of extracts administered to individuals. Table IV shows that: (1) the nature of the extract did not play any role, since an incidence of approximately 1:5 CIC-positivity was observed for each allergenic material used; (2) multiple immunotherapy did not affect this incidence. Of interest was the fact that none of the 9 patients receiving relatively unusual types of extracts, such as mixed molds (5 patients), insects (1 patient), or animal danders (2 patients), had increased CIC serum levels; only 1/6 patients on mixed bacterial vaccine had elevated CICs.

Effect of immunotherapy on rheumatoid factor activity

The possibility that immunotherapy might represent chronic antigenic stimulation, leading toward proliferation of immune parameters and generation of nonspecific factors such as RF, was examined by measurements of IgM-RF activity. Table V shows that there was a trend for the CIC-positive group to have enhanced RF activity, but statistical analysis revealed no difference compared with the CIC-negative group.

Effect of immunotherapy on serum immunoglobulin concentrations

Results of these measurements are presented in Table VI. When the immunotherapy-treated group

was compared with the nonimmunotherapy-treated group, no difference was noted for IgG, IgA, and IgM concentrations. However, IgE was significantly increased in the treated population as compared with the untreated group. This finding was further investigated by comparing IgE measurements in the CIC-positive and CIC-negative subgroups of the untreated and treated patients. Whereas a statistical difference in serum IgE concentrations was not obtained between treated and untreated subgroups, CIC-positive individuals receiving immunotherapy had significantly higher IgE values than the CIC-negative patients receiving immunotherapy.

DISCUSSION

Our results suggest that immunotherapy for allergic rhinitis and/or asthma is not necessarily associated with the development of CICs. Atopic individuals not receiving immunotherapy also have, as a group, increased serum concentrations of CIC, compared with healthy controls, although to a lesser extent than immunotherapy-treated patients.

This finding is similar to results obtained in our laboratories using a C1q deviation assay[3] but in contrast to other reports using the radiolabeled C1q precipitation assay.[23, 24] C1q methods are generally considered to detect CICs of relatively higher molecular weight than IgG type CICs.[24, 25] This is why we have concentrated solely on IgG CICs using a modified Raji cell assay, even though it does not measure the CIC containing IgG_3.

On the other hand, it is well documented that minimal variation in handling sera from the time of sampling to that of actual assay might induce a certain degree of precipitation of cryoglobulins that could give rise to artificially variable results even within individuals. For this reason we have allowed blood to clot overnight at 4° C to allow a uniform cryoprecipitation, if it occurs.

Studies of the present kind have some pitfalls in the choice of patient sampling. Thus, for the nonimmunotherapy group, the allergic symptoms are generally of earlier onset than for the immunotherapy-treated individuals. The period of actual "natural" exposure to the inhaled allergens may also differ. Furthermore, the clinical follow-up varies greatly among allergic patients: those not receiving immunotherapy tend to consult physicians during their severe symptomatic season. The "new" cases aim at immediate medical attention, which may not reveal instantaneously possible "cryptic" disorders other than allergy. This was exemplified during our investigation when 3 patients in the nonimmunotherapy group (46 patients) were found to have concomitant rheumatic disorders. The allergic individuals receiving maintenance immunotherapy are also a selected group of patients who already demonstrated benefit from treatment, and generally without major side effects; disease severity may also be different. Obviously, the optimal study would be a double-blind randomized, placebo versus allergen, immunotherapy. However, in the context of a medical condition not associated with known manifestations related to CICs,[3, 23, 24] it would appear unethical to proceed to such a study for the mere purpose of evaluating CICs. On the other hand, it is of great importance to better understand composite immune parameters that may lead to the development of "nonpathological" versus pathological CICs.[26]

Considering immunotherapy as a form of chronic antigenic stimulation, we have searched for possible clinical criteria and treatment characteristics that could be associated with the development of CICs. Age, sex, nature of the symptoms—allergic rhinitis versus asthma with or without rhinitis—did not appear to play any role. Similarly, the analysis of different therapeutic modalities—nature and total dose of allergens administered, number of different allergens used in some cases, as well as duration of immunotherapy and time from last injection—did not reveal any association with increased concentrations of serum CICs. Those patients who developed elevated CICs did not differ from CIC-negative individuals, whether receiving immunotherapy or not, as far as serum levels of IgG, IgA, IgM immunoglobulins, and RF activity. The only difference observed between CIC-positive and CIC-negative individuals was the concentration of serum IgE, which was elevated only for the group of patients receiving immunotherapy. This finding could not be explained.

The levels of CICs in some treated allergic individuals far exceeded those which could be expected from mere in vivo reaction between the allergen administered and the specifically induced antibodies. This, together with results from our group[2, 3] and those of others[23, 24] indicating that CICs are not a hallmark of immunotherapy, would suggest that a subpopulation of allergic people is prone to develop increased concentrations of CICs. We are presently investigating immunogenetic parameters that could potentially characterize this subpopulation.

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