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Nitrotyrosine production following the interaction between autologous human B lymphocytes and natural killer cells

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Abstract: It has been suggested that nitric oxide (NO) and superoxide anion may be involved in heterotypic interactions. We have now assessed nitrotyrosine, one of the products of peroxynitrite ($ONOO^-$), by a specific sandwich ELISA, in the supernatants of autologous B lymphocytes and natural killer (NK) cells that were cultured for 48 h alone or mixed in different ratios (5:1, 2.5:1 and 1:1). There were no significant differences in nitrotyrosine concentration independently of the conditions used. However, when cultures were established with allogeneic cells at a B: NK ratio of 1:1, nitrotyrosine levels markedly increased from 11.2 ± 3.5 up to 32.0 ± 4.8 ng/ml ($P < 0.0001$). We conclude that without allogeneic stimuli, there is no net increase in nitrotyrosine production, suggesting that cell survival may depend on peroxynitrate production.

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Keywords: B lymphocytes, natural killer cells, nitric oxide, nitrotyrosine, peroxynitrate

Introduction: Nitric oxide (NO), initially identified as an endothelial factor responsible for the relaxation of smooth muscle cells, is generated by the conversion of arginine to citrulline by nitric oxide synthase, with NADPH as a cofactor. The end-product is quickly transformed into a radical which, in the presence of superoxide radical ($O_2^{\bullet-}$), can be transformed into peroxynitrite ($ONOO^-$) [1,2]. This reacts with CO_2 to produce various noxious products that deplete antioxidants, and oxidize and nitrate proteins, lipids and DNA [1,2]. Peroxynitrate inhibits T lymphocyte activation and proliferation by impairing tyrosine phosphorylation and peroxynitrate-driven apopoptic death [3].

NADPH oxidase, superoxide anion producer, and the constitutive endothelial nitrogen oxide synthase, are present in B lymphocytes [4,5]. One may suggest that B cells produce peroxynitrate and consequently nitrotyrosine upon stimulation. Xiao *et al.* [6] have shown that the NO pathway may, in natural killer (NK) cells, mediate human cell cytotoxicity.

NK cells have been shown to cooperate with B cells in immunoglobulin production [7]. We wanted, therefore, to test the production of nitrotyrosine following the interaction of NK cells and B lymphocytes.

Materials and methods: B and NK cells were purified from peripheral blood mononuclear cells as described previously [7,8] and by negative selection using magnetic beads with a cocktail containing anti-CD3, anti-CD14 and anti-CD16 (for B cell purification) or anti-CD20 (for NK cell purification). Both cell types were > 95% positive for CD19 (B cells) and > 95% for CD56 (NK cells) as assessed by flow cytometry.

The cells were cultured at 37 °C for 48 h in RPMI 1640 media-10% fetal calf serum without stimuli or stimulated with a 1/200 dilution of pokeweed mitogen (PWM) (Gibco BRL, Gaithersburg, MD, USA).

The amount of total nitrotyrosine was determined by a standard sandwich ELISA assay as described by Ye and coworkers [9]. All the antibodies, the mouse IgG monoclonal for capturing the modified amino acid, the polyclonal against nitrotyrosine and the polyclonal goat anti-rabbit IgG-peroxidase, were obtained from Upstate Biotechnology (Lake Placid, New York, USA). Nitrotyrosine was quantified using a standard curve with known concentrations of nitrotyrosine from chemically modified bovine serum albumin as described previously [9]. The interassay and intraassay coefficients of variation were 8% and 11% respectively.

The results are expressed as means ± SD and the units are ng/ml. Student's *t*-test and ANOVA were used for statistical analysis; significance was considered when P values were < 0.05.

Results: Figure 1 illustrates a typical flow cytometry histogram of the purified cell populations. The samples were always > 95% pure in both populations, as assessed by anti-CD19 FITC (B cells) and anti-CD56 RD (NK cells).

Table 1 illustrates the nitrites produced by either the cells alone or mixed in B:NK cell ratios of 5:1, 2.5:1 and 1:1. In addition, a sample of both cell populations was stimulated with pokeweed mitogen (PWM). This showed no net

Table 1. Nitrotyrosine production following the interaction between autologous B lymphocytes and NK cells (means ± SD of eight different assays). There were no significant differences among the different groups (ANOVA, P > 0.8)

Cell type		Stimulus	Nitrotyrosine (ng/ml)
B	NK		
0	1×10^5	NO	13.1 ± 8.6
0	2.5×10^5	NO	14.1 ± 9.1
0	5×10^5	NO	12.7 ± 6.5
5×10^5	0	NO	13.9 ± 7.2
5×10^5	1×10^5	NO	12.4 ± 4.7
5×10^5	2.5×10^5	NO	14.3 ± 6.9
5×10^5	5×10^5	NO	11.6 ± 2.8
5×10^5	0	PWM	12.8 ± 7.2
0	5×10^5	PWM	9.6 ± 6.5

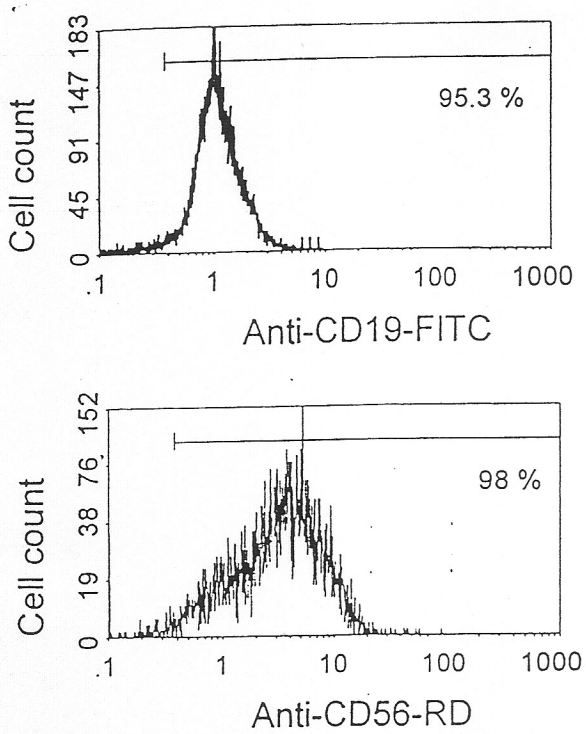

Figure 1. A flow cytometry analysis of the cell populations used for the assay. CD19 for B cells and CD56 for NK cells. The positiveness of the samples, recorded by the cursor, is illustrated in the up right-hand corner of the histograms. In all assays, the samples were > 95%.

increase in the production of nitrotyrosine as compared to non-stimulated cells.

In five experiments with allogeneic cells in a 1:1 ratio, B: NK, nitrotyrosine levels markedly increased from 11.2 ± 3.5 (cells cultivated separately) up to 32.0 ± 4.8 ng/ml ($P < 0.001$), paired Student's *t*-test.

Discussion: Assay of nitrotyrosine is one of the ways to assess peroxynitrate production. An increase in nitrotyrosine suggests cell activation either through the induction of NOS transcription or enhanced enzyme activity due to an increase in calcium and calmodulin activation [2] in parallel to an activated NADPH oxidase [3]. In these experiments nitrotyrosine levels were assessed to test the hypothesis of cell activation upon interaction.

Autologous B and NK cells cooperate in culture, leading to the production and switching of immunoglobulins (from IgM to either IgG, IgA or IgE secretion) [7]. However, the effect observed in allogeneic cultures is not the same since some authors have reported a lack of co-operation or inhibition [10].

In our experiments the production of nitrotyrosine was independent of cell number and of cell activation. This suggests that the nitrotyrosine production observed in the allogeneic cultures was responsible for the lack of co-operation reported. Future studies should assess the importance of co-operation, of NO and superoxide production in B cell imunoglobulin production and switching.

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