CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY 16, 245–253 (1980)

Circulating Immune Complexes May Be Associated with Increased Suppressor T-Cell Activity in Atopic Allergy

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Received August 27, 1979

Using two groups of atopics, one with abnormally elevated serum IC levels (IC+) the other with normal IC values (IC−) we have investigated the generation of suppressor cells after Con A stimulation in three different *in vitro* assays: PHA-induced lymphocyte transformation, one-way MLC cultures, and IgG synthesis after PWM stimulation of B cells. In all cases, lymphocytes derived from IC+ patients, after Con A pretreatment *in vitro*, exhibited enhanced suppressor activity compared to IC− and normals. Furthermore, lymphocytes from IC+ individuals tended to respond poorly to specific antigen stimulation. Both groups of atopics were closely matched for clinical and familial parameters as well as for treatment modalities and therapeutic results. HLA and B-cell alloantigen studies did not reveal any group segregation. These results show that an increased T-suppressor cell activity may be found together with abnormally elevated circulating immune complexes in nonvasculitic, atopic patients.

INTRODUCTION

Numerous reports have delineated a suppressor immunoregulatory defect in patients with vasculitis (SLE) (1–3). This was demonstrated qualitatively by a decreased ability of their lymphocytes, after Con A activation, to generate suppressor activity on different T- and B-cell responses (4, 5). More recently, a quantitative deficiency in the subpopulation of T cells with suppressor characteristics (Ts) was shown in systemic lupus erythematosus (SLE) (6). Elevated circulating immune complexes (IC) also appear to be involved in the pathogenicity of several arthridites (7–10). Considering immune complexes as a manifestation of autoimmunity, two theories have been considered: an absence of tolerance at the T-helper cell level for autologous determinants (11) and/or a mere defect within Ts (6). In this respect, it was of interest to know if an abnormal Ts immunoregulatory potential of lymphocytes could be demonstrated in patients with high serum IC values but without any sign of vasculitis.

In the present investigation, we have selected to study atopic individuals undergoing conventional immunotherapy for a relatively long period of time, because: (i) despite similar levels of IC in certain cases, compared to SLE, there is no

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0090-1229/80/060245-09$01.00/0

established detrimental role for elevated IC in atopy (12, 13); (ii) other than their allergic problems, they were perfectly healthy and; (iii) as shown in a previous report, there was no correlation with age and sex of the patients; localization of the allergic symptoms (rhinitis versus asthma); nature, multiplicity, and total dose of the extracts administered; period between the time of last injection and the blood sampling for IC measurements; duration of treatment; levels of serum IgG, IgA, IgM, and rheumatoid factor activity (13).

The results show that the Con A-inducible suppressor T-lymphocyte activity on both B- and T-cell functions was actually increased in the group of atopics with high serum IC values compared to that found in a matched population of patients.

1. MATERIALS AND METHODS

1.1. Patient Sampling

Of the 95 individuals tested in a previous study (13), 21 subjects who repeatedly had IC above 20 μg/ml (IC+) were matched for age, sex, familial history of atopy, type and duration of allergic complaints and of immunotherapy, with an equal number of IC-negative (IC $\leq$ 20 μg/ml) individuals (IC−). All patients were analyzed and coded by an allergist (N.G.) not involved in this study; they had no other medical problems and were not receiving systemic steroids. Retrospectively, it was found that, of the 21 IC+ individuals, only 12 consented to participate in the study whereas 11 of the matched IC− agreed to; despite this, there was no statistical difference in multiple clinical and therapeutic variables among both groups (Table 1). Furthermore, the percentage of symptomatic improvement, using the criteria proposed by the Johns Hopkins' Hospital (14), as well as the overall incidence of delayed, Arthus-like reactions (15) at the site of injection were identical (Table 1).

Three healthy, nonatopic volunteers and three documented SLE patients served as internal controls for assessing experimental variations within each test. These were representative of a much larger group of healthy and SLE subjects repeatedly tested in our laboratory. Also two healthy known blood donors (M and N) were used throughout to provide the "target cells" for the suppressor assays: they were selected because they did not share any known HLA or B-cell alloantigen; thus they could account for putative intrinsic effects induced by major histocompatibility differences in the coculture experiments. More importantly, as source of "target/effectors" in numerous pretests, they gave consistently similar results and optimal stimulation, at well-defined antigen/mitogen concentration and incubation conditions used in the present study.

1.2. Cell Suspensions

Thirty milliliters of heparinized blood from each individual was taken in early morning under identical conditions, in three different sets of experiments. Mononuclear cell suspensions were obtained by standard Ficoll−Hypaque gradient centrifugation, and washed four times in balanced salt solution (16).

Enriched B- and T-cell suspensions were prepared by E rosetting of lymphocytes with sheep erythrocytes (SRBC) followed by centrifugation onto Ficoll−Hypaque (17). The E-rosetted cells recuperated from the pellet were freed from SRBC by hypotonic lysis as described elsewhere (17). The B-enriched

TABLE 1
CHARACTERISTICS OF THE GROUPS/TREATMENTS

	IC+			IC−			
	No.	Years	%	No.	Years	%	*P*
Age		40.8 ± 13.9[a]			41.7 ± 12.2		NS
Treatment duration		4.1 ± 0.5			3.9 ± 0.4		NS
Sex							
Female	11		92	9		82	
Male	1		8	2		18	NS
Family history of atopy	6		50	6		55	NS
Immunotherapy[b]							NS
with R	6		50	6		55	
G	3		25	3		27	
D	3		25	3		27	
MI	6		50	4		36	
W	1		8	1		9	
Single:	6		50	6		55	
Clinical improvement			53.1 ± 23.6			64.6 ± 17	NS
Side effects			48.3			42.9	NS

[a] Arithmetic means ± standard deviation; NS, $P > 0.05$.
[b] R, ragweed extracts; G, mixed grasses extracts; D, dust extracts; MI, dust and feathers extracts; W, wormwood extracts.

population was recovered from the interface of the gradients. All cell preparations were washed again and resuspended at 2×10^6 cells/ml in RPMI 1640 (GIBCO). Viability as assessed by trypan blue exclusion always exceeded 90%. Enriched B lymphocytes contained 71% ± 10.7 (SD) surface-immunoglobulin (Ig) positive cells by immunofluorescence (18) with a range of 65 to 86% for all experiments. The final preparations of T cells never contained more than 1% surface-Ig positive cells.

Following these procedures, the recovery of B/T cells varied somewhat among samples; in some cases, not all the different lymphocyte transformation assays could be performed.

1.3. HLA and B-Cell Alloantigen Typing

Conventional HLA typing of the A and B loci was performed in Terasaki plates, essentially as described previously, using "nonseparated" mononuclear cell suspensions (19–21). B-cell alloantigen typing was done using sera collected by the Immunology Department of the Centre Hospitalier de l'Université Laval. These had been extensively absorbed with platelets to remove antibodies directed against HLA-A, -B, and -C determinants (22, 23). The assay was done otherwise as described for HLA except that enriched B and T cells were tested in parallel (24, 25). The relative incidence of the detected antigens for all typings did not differ between both atopic populations, and between these and 24 tested other nonatopic individuals (results not shown).

1.4. Lymphocyte Assays

All cell cultures were done in triplicate aliquots in RPMI supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 50 μg/ml streptomycin, and incubated in a 5% CO_2-containing humidified incubator.

For induction of T cells with suppressor characteristics, mononuclear cells at a density of 2×10^6/ml in Falcon tubes (No. 2054) were incubated in presence of Con A (Pharmacia) at a final concentration of 60 μg/ml (Con A+ cells), or without Con A (Con A−). After 48 hr, they were washed and treated as described elsewhere (6). Followingly Con A and unstimulated cells from the atopics, controls and SLE individuals were cocultured in the different PHA, MLC, and PWM assays. Source of donor cells, population, and number of cells added as well as incubation period are described in Table 2. All cell cultures were done at a density of 10^6/ml using tissue culture microplates (Falcon, flat bottom No. 3040) except for the PWM tests which were performed in tubes (as for the generation of Con A suppressor cells).

PHA and PWM (GIBCO) were used at the optimal concentration found in pretests for both donors of responder cells (M and N), at 1:500 and 1:600 final dilution, respectively. Antigen E was kindly supplied by the NIH and deglycerinated by passage through a Biogel P-2 column; optimal concentrations of AgE (1.25, 6.25, and 25 μg/ml) were used according to pretests (Goodfriend *et al.*, unpublished). For PHA, MLC, and antigen E assays, tritiated thymidine (50 μl = 1 μCi) (NEN, specific activity = 6.7 mCi/ml) was added to each well for the last 12 hr of incubation. Specific lymphocyte transformation was then obtained after subtraction of counts from corresponding cell cultures which did not receive PHA, antigen E, or stimulator cells for MLC. The quantitation of PWM-induced

TABLE 2
LYMPHOCYTE FUNCTIONAL ASSAYS USED[a]

Assay	Cells: Supp.	Responder cells	Stimulator cells	Incubation time (days)
PHA:donor	Test/c/SLE	M, N	—	3
Cell No.	10^5 X	10^5		
Source of H.L.	(Con A^+/Con A^-)	T	—	
MLC:donor	Test/c/SLE	Aut.	M, N	6
Cell No.	10^5 X	10^5	10^5 X	
Source of H.L.	(Con A^+/Con A^-)	T	W	
PWM:donor	Test/c/SLE	M, N	—	7
Cell No.	5.10^5 X	10^6	—	
Source of H.L.	(Con A^+/Con A^-)	B	—	
AgE:donor	—	Test/c/SLE	—	9
Cell No.		10^5		
Source of H.L.		W		

[a] Supp.: suppressor cells (Con A+/Con A−); X: irradiated; H.L.: human lymphocytes; B: B cells; T: T cells; W: whole lymphocytes; donor: Test (atopic), c (controls, SLE (lupus); M and N: normal donors; Aut.: autologous cells to the suppressor cells used in the corresponding assays (derived from the corresponding Con A− individual).

IgG synthesis was performed using radioiodinated protein A from *S. aureus* (I-pA) as described elsewhere (26). Cocultures without PWM were not included because this would have required huge quantities of purified B cells. Furthermore preliminary tests revealed minimal background values of protein A fixation by cocultured cells in the absence of PWM (less than 10% of that obtained with PWM). This could be explained by the fact that IgM is the predominant class of immunoglobulins expressed on resting B lymphocytes (27) whereas PWM induces B cells to produce and express primarily IgG with the incubation conditions chosen here (28).

1.5. Calculation of Results

The percentage of suppression by Con A-stimulated cells was obtained by comparison with the corresponding cocultures containing Con A− cells. In general the suppressor activity of Con A-stimulated lymphocytes from normal individuals ranges, in our laboratory, from 40 to 60% for T-cell functions (PHA and MLC induced blastogenesis) and from 5 to 10% for PWM-induced surface IgG synthesis. In the present study (and several other experiments not reported here) Con A-incubated T cells from active SLE patients had no significant effect on the different lymphoid cell functions tested; therefore their results (0% suppression) will be omitted. For each other group (IC+, IC−, and controls), arithmetic means of the percentage of abrogation in PHA and MLC blastogenesis for Con A+ versus Con A− cells was calculated and statistical analysis performed by Wilcoxon ranking test. For AgE-induced lymphocyte blastogenesis, the results are expressed as arithmetic means ± 1 SD of specific stimulation obtained in the presence of the optimal concentration of allergen.

2. RESULTS

2.1. Coculture Experiments

Suppressor activity generated after Con A stimulation of lymphocytes from both groups of atopics was analyzed in two T-cell functional assays: PHA (Fig. 1) and one-way MLC (Fig. 2). For the MLC assays, suppressor cells, and responder, enriched T cells were derived from the same donor. The average PHA stimulation of lymphocytes from donor M was 70,588 cpm ± 13,169 (SD) for all cocultures performed with Con A− cells, whereas it was 70,977 ± 18,602 for donor N. In MLC assays, the average transformation, in presence of Con A− cells, was much greater using as stimulator cells donor M (69,510 ± 12,237) compared to donor N (29,327 ± 25,458). Taking into account that donor N stimulated poorly and with great variability in MLC, the results strongly suggest that Con A-stimulated cells from the IC+ group were qualitatively much more efficient in suppressing both PHA (Fig. 1)- and MLC (Fig. 2)-induced lymphocyte transformation, compared to the IC− group and to normals.

Similar results were obtained when the suppressor activity of Con A-stimulated cells on the synthesis of surface IgG was studied using B cells incubated in presence of PWM (Fig. 3). Using B cells derived from donor M, the average fixation of I-pA on cell lymphocyte cocultures containing Con A− cells was 14,680 cpm ± 6,687 (SD) whereas it was 11,994 ± 2906 for donor N. Despite a lesser degree of suppression generated by Con A+ cells on this *in vitro* B-cell functional test,

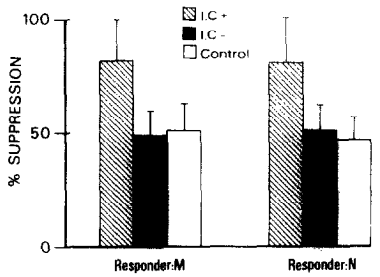

FIG. 1. Effect of coculture of Con A-treated cells from atopics with elevated IC (IC+), atopics with normal levels (IC−), and normals (C) on PHA-induced stimulation of lymphocytes. Data represent the mean suppression ± SD of three separate experiments; P values between IC+ (12) and IC− (11) were 0.07 and 0.03 for donor M and donor N, respectively; there was no difference between IC− and C.

compared to the previous results obtained in PHA and MLC assays, lymphocytes from the IC+ group also induced a greater nonresponsiveness of normal, B cells to PWM (Fig. 3). The broader standard deviation with donor M (45.5% of the mean) compared to donor N (24.2% of the mean) for all separate Con A− cocultures is a phenomenon described also by others (5, 29) which in part can explain the variability in the degree of statistical significance observed in such an assay.

2.2. Antigen E Lymphocyte Transformation

The final series of experiments were conducted to determine if specific antigen-induced lymphocyte transformation was different among both groups of atopics. Since ragweed allergens/pollen is the most frequent cause of allergic rhinitis in this region, we selected to study *in vitro* stimulation of lymphocytes by ragweed antigen E. As can be seen in Table 3, mononuclear cells from IC+ atopics undergoing ragweed immunotherapy were almost incapable of responding to AgE compared to cells from IC− atopics receiving the same treatment modality ($P = 0.025$). On the other hand, AgE-induced lymphoblastogenesis did not differ markedly between all IC+ and IC− atopics. These results would suggest a decreased responsiveness of lymphocytes to a specific, oligoclonal stimulus. In

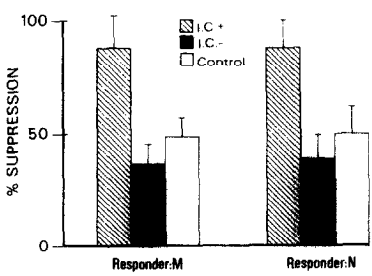

FIG. 2. Effect of coculture of Con A-treated cells from atopics with elevated IC (IC+), atopics with normal levels IC−, and normals (C) on MLC-induced stimulation of autologous lymphocytes by mononuclear cells from two unrelated individuals (M and N). Data represent the mean suppression ± SD of three separate experiments; P values between IC+ (12) and IC− (11) were 0.002 and 0.08 for donor M and donor N, respectively. IC− did not differ from C.

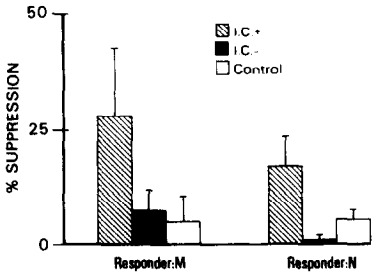

FIG. 3. Effect of coculture of Con A-treated cells from atopics with elevated IC (IC+) and atopics with normal IC levels (IC−) on PWM-induced IgG synthesis. Data represent the mean suppression ± SD of three separate experiments; P values between IC+ (12) and IC− (7) were 0.06 and 0.005, respectively.

analogy to experimental models, this phenomenon is compatible with increased suppressor cell activity toward specific antigens (6, 30, 31).

DISCUSSION

This study demonstrates that lymphocytes from a nonvasculitic group of atopic patients with comparable levels of circulating IC as found in SLE, are capable of being activated by Con A to express suppressor cell function in excess of what can be generated by lymphocytes from normals and from a matched group of patients without elevated IC. This was consistently demonstrated in several lymphocyte functional tests, T-cell related PHA- and MLC-induced blastogenesis, and B-cell PWM-induced IgG synthesis. In addition specific lymphocyte transformation was significantly lower in the IC+ group compared to the IC− group. This is in contrast with what has been observed in some arthritides entities with documented increased levels of IC (1−7).

Our finding of increased suppressor cell activity in a group of atopics undergoing immunotherapy is supported by the studies of Ishizaka in mice (30, 31). More interestingly, the present study has provided evidence that this was observed in the group of atopics who had abnormally high levels of serum-detectable IC. Despite a relatively small number of individuals tested, the findings were reproducible in three separate experiments. Of importance also is that both groups of atopics tested (IC+ and IC−) were very closely matched for all clinical, familial, and therapeutic parameters. Finally no genetic difference at the level of HLA-A and -B loci, nor at the level of B-cell alloantigens could be demonstrated. The

TABLE 3
AgE-INDUCED LYMPHOCYTE TRANSFORMATION

	IC+	IC−	P
All atopics[a]	455 ± 59	885 ± 652	(>0.1)
(No.)	(12)	(9)	
RW IT+[b]	273 ± 479	2400 ± 197	(0.025)
(No.)	(6)	(3)	

[a] cpm (arithmetic means ± 1 SD) for all atopic individuals tested.
[b] cpm (arithmetic means ± SD) for patients undergoing ragweed immunotherapy.

overall incidence of delayed, Arthus-like reactions, presumably due to IgG antibodies (15), was identical in the two groups.

It must be pointed out, however, that the nature of immune complexes may differ markedly in atopics and in vasculitides such as SLE. In the latter condition IC are presumably made of primarily autoreactive antibodies (11). In atopics undergoing immunotherapy, it could be argued that IC may contain a significant proportion of immunoglobulin complexed to the exogeneously administered antigens. However, the levels of IC do not reflect at all the total dose of extracts given (13).

What is more important to realize from this study is that a net increase of serum-detectable IC is not necessarily a reflection of deficiency of suppressor cell activity. Quite oppositely, increased T-suppressor cell activity may be found with abnormally elevated IC in atopy, for yet unknown reasons. Whether or not this phenomenon is actively involved in the regulation of IC in atopy remains to be elucidated. The fact that IC are also significantly elevated in atopics not undergoing immunotherapy, compared to nonatopics (13, 32) tends to favor that the results described herein are not secondary to immunotherapy per se.

ACKNOWLEDGMENT

We thank Dr. C. K. Osterland for his constructive criticisms; Dr. N. J. Gilmore for his clinical assessment of the patients; Miss Claudia Povajnuk for preparing this manuscript; Narciso R. Mejia and Ms. Mi Kim for their expert technical assistance. This study was supported in part by grants from Canadian Medical Research Council to G. D. and L. G.: and Canadian Arthritis and Rheumatism Society to G. D. and L. R. E. G. D. is a Quebec Medical Research Council Scholar. L. G. is a Canadian MRC Associate.

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