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Role of Nitric Oxide in Activation of Human T Lymphocytes Induced by Bacterial Superantigen

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The involvement of nitric oxide (NO) in the regulation of human T cell response to bacterial superantigen (staphylococcal enterotoxin B) was studied. It was shown that stimulated T lymphocytes are the main source of NO. This superantigen markedly increased NO production and triggered the proliferative response of mononuclear cells from healthy individuals; the degree of apoptosis was low. In patients with purulent surgical diseases with high spontaneous and induced NO production, superantigen enhanced apoptosis of lymphocytes and induced anergy of T cells to enterotoxins. Increasing the concentration of NO in cultured cells from healthy individuals in the presence of NO donors also stimulated apoptosis and inhibited proliferative activity. These data suggest that NO regulates T lymphocyte response to superantigens. The increased production of NO probably contributes to the development of immunosuppression during bacterial infection.

Key Words: *human T lymphocytes; staphylococcal enterotoxin B; nitric oxide; proliferation; apoptosis*

There is no doubt that activation of immunocompetent cells by exogenous and endogenous factors results in positive (cytokine production and proliferation) or negative (apoptosis and anergy) reactions [8,12]. The intensity and nature of activating and co-activating stimuli, cell-cell interactions, and growth factors determine the outcome of cell activation and the efficiency of the immune response.

The appearance of anergic T cells and intensive lymphocyte apoptosis in various infections [1,3] suggest that these factors contribute to immune disorders in patients with inflammatory and infectious diseases. Recent studies indicate that bacterial antigens (superantigens) induce anergy and apoptosis of T cells. Superantigens bind to specific V_β chains of T lymphocyte receptor complex and activate various clones of T cells. Hence, superantigens are potent polyclonal activators. Rapid injection of gram-negative bacterial superantigens, staphylococcal enterotoxins B (SEB) and A, causes hyperactivation of the immune system and massive release of antiinflammatory cytokines. This results in septic shock [10], acute respiratory distress syndrome [9], and death. At the same time, fractional administration of SEB induces apoptosis and anergy of V_β-positive T cells, which manifests in elimination of ligand-reactive T lymphocytes, inhibition of proliferation, and decreased production of interleukin-2.

The mechanisms of the negative response of T cells induced by bacterial superantigens are poorly understood. It was shown that primary stimulation with superantigens causes the release not only of cytokines produced by T1 helper cells, but also of other antiin-

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flammatory mediators. However, it remains unclear whether these mediators modulate the response of T cells during repeated stimulation with superantigens. Nitric oxide (NO) produced by mouse splenocytes in response to activation with T cell mitogens inhibits the activity of T1 helper cells. The balance of cytokines secreted by T1 and T2 helper cells is shifted, which probably impairs functional activity of T cells [4,7]. Here we studied the role of NO in SEB-induced activation of human T lymphocytes.

MATERIALS AND METHODS

The cells from 27 healthy individuals and 19 patients with purulent surgical diseases were studied. Bacteriological analyses of wound exudates revealed the presence of gram-positive bacteria and mixed infection (gram-positive and gram-negative bacteria) in 6 and 13 patients, respectively. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of heparinized venous blood in a Ficoll-Verografin density gradient. Adherent cells were removed by incubation of PBMC suspension on Petri dishes at 37°C for 45 min. T cell-enriched subpopulation was obtained by passing PBMC through nylon-wool columns [2]. Flow cytofluorometry on a FACScan device (Becton Dickinson) showed that the content of $CD3^+$ T lymphocytes was 93-96%. The cells were cultured in 96-well plates with U-shaped bottom (10^5/well) in RPMI-1640 medium (Sigma) containing 0.3 mg/ml L-glutamine, 5 mM HEPES, 100 μg/ml gentamicin, and 10% inactivated serum from donors of group AB (IV) at 37°C using CO_2 incubator. The cells were stimulated with 200 ng/ml SEB (Sigma). The intensity of proliferation was evaluated on day 3 by incorporation of ^{3}H-thymidine added 18 h before the end of culturing. The degree of apoptosis was analyzed by flow cytofluorometry after 24-h culturing. The cells were double stained with 0.2 μg/ml fluorescein diacetate (FDA, Sigma) and 4 μg/ml propidium iodide (PI, Sigma) [6]. Viable (FDA^+PI^-), dead (FDA^-PI^+), and apoptotic (FDA^-PI^-) cells were localized in the right lower, left upper, and left lower squares, respectively. NO production was evaluated on day 6-7 by accumulation of nitrites in supernatants [5]. Test supernatants (100 μl) were incubated for 10 min with an equivalent volume of Griess reagent in a 96-well plate (Greiner), and the intensity of staining was analyzed on a Behring multichannel spectrophotometer at 570 nm. We correlated our measurements with the standard calibration curve plotted by serial dilution of 1 mM sodium nitrite. To study NO production, the cells were cultured with an excess (0.5 mM) of L-NMMA (arginine analogue, Sigma) inhibiting NO production or D-NMMA (arginine derivative, Sigma) producing no inhibitory effect on NO synthesis. We also studied the effects of NO donor, S-nitroso-N-acetyl-penicillamine (SNAP, 1 mM, Sigma) on proliferation and apoptosis of PBMC from healthy individuals. The results were analyzed by descriptive, parametric, and nonparametric tests using Statistica 4.3 software.

RESULTS

PBMC from healthy individuals cultured for 6-7 days produced considerable amounts of NO even without stimulation. The intensity of spontaneous secretion allowed us to detect NO with Griess reagent (Table 1). Stimulation of PBMC with superantigen enhanced NO production. L-NMMA (NO synthase inhibitor) added to the culture medium inhibited spontaneous and SEB-stimulated NO production by 38 and 50%, respectively (data not shown). D-NMMA had no effect on NO production by PBMC.

Removal of adherent cells (monocytes and macrophages) from PBMC suspension did not affect NO production (Table 1). The data suggest that lymphoid cells, in particular, T lymphocytes expressing V_β chains of the receptor complex are the main producers of NO in response to stimulation with bacterial superantigen. The effect of SEB on NO production in cultures enriched with $CD3^+$ T cells was maximum and significantly differed ($p<0.01$) from that in PBMC before and after removal of monocytes and macrophages.

Treatment of PBMC from healthy individuals with SEB triggered the proliferative response, the rate of

TABLE 1. NO production by Blood Cells from Healthy Individuals ($M\pm m$, $n=5$)

Parameter		PBMC		T lymphocytes
		total	nonadherent	
NO production, μmol	spontaneous	18.1±0.2	19.5±5.7	13.0±1.1
	SEB-induced	23.3±1.5*	21.4±6.0	24.8±3.8*
Index of SEB effects		1.20±0.07	1.10±0.21	1.9±0.1

Note. *$p<0.01$ compared to spontaneous production (Student's t test for linked samples). The index of SEB effects was calculated as the ratio between NO production in SEB-stimulated cultures and spontaneous NO production.

TABLE 2. NO Production, Proliferation, and Apoptosis in Cultured PBMC from Healthy Individuals and Patients with Surgical Infections (*M±m*)

Parameter	Healthy individuals (*n*=16-23)		Patients (*n*=19)	
	control	SEB	control	SEB
NO production, μmol	18.6±1.8 (14.8-22.4)	24.3±2.5 (19-29)	36±10	48.0±12.6
Apoptosis, %	6.5±0.6 (1-11)	7.8±1.3 (2-11)	19.4±2.2*	29.4±3.3*
Proliferation, cpm	1220±380 (520-1930)	36,450±2770 (18,800-63,600)	1570±170	15,140±2440*

Note. Range of means (min-max) in 95% healthy individuals is shown in parentheses. *$p<0.01$ compared to healthy individuals (Mann—Whitney test).

apoptosis was low (Table 2). Unlike PBMC from healthy individuals, PBMC from patients with purulent surgical diseases were characterized by increased spontaneous and SEB-induced NO production and intensive apoptosis. The content of apoptotic lymphocytes in patients surpassed the normal in both the absence or presence of SEB (Table 2). The intensity of SEB-induced proliferative response in patients sharply decreased and was below the lower normal limit.

The individual analysis revealed different sensitivity of cells from patients to stimulatory effects of SEB (Table 3). In the majority of patients (*n*=14), cultured PBMC were characterized by low proliferative response to SEB (below 18,800 cpm), while in 5 patients, the sensitivity of cells to this superantigen did not differ from normal. NO production and the degree of apoptosis in SEB-stimulated cultures differed between these patients. In patients with preserved sensitivity to SEB, NO production did not differ from that in healthy individuals, while the rate of apoptosis 2-fold surpassed the control (16.8±2.9 *vs.* 7.8±1.3%, respectively). In patients with low proliferative response to superantigen, NO production markedly increased, and the rate of apoptosis 4-fold surpassed that in healthy individuals. Thus, high NO production in SEB-stimulated PBMC from patients with purulent surgical diseases was accompanied by a negative response to superantigen manifested in considerable apoptosis and suppressed proliferative response of T lymphocytes. It should be emphasized that the intense production of NO is not the only mechanism of apoptosis, because high-intensity apoptosis of lymphocytes can accompany normal NO production.

To confirm the role of enhanced NO production in the negative response of activated cells, we studied the effects of SNAP on apoptosis and proliferation of SEB-stimulated PBMC from healthy donors. SNAP (1 mM) increased the relative content of apoptotic lymphocytes and sharply inhibited the proliferative response of PBMC stimulated with bacterial superantigen (Fig. 1).

Our findings indicate that NO is involved in the regulation of SEB-induced activation of human T cells. Since T lymphocytes are the major source of NO under conditions of stimulation with SEB, NO probably acts as not only antiinflammatory mediator, but also as anS autocrine regulatory factor. In physiological concentrations NO acts as a positive regulator of cultured PBMC from healthy individuals, because its intense production in response to SEB is accompanied by induction of the proliferative response without activation of apoptosis. In patients with purulent surgical diseases, whose cells were probably in contact with antigens of gram-positive and/or gram-negative bacte-

TABLE 3. NO Production, Proliferation, and Apoptosis in Patients with Preserved (Group 1) and Reduced (Group 2) Sensitivities to SEB (*M±m*)

Parameter	Group 1 (*n*=5)		Group 2 (*n*=14)	
	control	SEB	control	SEB
NO production, μmol	14.6±1.6	19.6±1.9	43.5±13.5	58.5±16.4**
Apoptosis, %	16.0±1.9	16.8±2.9	20.6±2.8	34.0±3.8*
Proliferation, cpm	2360±420	30,400±3210	1290±110	9680±1180*

Note. *$p<0.01$ and **$p<0.05$ compared to group 1 (*U* test).

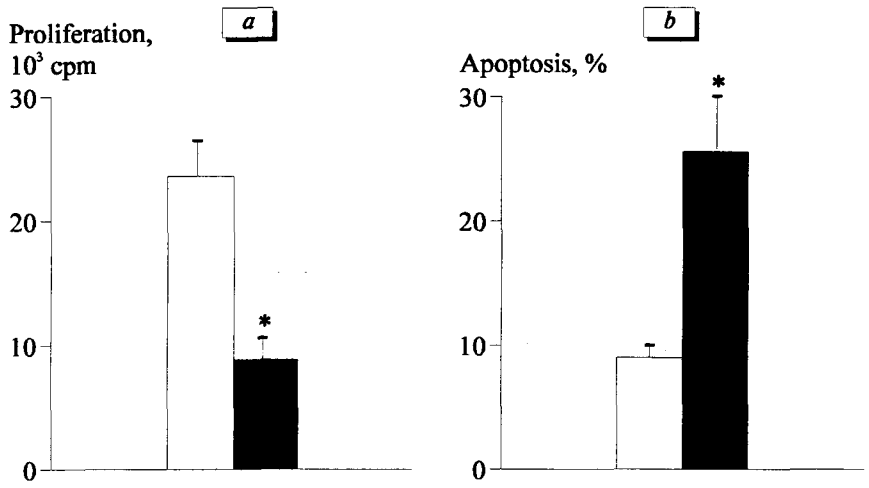

Fig. 1. SEB-induced proliferation (*a*) and apoptosis (*b*) in cultured peripheral blood mononuclear cells from healthy individuals (n=6) with (dark bars) and without SNAP (light bars). *p<0.01 compared to parameters without stimulation.

ria *in vivo*, high spontaneous and SEB-induced NO production determined the shift of cell activation towards the negative response. This resulted in intensive lymphocyte apoptosis and decreased proliferative activity of T cells in response to SEB. Only in 5 of 19 patients, T cells responded to SEB; in these patients, the intensity of NO production did not differ from that in healthy individuals. Previous studies with high concentrations of NO in PBMC cultures from healthy individuals demonstrated its role in determining the negative response of activated cells. Under these conditions 1 mM SNAP caused cell death and inhibited their proliferative activity.

Our findings are consistent with published data [11] on a positive role of NO in the regulation of the response of PBMC from healthy individuals to bacterial superantigens (streptococcal enterotoxin A or toxic shock syndrome toxin-1). The decrease in basal production of NO induced by L-NMMA inhibited cell proliferation and secretion of some cytokines. A. W. Taylor-Robinson [13] showed a dual role of NO in the regulation of proliferative activity of cloned mouse T lymphocytes. NO in physiological concentrations increased this activity, while in low concentrations NO blocked expansion of cloned T1 helper cells.

In conclusion, stimulation of lymphoid cells from patients with purulent surgical diseases with bacterial superantigen markedly enhanced production of endogenous NO, which promoted their negative response to activating stimuli (apoptosis and anergy) and prevents immunosuppression.

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