Lymphocyte Markers and Proliferative Responses to Microbial Antigens in Patients with Allergic Rhinitis

Allerjik Rinitli Hastalarda Lenfosit Belirteçleri ve Mikrobiyal Antijenlere Karşı Proliferatif Yanıtlar

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ABSTRACT Objective: Atopy is a condition of predisposition to allergic reaction to environmental allergens, and T cells have a critical role in initiating and ending allergic responses. This study was conducted to evaluate the T cell responses of atopic patients with allergic rhinitis who have allergen-hyperreactive memory CD4 T cells in vitro. **Material and Methods:** Cell surface markers (CD3, CD4, CD8, CD19, CD28, CD45RA, CD45RO, CD95, HLA-DR) were analyzed for T and B lymphocytes by flow cytometry using fluorescein isothiocyanate (FITC) or phycoerythrin (PE) labeled monoclonal antibodies. T cell proliferative response assessing pokeweed mitogen (PWM), tetanus toxoid (TT), purified protein derivative of mycobacterium (PPD) and cytomegalovirus antigen (CMV) were examined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay. **Results:** Immune profile and lymphoproliferative responses of 19 allergic rhinitis (AR) patients with positive prick skin test and nasal blockage, discharge, sneeze, nasal or ocular itching (mean age 33.2 ± 8.4), and 10 healthy controls (mean age 31.6 ± 9.1) were evaluated. CD3 and CD4 expression was higher in AR patients than in healthy controls. Memory (CD45RO) and activated (CD28) T cell levels were higher, but lymphoproliferation to PWM, TT, PPD, and CMV was decreased in AR patients. **Conclusion:** The high CD28 and CD45RO expression associated with atopy symptoms indicated that immune reactions in AR patients tended to show an undesirable shift toward Th2 skewed with high levels of allergen-reactive memory T cells. Consequently, the reduced lymphoproliferation to non-allergenic stimulants such as mitogens, bacterial and viral antigens in AR patients may lead to reduced immune response capability to infectious agents.

Key Words: Allergy and immunology; antigens, CD4; lymphocyte proliferation potentiating factors

ÖZET Amaç: Atopi çevresel allerjenlere karşı allerjik reaksiyonlara yatkınlık durumudur ve allerjik reaksiyonların başlatılması ve sona erdirilmesinde T hücreleri kritik role sahiptir. Bu çalışma allerjene hiperreaktif bellekli CD4 T hücrelerine sahip allerjik rinitli atopik hastaların in vitro T hücre cevaplarını değerlendirmek için gerçekleştirilmiştir. **Gereç ve Yöntemler:** B ve T lenfositleri için hücre yüzey belirteçleri (CD3, CD4, CD8, CD19, CD28, CD45RA, CD45RO, CD95, HLA-DR) flow sitometride floresan izotiosiyanat (FITC) veya fikoeritrin (PE) işaretli monoklonal antikorlar kullanılarak analiz edildi. T hücrelerinin proliferatif yanıtı pokeweed mitojen (PWM), tetanoz toksoidi, mikobakteri pürifiye protein derivesi (PPD) ve sitomegalavirüs antijeni (CMV) kullanılarak 3-[4.5-dimetiltiyazol-2-yl]-2.5-difeniltetrazolium bromid (MTT) indirgeme metodu ile değerlendirildi. **Bulgular:** Burun tıkanıklığı, akıntı, hapşırma, burun veya gözde kaşınma sorunları olan ve deri prick testi pozitif 19 allerjik rinit hastası (yaş ortalamaları 33.2 ± 8.4) ile 10 sağlıklı kontrolün (yaş ortalamaları 31.6 ± 9.1) immün profilleri ve lenfosit proliferasyon yanıtları değerlendirildi. CD3 ve CD4 ekspresyonu allerjik rinit hastalarında sağlıklı kontrollerdekinden daha yüksek bulundu. Allerjik rinit hastalarında bellekli (CD45RO) ve aktive olmuş (CD28) T lenfositi düzeyleri yüksek, fakat PWM, TT, PPD ve CMV'ye karşı lenfoproliferatif yanıtlar düşük bulundu. **Sonuç:** Atopi belirtileriyle birlikte yüksek CD28 ve CD45RO ekspresyonu, allerjik rinit hastalarındaki immün reaksiyonların, yüksek düzeydeki allerjen-reaktif bellekli T lenfositleriyle tercih edilmeyen Th2 yönüne kaydığını göstermiştir. Sonuç olarak, mitojenik, bakteriyel ve viral antijenler gibi allerjenik olmayan uyaranlara karşı lenfoproliferatif yanıtta gözlenen düşüş, allerjik rinit hastalarında enfeksiyon etkenlerine karşı immün yanıt kapasitesinde düşüşe yol açabilmektedir.

Anahtar Kelimeler: Allerji ve immünoloji; CD4 antijeni; lenfosit stimule edici faktör

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Atopy is a chronic disorder of the nose induced by immunoglobulin-E (IgE) derived reactions against environmental allergens.[1,2] IgE-mediated inflammation of the nasal mucosa after exposure to one or more allergens is a characteristic feature of allergic rhinitis.[3] The relevant symptoms are sneezing, clear and watery rhinorrhea, nasal congestion, nasal or ocular itching. T cells have a critical role in initiating and ending the allergic responses.[4] The beginning of T cell activation functions as a trigger for production of critical cytokines and especially IgE, which start and expand the inflammation. The studies have shown that Type 2 (Th2) cell subpopulation of $CD4^+$ T cells have a major role in atopy and Th2 patterns of cytokines produced by T lymphocytes has been accepted as the primary abnormality of atopic patients. The main cytokines produced by Th2 subgroups of activated T cells are interleukin (IL)-4, IL-5 and IL-13.[5,6] This immune response is also characterized by a defective production of interferon-γ (IFN-γ). On the contrary, activation of Th1 subgroups cause production of IL-2 and IFN-γ, which initiate the killing of viruses and other intracellular organisms by activating macrophages and cytotoxic T cells.[7]

Considering these data, it is possible to postulate that a lower immune response might occur against viral and microbial stimulations in allergic individuals in whom Th1 activation is suppressed. Few studies have drawn attention to this issue so far.[8,9]

The incubation of blood lymphocytes of atopic patients with inhalant and food allergens leads to a proliferative response, which indicates the presence of a specific T cell mediated hypersensitivity.[10] Different pathways of T lymphocyte activation may be involved after stimulation with different source of allergenic and non-allergic stimulants such as mitogens, bacterial and viral antigens in allegic rhinitis (AR) patients.

In this study, to investigate the immune status of AR patients, immune profile and lymphoproliferative response to bacterial, viral and mitogenic stimulants were examined.

MATERIAL AND METHODS

STUDY POPULATION

AR outpatients with nasal blockage, discharge, sneeze, nasal or ocular itching and healthy individuals were informed about the study and were invited to participate as volunteers. The procedure was approved by the Local Ethics Committee approval. Nineteen voluteer AR patients [7 males, 12 females; mean age 33.2 ± 8.4 (18-46)], and ten healthy controls [4 males, 6 females; mean age 31.6 ± 9.1 (18-45)] with positive and negative skin prick test, respectively, were recruited to the study.

DIAGNOSTIC CRITERIA

Skin prick tests (SPT) were performed in AR patients and healthy controls with inhalant allergens; *D. ptergynissinus*, *D. farinae*, *Alternaria*, *Cladosporium*, grass pollen, weed pollen and tree pollen, (Stallergen®, Paris, France), and histamine hydrochloride (10 mg/mL) were used as positive, and saline 0.9% as negative control. The wheal size was marked with a filter pen after 20 min and measured on a micropore tape. The SPTs were considered positive if the mean wheal diameter was at least 3 mm larger than negative or 50% larger than positive controls, respectively.[11] The diagnosis of atopy was allowed if at least one skin prick test to a common environmental aeroallergen had been positive.[12]

EXCLUSION CRITERIA

Severe asthma, use of corticosteroids or other immunosuppressive drugs, immunotherapy, chronic and acute infections, especially of the upper respiratory tract within the last month was exclusion criteria from the study. The same criteria were valid also for healthy controls.

BLOOD SAMPLES

Blood was drawn from 19 AR patients and 10 healthy controls have been taken in August in which less seasonal exposure to pollens or chronic exposure to perennial allergens was minimal. Blood samples were obtained within a period of 15 days and each sample was analyzed as soon as drawn from the subjects.

CELL PURIFICATION

Peripheral blood mononuclear cells ($PBMC_s$) were obtained from heparinized blood by density gradient centrifugation over Ficoll (Sigma Chemical Co., St. Louis, MO, USA) and were suspended in Iscove's Modified Dulbecco's Medium (IMDM, Sigma) in the presence of 10% fetal calf serum (FCS). Cell viability was measured by trypan blue (Sigma) dye exclusion and was over 96% in all samples.

FLOW CYTOMETRIC ANALYSIS

For flow cytometric analyses, $5x10^4$ cells were stained with fluoroscein isothiocyanate (FICT)-labeled anti-CD4, anti-CD3, anti-CD45RA, anti-HLA--DR, anti-CD95 and Phycoerythrin (PE)-labeled anti-CD8, anti-CD19, anti-CD45RO, anti-CD28. The controls were FITC or PE-labeled mouse IgG1 (all purchased from Becton Dickinson cytometry systems, USA). Stained cells were fixed in 2% paraformaldehyde. Flow cytometric analyses were performed with a FacsCalibur (Becton Dickinson cytometry systems, USA). At least 10.000 events were acquired for each analysis and the data were analyzed using the Cellquest® software.

COLORIMETRIC MTT ASSAY

Lymphoproliferative response was determined by stimulation of $1x10^5$ PBMC for 72 h with mitogen and antigens in 100 mL of medium in 96 well flat-bottom tissue culture plates in triplicates. Cells were incubated in the presence of 20 μg/mL pokeweed mitogen (PWM), 6 mg/mL tetanus toxoid (TT, Aventis Pasteur SA, Lyon, France), 10 mg/mL purified protein derivative of mycobacterium (PPD, B-NCIPD Ltd. Sofia, Bulgaria), 1.25 mg/mL cytomegalovirus (CMV, Abbott Lab. Abbott park, IL). After 72 hours of incubation the cultures were pulsed with 10 mL of 5 mg/mL methyl-thiasole-tetrazolium salt (MTT, Sigma) for 4 hours. Plates were centrifuged and the untransformed MTT was removed carefully by an Eppendorf pipette. To dissolve MTT-formazan, formed by the cells from MTT, dimethyl sulfoxide (DMSO, Sigma) was added to the wells and they were incubated overnight at room temperature in a dark place.[13] The spectrophotometric values were obtained in ELISA plate reader (Organon Technica Reader 530) at 540 nm wavelength and a reference wavelength of 620 nm.

STATISTICAL ANALYSES

Data were expressed as median, minimum-maximum and interquartile range. Statistical analysis was performed by Mann-Whitney U test. A "*p*" value of less than 0.05 was considered significant.

RESULTS

Nineteen AR patients with positive prick skin test, mean age [33.2 ± 8.4 (18-46)] and nasal blockage, discharge, sneeze, nasal or ocular itching, and 10 skin prick test negative healthy control individuals [mean age 31.6 ± 9.1 (18-45)] were evaluated.

Cell surface antigens assessed by flow cytometry using specific fluorescein-labeled monoclonal antibodies are shown in table and graphic forms, separately (Table 1, Figure 1).

T CELL ASSOCIATED SURFACE ANTIGENS

CD4 expression was significantly higher in AR patients than in healthy controls (p= 0.0001), whereas CD8 was expressed almost identically in both groups (p= 0.573). The expression of CD3, a marker of the total T lymphocytes, in conformity with increased CD4 expression, was higher in AR patients than in healthy controls (p= 0.0001).

B CELL ASSOCIATED SURFACE ANTIGEN

As B lymphocyte marker, CD19 expression was similar in AR patients and healthy controls (p= 0.804).

ACTIVATED T CELLS

As an activated T cell marker, CD28 expression was significantly higher in AR patients than in healthy controls (p= 0.002).

NAIVE AND MEMORY T CELLS

CD45RA expression, as a naive T cell marker, was similar in AR patients and healthy controls (p= 0.769), but CD45RO expression, as a memory T cell marker, was significantly higher in AR patients than in healthy controls (p= 0.0001).

TABLE 1: Flow cytometric analyses of cell surface antigens in healthy controls and allergic rhinitis patients.

		Cell surface antigen expression (%)			
	Groups	**Median**	**Min-Max**	**Interquartile range**	**Significance**
CD4	Healthy Control	31.87	23.81 - 46.89	8.02	p= 0.0001
	Patients with AR	45.98	33.83 - 55.64	9.94	
CD8	Healthy Control	28.49	16.94 - 36.47	11.41	p= 0.573
	Patients with AR	27.30	16.45 - 37.78	6.54	
CD3	Healthy Control	56.59	32.31 - 70.00	18.47	p= 0.0001
	Patients with AR	71.41	60.90 - 78.76	8.79	
CD19	Healthy Control	8.59	6.28 - 22.63	5.39	p= 0.804
	Patients with AR	9.36	5.90 - 16.72	5.10	
CD45RA	Healthy Control	38.85	23.82 - 57.36	13.58	p= 0.769
	Patients with AR	38.93	28.91 - 58.98	9.05	
CD45RO	Healthy Control	24.16	17.93 - 32.32	7.12	p= 0.0001
	Patients with AR	34.49	20.96 - 45.59	15.25	
CD28	Healthy Control	39.97	31.04 - 51.64	14.45	p= 0.002
	Patients with AR	55.02	19.74 - 74.89	13.29	
HLA-DR	Healthy Control	14.17	9.24 - 27.50	7.08	p= 0.875
	Patients with AR	15.09	7.85 - 25.05	5.73	
CD95	Healthy Control	25.29	16.83 - 36.12	8.44	p= 0.205
	Patients with AR	26.85	12.36 - 47.32	8.43	

AR= Allergic rhinitis, CD= Cluster of differentiation, HLA= Human leukocyte antigen.

HLA-DR AND CD95 (FAS) EXPRESSIONS

HLA-DR expression was similar in AR patients and healthy controls (p= 0.875). CD95 (Fas) expression was higher in AR patients than in healthy controls (p= 0.205), but this difference was not significant.

Lymphoproliferative Response to Mitogen, Bacterial and Viral Antigens

Lymphoproliferative response to mitogen, bacterial and viral antigens assessed by colorimetric MTT assay are shown in table and graphic forms, separately (Table 2, Figure2).

AR patients exhibited lower lymphoproliferative response to PWM than healthy controls (p= 0.002). Compared to healthy controls AR patients exhibited lower lymphoproliferative response to TT, (p= 0.016) PPD (p= 0.007) and CMV (p= 0.005).

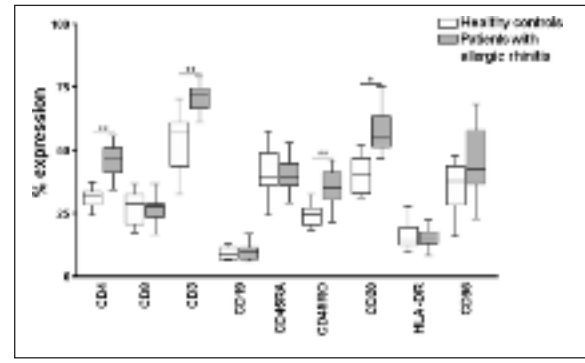

FIGURE 1: Cell surface antigen expression of atopic patients with allergic rhinitis and healthy controls.

Freshly purified cells were labeled with FITC/PE conjugated B and T cell subsets surface monoclonal antibodies (mAbs) and were analyzed by flow cytometry. Comparisons of the groups were performed by Mann-Whitney U-test.

*p< 0.05, **p< 0.001.

CD= Cluster of differentiation, HLA= Human leukocyte antigen.

DISCUSSION

In this study, lymphoproliferative responses to non-allergic stimulants and immunphenotypic chararteristics of AR patients were investigated in order to determine their immune status. We determined significant increase in CD4+ Th cells in atopic patients with AR. Several distinct T helper (Th) subsets have been identified, based on cyto-

TABLE 2: Lymphoproliferative responses of mononuclear cells in healthy controls and allergic rhinits patients against PWM and various antigenic stimulants.

		Lymphoproliferative responses (Mean OD)			
	Groups	**Median**	**Min-Max.**	**Interquartile range**	**Significance**
PWM	Healthy Control	234	207 - 302	47	p= 0.002
	Patients with AR	200	87 - 266	105	
TT	Healthy Control	233	217 - 298	46	p= 0.016
	Patients with AR	204	109 - 305	82	
PPD	Healthy Control	228	186 - 289	51	p= 0.007
	Patients with AR	170	106 - 294	110	
CMV	Healthy Control	273	221 - 292	43	p= 0.005
	Patients with AR	215	103 - 304	123	

PWM= Pokeweed mitogen, PPD= Purified protein derivative, TT= Tetanus toxoid, CMV= Cytomegalovirus, AR= Allergic rhinitis, OD= Optical density.

kine secretion by cells.[14] Chambers et al suggest the presence of changes in subsets of Th cells specific for microbial antigens as well as allergens in atopics, which may have important implications for the aetiology of atopic diseases including AR.[15] The key role of allergen specific $CD4^+$ T lymphocytes in atopic patients with AR is well-established. Rimmaniol et al stimulated the peripheral blood mononuclear cells with *P. pratense*, or with recall antigens, and by determining CD4+/CD3+ T-cells in grass pollen sensitive atopic patients showed that they had enhanced CD4+ T-cell reactivity.[10] Varga et al reported that AR was associated with increased numbers of submucosal CD3+ T cells.[16] The results suggest that the CD+ T cells proliferate and migrate to nasal mucosa. In our study, in accordance with these results, the finding of high level of CD4 and CD3 expressions reflects high Th activity in atopic patient with AR.

CD28 costimulates T cell proliferation and cytokine production. CD28 stimulation is critical for Th2 differentiation. Zdolsek et al showed that high CD28 in children with positive prick skin test and atopic symptoms might possibly be a consequence of a Th2-skewed immune system.[17] Ir was not possible to judge whether $CD4^+$ cells belonged to any of Th1 or Th2 subgroups due to the lack of cytokine panel in our study; however, the high CD4 expression together with high CD28 molecules reflects that the atopic patients with AR have Th2-skewed immune responses. This may account for the lower lymphoproliferation to non-allergenic stimulants explained by preclusion of Th1 lymphocytes and insufficiency of immune response capabilities in AR patients.

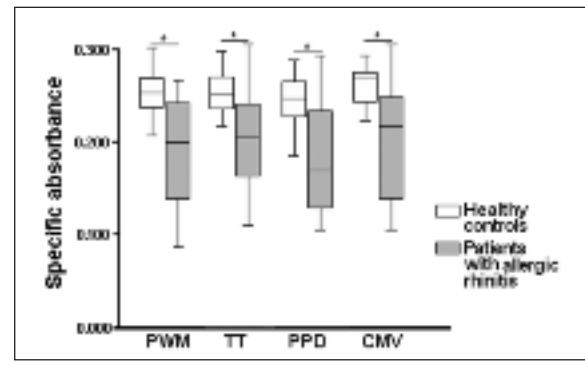

FIGURE 2: Mitogen- and antigen-induced lymphoproliferation of atopic patients with allergic rhinitis and healthy controls.
Peripheral blood samples were obtained from each subject and were cultured in triplicate in the presence of PWM (10 μg/mL), TT (6 μg/mL), PPD (10 μg/mL) and CMV (1.25 μg/mL). Following 72 h of culture, the proliferation was assessed by MTT (methyl-thiazole-tetrazolium) reduction. Comparisons of the groups were performed by Mann-Whitney U-test. *p< 0.05.
PWM= Pokeweed mitogen, TT= Tetanus toxoid, PPD= Purified protein derivative, CMV= Cytomegalovirus.

Akdis et al showed Th2 subgroup predominance in atopic diseases by preferential apoptosis of circulating memory/effector Th1 cells.[18] Barkow et al suggested that chronic immune activation leads to hyporesponsiveness and deficiency of the immune cells.[8] Furthermore, these authors suggested that hyporesponsiveness or anergy of the

immune cells cause a diminished capacity to cope with infection and to build an effective immune response after vaccination. Taking into consideration that Th2 dominating situations; our findings seem to be in accordance with some aspects of the studies mentioned above.

Previously lymphocyte responses to mitogens were studied in vitro by some investigators in allergic diseases. Kinikli et al showed that lymphoproliferative responses to the PWM was low in atopic patients with AR and recovered by immunotherapy.[19] Avila et al demonstrated that the negative tuberculin response to PPD was a consequence of reduced cell response and recommended revaccination of allergic children with BCG.[20] Similar to this study, Gentile et al showed differential Th cytokine responses in atopic patients with AR after immunization with tetanus toxoid due to the lower levels of IFN-γ, a proinflammatory cytokine, found at lower levels in AR patients after immunization by TT.[21] These findings suggest that atopics have lower immune response capability to mitogenic and some bacterial antigens, supporting the results of our study.

It is well-established that viral infections have a major role in the pathogenesis of atopic diseases. Dökce et al suggested that subclinical CMV infections occur more frequently in patients with atopic diseases.[22] However, researchers showed a significant increase in CMV reactive T lymphocytes producing IFN-γ, a finding that seems in discrepancy with our results.

To conclude, in our study, the decreased lymphoproliferative response to mitogens, TT, PPD and CMV antigens in vitro suggest that persistent immune activation in atopic patients with AR might cause hyporesponsiveness of the T lymphocytes due to Th2 skewed immune activation to some microbial antigens stimulating the immune system on Th1 pathway.

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