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Bystander T Cells Participate in Specific Response to Cockroach Antigen (CR) In Vitro

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ABSTRACT

Allergic reactions due to whole body, body parts and fecal products of cockroach (CR) are characterized by inflammatory reaction that may lead to symptoms of rhinitis or asthma in atopic individuals. Although the majority of T cells at the site of CR hypersensitivity are not antigen specific, the cellular subset and cytokine receptors that participate and control the outcome of the reaction have not been fully studied. In this study, we have used fluorescent activated cell sorter (FACS) analysis to characterize the activation marker and cytokine profile of antigen specific and bystander T cells after in vitro stimulation of peripheral blood lymphocytes with whole body extract of CR antigen. There was significant enhancement of CD69 on blast and bystander T cells in all atopic subjects compared to non-atopics. Both antigen specific and bystander T cells showed increased expression of HLA-DR, CD25 and CD71 in 9 of 11 atopic patients compared to control. There was also an increase in CD45RA+ and a decrease in CD45RO+ cells following antigen stimulation. These results correlated with the increase in the early apoptotic cells observed in patients as measured by Annexin V stain. Our data revealed that there was no difference in the expression of CD95 in both stimulated and bystander T cells. However, there was enhancement of FasL by CR antigen, suggesting that the increased apoptosis that was observed was probably due to the Fas/FasL interaction. Positive intracellular IL2, IL-4 and IFN- γ in T cells were observed in only the antigen specific blast cells in 83% of patients studied.

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These results suggest interplay of memory T cell response, apoptosis, and activated bystander T cells activities in maintaining cellular homeostasis during allergic reaction in cockroach sensitive atopic subjects.

Key Words: Bystander T cells; Cockroach antigen; Hypersensitivity.

INTRODUCTION

Several million Americans suffer from allergic reactions, which include asthma, allergic rhinitis, hay fever and atopic dermatitis. Polar et al. (1988) reported that 23 of 25 individuals with asthma had cockroach allergen specific IgE. Rosenstreich et al. (1997) reported that of 476 asthmatic children from eight inner-city areas in USA, 38.8% were allergic to cockroaches.

The functional outcomes of antigenic engagement of T cell receptor (TCR) include activation, anergy, and apoptosis of T lymphocytes. In cockroach allergen induced asthma, there is chronic inflammation of the bronchial mucosa. There are several reports of the involvement of T cells in inducing the disease (Djukanovic et al., 1990). Therefore, the disease process results in increase and accumulation of T cells as a result of cellular entry, proliferation, egress, and cell death. Allergic rhinitis and asthma are associated with IgE production and most studies have assessed sensitivity to cockroach allergen by measuring specific IgE or by skin prick test (Kang et al., 1993; Sastre et al., 1996). Although the role of T cells in hypersensitivity has not previously been well studied, in recent years, their role has been brought into focus. Jeng et al. (1996) showed that a significantly higher stimulation index was induced by CR antigen in the cockroach sensitive atopic individuals than in non-atopic subjects. They also demonstrated CR antigen specific induction of IL-4 mRNA in the T cells from atopic individuals.

CD69 is one of the earliest activation molecules expressed on T cells undergoing activation (Cebrian et al., 1988; Lanier et al., 1988; Testi et al., 1989). The data obtained from measurement of CD69 expression is comparable to that obtained by measuring H^3 -thymidine uptake following anti-CD3 stimulation (Mardiney et al., 1996). This surface antigen is expressed within 2 hours and peaks between 18 and 24 hours of activation. It has been suggested that the expression of CD69 signals an activation process that leads to production of other immunoregulatory molecules during an immune response. Apoptosis resulting from the engagement of CD95 receptor by monoclonal antibodies or soluble factors has been demonstrated in many systems, including mouse and human T cells (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). However, the process of apoptosis in activated T cells is less clear as there are contradictory reports suggesting both resistance and sensitivity to CD95 mediated apoptosis (Klas et al., 1993; Miyawaki et al., 1993). Studies are needed to identify and characterize cockroach sensitive T cells from atopic individuals as well as the role of CR specific and activated bystander T cells in modulating the disease process. Such studies would provide a better understanding of the mechanisms involved in terminating T cell driven processes and lead to improved management strategies of the disease.

The study reported here was designed to detect specific activated T cells as well as activated bystander T lymphocytes in cockroach sensitive atopic individuals. The aims were to 1) use early activation (CD69), late activation (HLA-DR, CD25, CD71), and



FACS analysis to detect sensitized T cells from CR antigen skin test positive and negative individuals, 2) to evaluate the presence and the role of activated bystander T cells during CR antigen response, and 3) to examine the modulation of CD95 and CD95L, and production of IL-2, IL-4, and IFN- γ in CR specific and bystander T cells in controlling and effecting the resolution of CR induced allergic reactions in atopic patients.

MATERIALS AND METHODS

Subjects

After obtaining approval from the Howard University Institutional Review Board (IRB), atopic subjects of both sexes age ranging from 9 to 53 years (mean age, 41 ± 4 years) were recruited from patients visiting the allergy clinic at Howard University Hospital. Patients were determined to be atopic if they suffered from clinical symptoms of allergic rhinitis or asthma and had positive skin prick tests to a panel of common aeroallergens. They were cockroach positive if the skin prick test was ≥ 3 mm wheal to whole body cockroach extract. Patients were selected according to routine protocols used for atopics in allergy service. The control group consists of volunteers and the staff of Howard University (14–50 years of age) who have no history or symptoms of atopic disease. Each individual participating in the study was skin tested with whole body extract of German and American cockroaches (ALK Lab Inc. Wallingford, CT.) after informed consent was obtained. Ten-milliliter blood samples were collected from each skin test positive (≥ 3 mm) or skin test negative (< 3 mm) participant into sterile green top Vacutainer tubes containing sodium heparin anticoagulant (Becton Dickinson, Franklin Lakes, NJ). All blood samples were processed within 6 hours for whole blood assays. Peripheral blood lymphocytes (PBL) were isolated from heparinized blood using lymphocyte separation medium (LSM) and the manufacturer's instructions (Organon Teknika Corp., NC).

Monoclonal Antibodies

The following fluorescent labeled monoclonal antibodies were purchased from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA). The FASTIM-MUNE 3-color open reagent system consisting of Fluorescein Isothiocyanate (FITC), Phycoerythrin (PE), and Peridinin Chlorophyll Protein (PerCP) labeled respectively, CD4/CD69/CD3, CD8/CD69/CD3, $\gamma 1/\gamma 1$ /CD3 (isotype control) for early activation markers, and FITC-labeled anti-HLA-DR, anti-CD25, anti CD71 for late activation markers were used in the assays. Intracellular formulation of IL-2-PE, IL-4-PE and IFN- γ -FITC and the corresponding isotype controls were also purchased from BDIS. Anti-CD45RA-FITC, CD45RO-PE, CD28-PE, CD95-PE, CD95L-APC, and Annexin V-FITC/PI were purchased from Pharmingen (San Diego, CA).

Whole-Blood Activation

Briefly, 500 μ l of whole heparinized blood was added to separate sterile polystyrene tubes with or without 120 protein nitrogen units (PNU/ml) of cockroach whole body

extract, 10 µg/ml of phytohemagglutinin (PHA), 10µg/ml of pokeweed mitogen (Sigma Chemicals, St. Louis, MO) or 5 µl of monoclonal antibodies against the CD2/R receptor as positive control. Tubes were incubated at 37 °C in 5% CO₂ for 6h and the cells were analyzed for early activation antigen CD69. Using the method described by Suni et al. (1998) to detect intracellular cytokines, Brefeldin A was added in the last 4h of a 6h incubation period in separate tubes of whole blood with or without CR antigen or PHA. For the expression of late activation markers, naive/memory cells, CD95, CD95L, or apoptosis, washed PBL at a concentration 1×10^6 lymphocytes/ml were incubated in complete RPMI supplemented with 5% FBS and 2% glutamate with or without the same concentrations of CR antigen or mitogens and incubated under similar conditions for up to 96 hours.

Staining and Flow Cytometric Analysis

After 6-hour activation, 50 µl of whole blood was added to 20 µl of FASTIMMUNE reagents. Samples were gently vortexed and incubated in the dark at room temperature for 20 minutes after which 450 µl of FACS lysing solution (Becton Dickinson, San Jose, CA) was added, vortexed and incubated in the dark at room temperature for at least 30 min to lyse red cells. For late activation markers, 50 µl of PBL in RPMI was added to each appropriately labeled tube containing 20 µl of FITC-labeled HLA-DR CD71, CD25, CD45RA, or PE-labeled CD95, CD45RO, or APC-labeled CD95L. The tubes were incubated in the dark at 4 °C for 30 minutes. The cells were washed once in phosphate buffered saline (PBS) containing 0.1% sodium azide and suspended in 0.5 ml 1% paraformaldehyde. Appropriate isotype control were also prepared and processed accordingly. For intracellular cytokines, 50 µl of activated whole blood was surface stained with 20µl of anti-CD3-PerCP as already described. The red cells were lysed with FACS lysing solution, and the remaining cells were washed and permeabilized (BDIS) for 30 min. The permeabilized cells were stained with intracellular formulation of IL-2, IL-4, IFN-γ or isotype matched controls for 30 min in the dark at 4 °C, washed once in PBS containing 0.5% BSA and suspended in 0.5 ml 1% paraformaldehyde. Externalization of phosphatidylserine was measured by the Annexin V/PI binding assay (Pharmingen, San Diego, CA) following the manufacturer's instruction to detect apoptosis. In addition, PE labeled anti-CD95 antibodies were used to stain activated lymphocytes to determine the percent changes in the expression of CD95. Flow cytometry analysis was performed using FACSCalibur (Becton Dickinson Immunocytometry System, San Jose, CA) to collect 10,000 events on activated cells with gates set on CD3 + T lymphocytes with a laser excitation at 488 nm or 635 nm wavelength. Data analysis was performed by using the CellQuest software (BDIS).

Statistical Analysis

All statistical analyses were performed by using the student's *t-test*. Accordingly, $p \leq 0.05$ was considered significant.

RESULTS

Primary Response by CR activated T cells are in atopic subjects. In the presence of cockroach antigens, CD69 was upregulated in atopic subjects compared to non-atopic individuals using flow cytometry. As shown in Figure 1a, over 4 fold increase in the expression of CD69 was observed in atopic subjects compared to non atopic volunteers ($p < 0.05$). Furthermore, we observed that higher number of CD4 + T cells responded with increased expression of CD69 than CD8 + cells (Figure 1b). The late activation

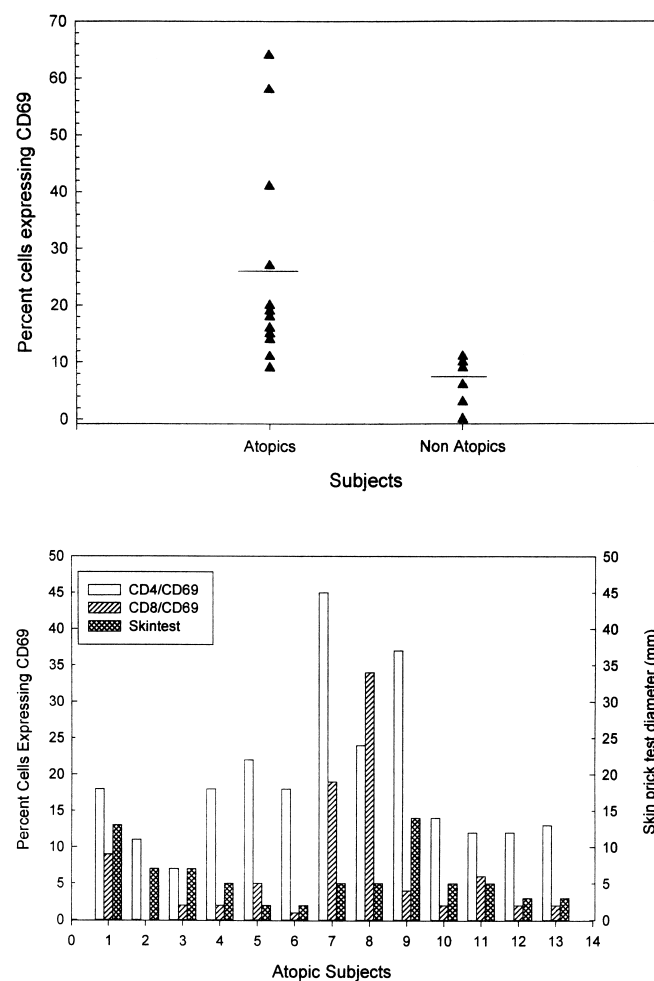


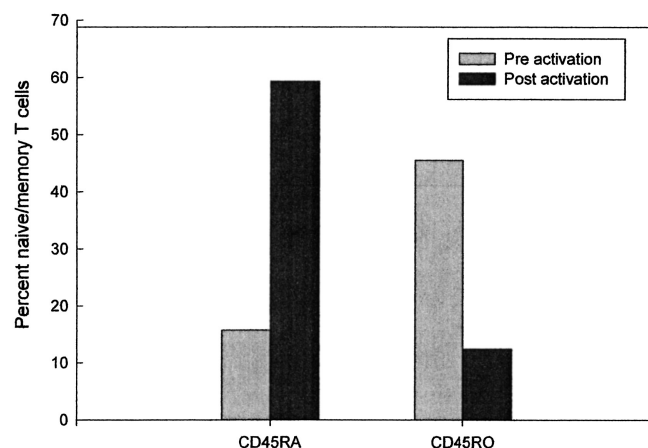
Figure 1. Surface expression of CD69 on T cells from atopic and non-atopic subjects. Whole blood from atopic and non-atopic was cultured with whole body extract of CR antigen for 6 h. Cells were stained with fluorescent labeled (a) CD69/CD3 and (b) CD4/CD69/CD3, CD8/CD69/CD3 monoclonal antibodies to assess the differences in response by the two T cell subsets and skin prick test diameter. Horizontal lines represent the mean values. $p < 0.05$.

Table 1. Expression of HLA-DR and CD71 on T cells from atopic and non-atopic subjects after in vitro treatments.

Treatment	Percent T cells expressing late activation surface markers			
	CR+ atopic subjects		Non atopic subjects	
	HLA-DR	CD71	HLA-DR	CD71
T cells + CR antigen	15.9 ± 2.1	6.3 ± 1.1	5.5 ± 1.8	2.3 ± 0.9
T cells + PHA	39.2 ± 5.1	15.8 ± 1.9	21.1 ± 3.4	9.4 ± 1.7
T cells + Media	6.4 ± 3.2	3.3 ± 0.9	1.2 ± 0.1	1.9 ± 0.8

Isolated PBL obtained from CR antigen skin test positive (atopic) and negative (non atopic) subjects was incubated with 120 PNU/ml of CR antigen, 10 µg/ml of PHA or media alone at 37 °C for 96h. After activation, cells were stained with fluorescent-labeled CD3, HLA-DR, and CD71. Ten thousand events were acquired and analyzed as described. Data represent mean ± SE of 13 atopics and 7 non-atopics ($p < 0.05$).

markers, HLA-DR and CD71 were observed to increase in 9 of 11 patients with ≥ 3 mm cockroach skin test positive results compared to non-atopic controls ($p < 0.05$). Increased expression of HLA-DR and CD71 suggests that CR antigen does not only induce activation but drives the cells to proliferate in such individuals (Table 1). There was an increase in the number of cells expressing CD45RA + (naive) antigens compared to cells that were not exposed to CR antigens for 96 hours. Corresponding cells expressing CD45RO+ antigens (memory) in the same individuals were decreased after similar treatment and incubation period in 8 of 9 (89%) of tested atopic subjects (Figure 2).

**Figure 2.** The effect of CR antigen on the naive/memory T cell ratio before and after culturing isolated T cells from CR+ atopic patients with antigen. Isolated T cells were exposed to CR antigen for 96 h and stained with labeled anti-CD45RA and anti-CD45RO. $p < 0.001$.

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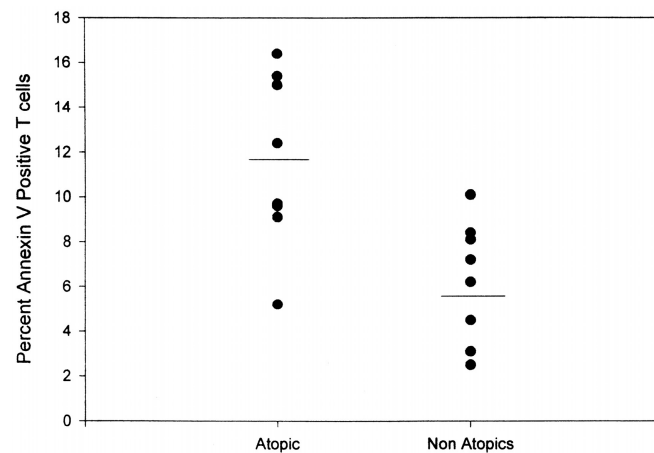


Figure 3. T cells from CR+ atopic subjects are more susceptible to apoptosis than non-atopics. Isolated PBL in complete medium were exposed to CR antigen for 96 h and stained for the expression Annexin V. Ten thousand cells were acquired and data analyzed using Cell Quest. Horizontal lines represent the mean values. $p \leq 0.05$.

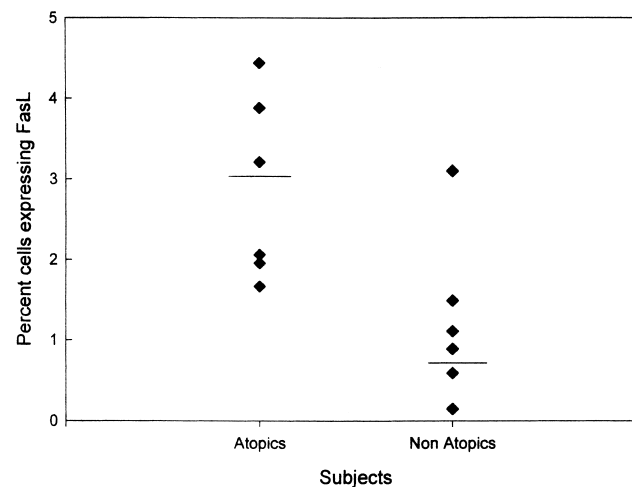


Figure 4. Enhancement of FasL on T cells from atopics after exposure to CR antigen. Isolated PBL from atopic and non atopic subjects was exposed to CR antigen for 96 and stained with APC-labeled anti-FasL. Ten thousand stained cells were acquired and analyzed using flow cytometer and Cell Quest data analysis program. Horizontal lines represent the mean values. There was significant difference between CR+ atopic subjects and non-atopics. $p \leq 0.01$.

Detection of Apoptosis in PBL

There have been conflicting reports on the relationship between Fas/FasL mediated apoptosis in allergic response and asthma (Jayaraman et al., 1999; Krug et al., 1999; Milik et al., 1997). Therefore, we examined the effect of CR antigen in mediating apoptosis in vitro. When the cells were tested to determine if CR antigen was capable of inducing apoptosis, a greater number of atopic subjects showed an increase in the number of early apoptotic cells as measured by the Annexin V/PI staining method. The observation here correlated with the low number of memory cells (CD45RO +) seen after activation with CR antigen, suggesting that the cells may have been eliminated through apoptosis (Figure 3). In an effort to determine if the observed increase in early apoptotic cells was due to Fas/FasL mediation, expression of CD95 was measured on cells exposed to CR antigen under the same conditions described previously. There was no significant difference in the number of cells expressing CD95 compared to untreated control (data not shown.). However, there was significant difference in the enhancement of CD95L in atopics compared to non atopic controls, suggesting that the increased number of early apoptotic cells was probably due to Fas/FasL interaction (Figure 4).

Enhancement of Activation Markers on Bystander Activated Cells

Under normal conditions of stimulation by antigens, only a small percentage of T cells that express the appropriate TCR rearrangements would be predicted to respond to stimulation. To identify cells that have undergone CR induced early activation, FACS analysis was used to differentiate between cells with higher forward and side scattergram (Figure 5A, R3), which appeared to identify cells that have been activated by CR antigen and small cells with low forward and side scattergram (Figure 5A, R2), which may be considered bystander cells. To confirm this hypothesis, the surface markers of the two populations were compared. The large scattering cells (Figure 5B, R3) exhibited the characteristics of activated cells by increased expression of CD69, HLA-DR, and CD25 compared with low scattering cells (Figure 5b, R2). In lower scattering cells, which have been shown not to be antigenically activated, the same analysis revealed the expression of CD25 and HLA-DR although, at a lower level in R2 than in R3 cells (Figure 5b). The surprising observation was that in CR sensitive atopic subjects, cells in R2 (bystander T cells) showed enhancement of CD69, HLA-DR, and CD25 over unstimulated control (Figure 5b, 0), including an identical level of expression of CD95 when compared with antigen activated T cells (R3). This observation was not seen in non-atopic subjects since they did not respond to CR antigen (data not shown). Among the individuals tested for the presence of intracellular cytokines, 55% were shown to have higher intracellular IL-2, IL-4 and IFN- γ in cockroach sensitive individuals than in control cells. Using the scattergrams (Figure 6A), it was observed that although the low scatter cells expressed CD69, CD25, and HLA-DR, no cytokine was detected in those cells whereas large numbers of blasts expressed IFN- γ , IL-4 and IL-2 (Figure 6B). These findings suggest that the immune processes that occur in atopic individuals to produce cytokines may not involve activated bystander T cells, although these cells express activation markers during allergic reaction to CR antigens.

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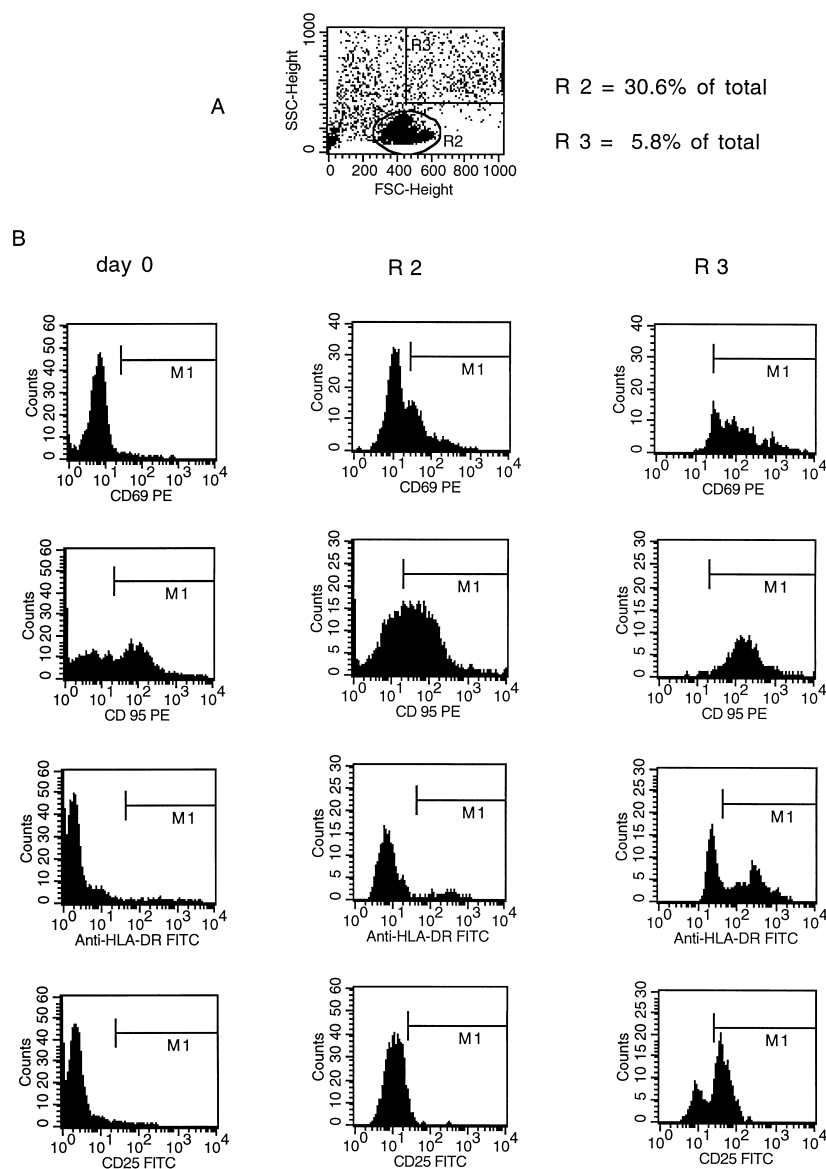


Figure 5. Characterization of CR antigen activated blast culture. A) Forward and side scatter analysis of 18–96 h CR blast culture showing distinct T cell populations; R3 (large cells) and R2 (small cells) regions. B) Surface phenotype of unstimulated PBL (day 0) compared with CR-stimulated cells with R2 and R3 regions as assessed by using flow cytometry. Data is a representative of 7 separate experiments with similar results.

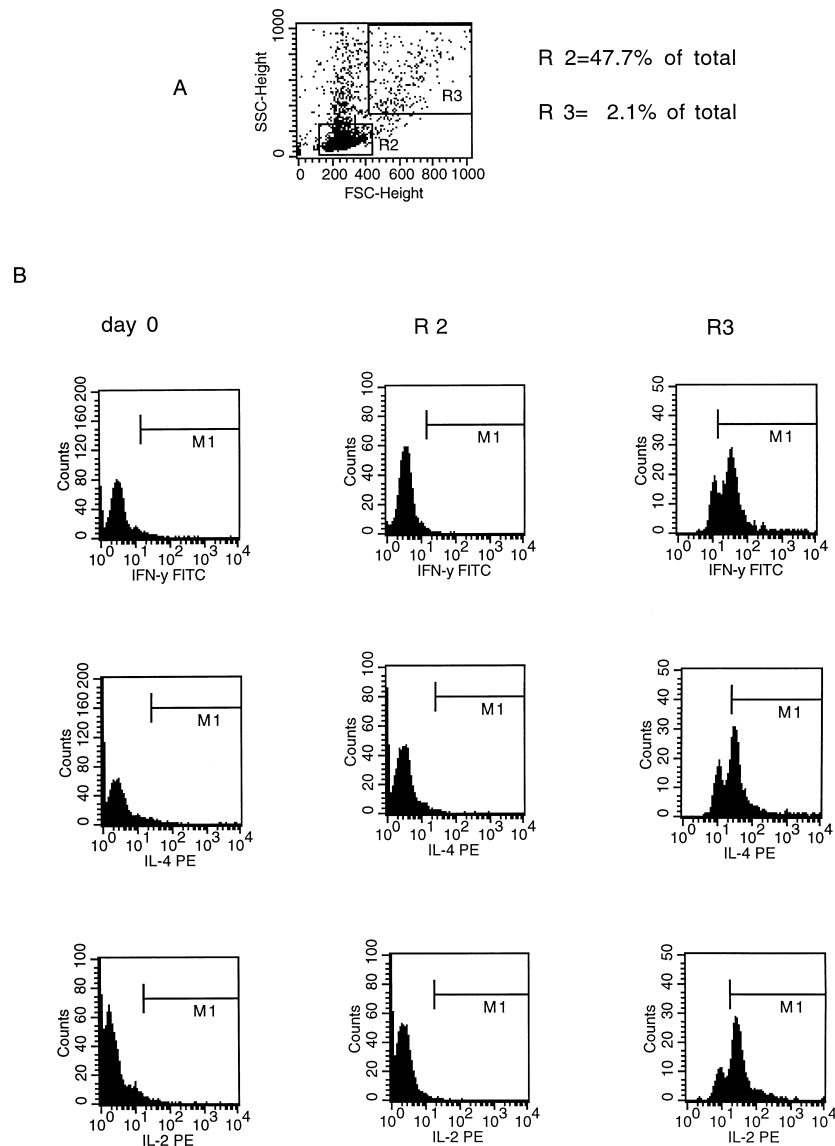


Figure 6. Cytokine profile characteristics of CR antigen-specific activated blast cells (high scatter R3) versus low scatter (R2). T cells from CR+ atopic subjects were cultured with CR antigen for 6–12 h and stained with fluorescent labeled intracellular formulation of cytokines as described in the Materials and Methods section.



DISCUSSION

A well recognized feature of hypersensitivity to cockroach antigen in atopic individuals include T cell recruitment and activation, but the mechanisms in which large number of activated T cells are observed has not been fully explored. Successful engagement of allergen to T cell through the T-cell receptor can lead to antigen recognition, clonal proliferation and activation induced death via apoptosis (Ucker et al., 1992). In this study, we used the ability of the flow cytometer to distinguish cells with large forward scatter (FSC) and side scatter (SSC), characteristics that appear to identify cells that have been activated by CR antigen, as well as specific monoclonal antibody for early (CD69) and late (HLA-DR, CD71, and CD25) activation molecules to identify T cell responsiveness to cockroach antigen in atopic and non atopic individuals. Two reports Touch et al. (1996) and Ehl et al. (1997) have suggested that during antiviral responses, most proliferating cells are not antigen specific but are stimulated by lymphokines resulting in a bystander response. We therefore used the FACS analysis to determine and characterize the regulation of cell surface markers on bystander T cells in the atopic population in response to in vitro CR allergen challenge. Expression of CD69 is a sensitive marker for T cell activation (Mardiney et al., 1996) and was a valuable tool for detecting cockroach sensitized individuals since all atopic subjects showed enhancement of the marker. The size of the skin test did not correlate with the percent of cells expressing CD69, as some individuals with less than 2 mm reaction gave a strong response as measured by the percentage of cells expressing CD69 following stimulation. These observations are in agreement with Leonard et al. (1997) who showed that although different groups of atopic patients exhibit different patterns of allergen-induced cytokines, the production of such cytokines did not correlate with the severity of the disease. There is considerable experimental evidence indicating that the pathogenesis of asthma is mediated by Th1 and Th2 CD4 + T cells (Umetsu et al., 1997). However, there was one individual in this study whose CD8 + subset showed greater CD69 expression than in the CD4 + subset (patient #8 in Figure 1b). Although we could not assign any obvious reason for this individual, CD8 + T cells have been shown to produce IL-4 to promote IgE synthesis (Meissner et al., 1997; Punnonen et al., 1997). Umetsu et al. (1985) and Kisaki et al. (1988) have demonstrated the importance of activated bystander T cells in providing B cells from atopic individuals with what was termed 'a binding factor' to enhance IgE synthesis.

Altogether, FACS analysis and monoclonal antibodies have been used to show that upon exposure to CR antigen, the inflammatory responses in atopic subjects, its immunopathology and resolution may involve activated bystander T cells. Although these activated cells have not been shown to secrete the cytokines studied here, their presence in activated state via the expression of activation markers is noteworthy. This suggests that they may play a role in the allergic process. The data presented here makes it tempting to speculate that these cells proliferate independently of antigen stimulation to maintain homeostasis and thereby compensate for the loss of lymphocytes through activation induced apoptosis. This is a credible speculation in that Unutmaz et al. (1994) have shown an antigen independent activation of naive and memory resting T cells by a cytokine combination of IL-2, TNF- α , and IL-6. Their results suggested that such cytokine combinations could induce proliferation of naive T cells without a switch to memory phenotype to help maintain the peripheral pool of

naive T cells. The recent study by Guerra et al. (1999) showed that allergens induced increased apoptosis in peripheral blood lymphocytes from atopic patients, which was duplicated in our study.

All the above referenced studies indicate that activated bystander T cells may play a role in allergic and other diseases, and collectively point to a need for additional well designed studies to elucidate this phenomenon.

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