Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2010 Jun; 154(2):163–167.

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NITRIC OXIDE PRODUCTION DISORDERS IN LEUKOCYTES OF PATIENTS WITH RECURRENT FURUNCULOSIS

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Received: October 12, 2009; Accepted: May 16, 2010

Key words: Nitric oxide/Recurrent furunculosis/Staphylococcus aureus/Leukocyte functions

Aim. The propensity for certain individuals to develop staphylococcal recurrent furunculosis (RF) is not fully understood. But among the reasons of its development the immune system dysfunctions are described. As in the staphylococcus elimination the main role is played by neutrophils, the objective of this study was to determine nitric oxide (NO) and reactive oxygen species (ROS) production by polymorphonuclear leukocytes (PMNs) of patients with RF and to compare them with the response of normal cells to stimulation.

Materials and methods. The spontaneous and pyrogenal-stimulated nitric oxide production was evaluated in leukocyte cell cultures by Griess reaction, and ROS generation was determined in the stimulated and unstimulated NBT-test.

Results. In this study we have demonstrated that leukocytes of healthy subjects respond on stimulation by the increase of both NO and ROS production. In contrast, leukocytes of patients with RF react by depression of NO formation at stimulation, and are characterized by decrease of ROS production reserve with the increasing of spontaneous ROS generation. Described disorders are revealed in the remission period as well as at exacerbation of furunculosis.

Conclusion. The present study suggests that leukocytes of patients with RF have stable defect of stimulated NO production increase, which can be the reason for recurrent and severe course of furunculosis.

INTRODUCTION

Furunculosis is a deep infection of hair follicles caused by *Staphylococcus aureus* (*S. aureus*). The propensity for certain individuals to develop staphylococcal RF is not fully understood. It often occurs in individuals who are persistent carriers of *S. aureus* in the anterior nares or perineum. Frequency of *S. aureus* revealing, by different authors, ranges from 60 to 97% (ref.[1,2]).

Several underlying host factors, including obesity, poor hygiene, microorganism virulence and integrity of skin and mucosal barriers may be important in some instances, but as in any type of recurrent infection, an abnormality of the immune response must be suspected. Factors such as immunoglobulin deficiency states, immunosuppression, and disorders of neutrophil chemotaxis and function have been associated with furunculosis[2,3].

Neutrophils are the first to migrate into the skin in response to invading of pathogens. Among the roles played by neutrophils in inflammatory and immune responses are phagocytosis and killing of bacteria via the release of lytic enzymes stored in granules[4,5], the generation of ROS[6,7] and release of nitric oxide[8,9]. Host defense functions of NO are known for variety of bacterial infections[10,11], but its role is not fully understood at *S. aureus* – triggered RF. The major aim of the present study was to assess the leukocytes nitric oxide production during recurrent furunculosis.

MATERIALS AND METHODS

Patients. Sixty eight patients with recurrent furunculosis (24 men and 44 women at the age of 18 to 48) and bacteriologically confirmed staphylococcal etiology were examined. Among them 58 patients were in the remission period and 10 – at the exacerbation of RF. Twenty eight healthy volunteers matched for age with no history of *S. aureus* infection were taken as a control group. Intensity of NO and ROS production by leukocytes and also the phagocytosis activity were evaluated.

Blood was obtained by venipuncture of the forearm vein from healthy volunteers and patients with RF who had taken no medication for at least 10 days before the day of sampling in cases of remission or before the antibacterial therapy in cases of exacerbation. The study was approved by the ethics committee of the Gomel state medical university, and all study participants gave informed consent

Isolation of leukocytes. As NO production of PMNs depends to a large extent on regulatory influence of mononuclear cells, cultures of the leukocytes not parted on fractions were examined.

Blood samples were drawn into heparinized (10 U/ml) plastic tubes. Platelet-rich plasma was removed by centrifugation at 250 g for 20 minutes at 20 °C and the buffy coat was subjected to 6% dextran sedimentation for 30 minutes at 37 °C. Leukocytes from supernatant after hypotonic lysis of contaminating red cells were washed twice with phosphate-buffered saline (PBS: Na_2HPO_4 8.1 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM, NaCl 137 mM,

pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid and were suspended in culture medium. Leukocyte suspensions containing 70±10% of PMNs were used for investigation. Monocytes were lost during this method of leukocytes isolation, and their amount was <2% in the final suspension. Cells viability (>95%) and composition were assessed by trypan blue exclusion and May–Grunwald–Giemsa staining, respectively.

Culture condition and Cell Stimulation. For definition of NO production we used Leibovitz L-15 medium with glutamine (Flow Lab, UK), supplemented with 100 U/ml penicillin and with 100 μg/ml streptomycin. Cell suspensions were standardized by PMNs till the concentration 5×10^6 /ml without taking into account the lymphocytes. Incubation of cells was performed in final volume 0.3 ml at 37 °C for 3 hours in 96-well flat-bottom tissue culture plates (Nunc, Denmark). Leukocyte cultures with no further additions served as a control, while pyrogenal (lipopolysaccharide from *Salmonella typhi*) was added at 7 μg/ml as the universal inductor of leukocyte NO production. Each cell culture was investigated in duplicate.

Measurement of nitric oxide production. At the end of the incubation period, supernatants were collected and nitric oxide production was quantified by the accumulation of nitrite (as stable metabolite of NO) using the standard Griess reaction. An aliquot of culture supernatant (0.2 ml) was mixed with an equal volume of Greiss reagent and incubated for 20 min at room temperature with shaking. The reaction products were colorimetrically quantitated at 540 nm with background subtraction at 630 nm against reagent blank[12].

Conversion of absorbance to micromolar concentrations of NO per liter was deduced from a standard curve using a known concentration of $NaNO_2$ (0.1–1.6 μM/l) prepared in medium reacted with Greiss reagent under the same conditions. Correction of the NO concentration was not performed because there was no significant difference in the cell number and viability in all experiments. Amount of NO was expressed in μM per liter after the appropriate recalculation. The functional reserve of NO production was evaluated by calculation of the stimulation index (SI_{NO}) as NO_s / NO_b ratio, where NO_b – the level of basal NO production, NO_s – the level of NO production at pyrogenal stimulation.

Phagocytosis assay. For definition of neutrophil phagocytosis activity the heat-killed *S. aureus*, strain ATCC 25923 in concentration 10^8 cells/ml was used. Leukocytes from buffy coat were mixed with *S. aureus* in ratio 1:10 in U-bottom plates with final volume 0.2 ml, incubated for 0.5 hours at 37 °C and then slides were prepared with May–Grunwald–Giemsa staining. Bacterial ingestion was determined by light microscopy under oil immersion. Results are presented, as percentage of neutrophils that internalized at least one *S. aureus* (phagocytosis percent, PP).

ROS generation by NBT-reduction microscopic assay. The stimulated and unstimulated reduction of nitroblue tetrazolium chloride to diformazan (NBT-test) was performed directly on whole blood for evaluation of leukocyte respiratory burst[13].

The stimulated NBT-test (NBT_s) was performed by mixing 0.1 ml of whole blood with 0.1 ml of *S. aureus* suspension (10^8 cell/ml) and 0.1 ml of 0.1% NBT in PBS, pH 7.4. The mixture was kept at room temperature for 15 min and subsequently incubated at 37 °C for additional 15 min.

The unstimulated NBT test (NBT_b) was performed by mixing 0.1 ml of whole blood with 0.1 ml of 0.1% NBT and 0.1 ml of PBS. After incubation slides was prepared and stained with 0.1% safranin for microscopic examination. The percentage of cells containing formazan crystals was recorded.

The ability of PMNs to increase the basal ROS-production at the additional stimulation (reserve of ROS production) was evaluated by the index of the phagocytal reserve (IPR), counted under formula IPR = (NBT_s – NBT_b) / NBT_s.

Statistics. Data are presented as means ± standard errors of the means (SEM). Statistical analyses were performed using the nonparametric Mann-Whitney U test, or the Wilcoxon matched pairs test. Correlation data were analyzed using Spearman rank-order correlation coefficient. A p value of less than 0.05 was considered statistically significant.

RESULTS

Nitric oxide production by leukocytes of patients with RF. In unstimulated cultures the leukocytes of patients with RF in remission and healthy subjects showed similar NO-producing activity (Table 1). Stimulation of healthy subjects' leukocytes by pyrogenal resulted in significant augmentation of NO production (0.48±0.06 in unstimulated cultures, 0.69±0.08 – after stimulation; $p<0.001$). However leukocytes of patients with RF did not react by NO-production increasing in response to stimulation (0.43±0.04 before, and 0.40±0.03 after stimulation; $p>0.05$).

Decrease of NO production by leukocytes of patients in response to stimulation was observed in 44 patients (76%), and at 60% of patients the "negative" NO-producing answer was revealed in the form of reduction of stimulated NO production lower than the basal level. SI_{NO} reflecting the functional reserve of NO production was 1.03±0.07 in the group of patients with RF as a whole, that was significantly lower than the corresponding value of control group (2.09±0.57; $p<0.001$). Interestingly, in the patients with the "negative" NO-producing answer of leukocytes the index of the phagocytal reserve was lower in comparison with other patients (0.53 and 0.62 accordingly, $p=0.012$).

NBT-reduction microscopic assay. We have carried out analysis of ROS production by neutrophils in spontaneous and stimulated variants of NBT-test.

Increase of spontaneous NBT-test values in 38 (65%) patients and suppression of stimulated NBT-test values

Table 1. Functional activity of leukocytes in patients with RF in remission compared with control group.

Parameters	Control group, n=28	RF in remission, n=58	p-value, Mann-Whitney U test
NO_b, μM/l	0.48 ± 0.06	0.43 ± 0.04	not significant
NO_s, μM/l	0.69 ± 0.08	0.40 ± 0.03	< 0.05
SI_{NO}	2.09 ± 0.57	1.03 ± 0.07	< 0.001
NBT_b, %	12.2 ± 1.2	21.7 ± 1.5	< 0.001
NBT_s, %	54.9 ± 2.0	48.7 ± 1.8	< 0.05
IPR	0.78 ± 0.02	0.57 ± 0.02	< 0.001
PP, %	71.9±1.5	69.0±1.6	not significant

NO_b, NO_s, basal and stimulated NO production; SI_{NO}, the stimulation index of NO production; NBT_b, NBT_s basal and stimulated NBT-test; IPR, the index of the phagocytal reserve; PP, phagocytosis percent.

in 24 (41%) patients with RF was observed in spite of the fact that they were examined in remission period. NBT_b was 21.7±1.5 that was significantly higher than the corresponding value of the control group (12.2±1.2; p<0.001). Simultaneously the suppression of stimulated NBT-test values (48.7±1.8; p=0.037) was registered.

Therefore ROS production reserve was reduced as a whole in the group of patients with RF in remission (IPR 0.57±0.02; p<0.001 in comparison with control group). Also patients with decreased IPR (n=39, 67%) simultaneously had suppression of both SI_{NO} (p=0.002) and neutrophils phagocytosis activity (p=0.017) in comparison with other patients.

Phagocytosis. Decrease of PP was observed in 14 patients (23%). However this parameter as a whole in the group of patients with RF in remission did not differ from the corresponding values in control group. PP was 69.0±1.6 in the group of patients and 71.9±1.5 in control group.

Leukocytes functional activity at RF exacerbation. We compared indexes of leukocyte functional activity in 10 patients with RF depending on a period of the disease (remission and exacerbation).

The essential changes of the examined leukocyte functional properties were not revealed in patients at the disease exacerbation in comparison with remission, except for reduction of PMNs phagocytal activity. PP was 58.4±3.3 at exacerbation and 67.6±3.2 in remission (p=0.043).

NO - ROS correlation. We carried out analysis of correlations between the level of ROS production, examined by NBT-test, and NO-producing activity of leukocytes of healthy subjects and patients using the Spearman rank-order correlation test. In the control group positive correlation between the value of stimulated NBT-test and the level of basal (r=0.57; p=0.003) and stimulated NO production (r=0.54; p=0.005) was revealed. In the group of patients in remission the above mentioned correlations were not observed. However positive correlation between the level of basal NO production and the value of spontaneous (r=0.78; p=0.039) and stimulated NBT-test (r=0.84; p=0.019) was revealed in patients at the exacerbation of furunculosis.

DISCUSSION

For the evaluation of cell NO production various incubation terms for cell cultures can be used. It is obvious, that nitrite accumulation increases with duration of the incubation. But simultaneously nonspecific activation of cells and decrease of their viability can be observed. The 3 hours incubation term allowed receiving sufficient amount of NO for photometric definition without decrease of cells viability. Also we suppose, that reduction of incubation time of leukocytes to 3 hours allows to estimate separately activation of NO production under the influence of antigen stimulants *in vivo*, and the promoting effect of an inductor added *in vitro*. Such solution is based on the fact that in leukocytes the enzyme NO-synthase is found in small amount or misses, and several hours after stimulant addition are necessary for the gene activation, synthesis of NO-synthase m-RNA, and the enzyme itself[14,15]. As a result at the long-term incubation the effects of endogenous stimulation of NO production and an inductor added in vitro overlap and are indistinguishable. Therefore the 3-hours incubation period was chosen as optimal for our examinations. As the PMNs functional activity including NO production in many aspects depends on the regulatory influence of mononuclear cells[15-17], the cultures of leukocytes not parted on fractions were used. It allows to evaluate NO production in view of mutual cellular regulation, that adapts behavior of cells in culture for the processes taking place *in vivo*.

The normal functional condition of PMNs is a determinative requirement of resistance to cutaneous infection caused by *S. aureus*[4]. We have shown, that leukocytes of patients with RF both in remission, and in an exacerbation, have the defective NO-producing answer to lipopolysaccharide stimulation

The results obtained in the present investigation show that unstimulated leukocytes of patients with RF in remis-

sion and control group produced comparable amount of NO, in contrast, stimulation of cells resulted in the opposite effect. Under the influence of pyrogenal significant augmentation of NO production by leukocytes of healthy subjects was observed, that corresponds to data of other investigators[18], but the leukocytes of patients did not respond to stimulation by increase of NO production.

In the examined patients the reduced NO-producing answer of leukocytes predominantly was revealed, and in most cases "negative" response of leukocytes was observed in the form of suppression of NO production after inductor addition and as consequence the decrease of SI_{NO}. It is necessary to notice, that patients with the "negative" NO-producing answer differed from other patients by low index of the phagocytal reserve that indicated the connection between reactive oxygen and nitrogen species production disorders.

Thus, the simultaneous definition of basal and stimulated NO-producing activity of leukocytes carried out in our investigations allowed to estimate to a certain degree their functional reserve of NO production. Absence of such reserve in leukocytes of patients with recurrent furunculosis is revealed, while the cells of healthy subjects react by augmentation of nitric oxide production in response to stimulation.

As is known, in addition to NO production, phagocytes, carrying out oxygen-dependent bactericidal action, produce ROS (O_2^-, H_2O_2, $OH^\times$, etc.) which are the important factors for intracellular killing of *S. aureus*[19]. These radicals may react with NO to form peroxynitrite and other intermediates possessing more expressed microbicidal effect. Intensifying of cytotoxic effect of human neutrophils is observed at addition of nitric oxide donors into a culture medium[20]. At examination of bactericidal activity of reactive nitrogen and oxygen species on *S. aureus* Kaplan and co-authors showed, what exactly the combined action of NO and O_2^- results in prolonged, but complete destruction of *S. aureus*, whereas separate influence of these molecules is transient and ineffective[21]. Predominance of one of the mentioned bactericidal systems depending on both a type and degree of pathogen virulence is also described[19]. Apparently, only at maintenance of optimal balance between the levels of ROS and NO production and antigen influence the adequate realization of phagocyte bactericidal action is possible.

In view of the above-mentioned facts, we analyzed the results of NBT-test values and the phagocyte activity of granulocytes in the examined patients.

In most patients with RF in remission the increase of spontaneous NBT-test values with simultaneous reduction of stimulated NBT-test values was noted, which finally lead to significantly reduced reserve of ROS production. Such changes are described by other authors and can be a consequence of pathogen persistence in disease remission[22].

However at 1/3 of patients with RF in remission IPR was not changed and that was possible to consider as the state of compensation of oxygen – dependent bactericidal neutrophil function. At the same time most of them had the defect of NO production in the form of decreased SI_{NO}. It is important to notice, that reduction of the ROI production reserve by leukocytes of patients with RF was simultaneously accompanied by decrease of the NO production reserve and suppression of neutrophils phagocyte activity. It testifies that, on the one hand, disorders of leukocytes activity at RF simultaneously concern several their basic functions, and on the other hand, the mentioned changes can be triggered by the general provoking factor, probably, *S. aureus* extracellular products which can downregulate receptor expression and PMNs function[23–25].

At carrying out of correlation analysis (Spearman test) between values of NBT-test and NO-producing activity of leukocytes, in group of healthy subjects the statistically significant positive correlation was found between stimulated NBT-test values and the level of basal and stimulated NO production. Apparently, the mentioned correlations reflect the mutual regulation of nitrogen and oxygen radicals formation in the activated PMNs. In the literature there are data on possible opposing character of NO and ROS interrelations as a result of the concurrence for participation of NADPH in formation of these radicals[20,21]. In investigations with murine leukocyte cultures suppression of oxygen radical production at intensifying of NO synthesis is revealed[26]. It is known that the physiological concentrations of NO produced by the activated granulocytes lead to increase of peroxynitrite formation, and this potentiates O_2^- formation while high NO levels suppress it[27].

In contrast to the control group, in patients with RF in remission mentioned interrelations were absent. Taking into account disorders of NO and ROS production, total change of character of the mentioned correlations in RF remission can be regarded as disturbance of NO / ROS balance owing to the long-term stimulation of leukocytes by bacterial products of persistent pathogen. Correlations of the examined parameters in patients with RF in an exacerbation as a whole are similar to those in healthy subjects that it is possible to regard as the tendency for normalization of NO / ROS bactericidal state in leukocytes during the reactivation of this chronic disease.

The suppression of phagocytal activity revealed at RF exacerbation can be connected with activation of leukopoiesis and appearance of immature neutrophils with the reduced functional activity in peripheral blood. Thus, in series of investigations it is underscored that at pyoinflammatory diseases the dysfunction of phagocytes is observed at the active process, but is absent in remission period[3,22]. However as a whole in the stage of RF exacerbation the defect of NO and ROS production by leukocytes remains. This points at the constant leukocyte function disorders, but on the other hand, it can be explained by continuously-recurrent disease course and the unstable period of remission during which there is no essential normalization of leukocyte functions.

CONCLUSION

In summary, we showed that the functional state of leukocytes in patients with RF in remission is characterized by dysfunction in formation of the major bactericidal factors – nitric oxide and reactive oxygen species. Reduction of leukocyte NO-production reserve owing to suppression of NO release in response to cell stimulation, and also decrease of ROS production reserve with its simultaneous increased spontaneous formation was observed. Such disorders were revealed also at the exacerbation of disease.

In response to stimulation by bacterial antigens the leukocytes of healthy subjects react by simultaneous augmentation of nitric oxide and oxygen radicals production (probably owing to sufficient availability of NADPH and to absence of the concurrence of NO-synthase and NADPH-oxydase for this substrate), that finally enlarges their bactericidal potential. In case of the patients with RF predominant ROS formation and «negative» NO-production of stimulated leukocytes is observed that can be sufficient only for killing O_2^- – sensitive catalase-negative microorganisms. The described dysfunction of leukocytes can be one of the reasons for stable *S. aureus* persistence and recurrent severe course of furunculosis.

ACKNOWLEDGMENTS

We thank Dr. A. Salivonchik (Republican Research Center of Radiation Medicine and Human Ecology) for assistance with the observation of patients and Dr. E. Voropaev (Gomel state medical university, Central Research Laboratory) for providing reagents.

Author contributions to the submitted work were as follow: clinical observation of patients and reception of the informed consent – Irina A. Novikova, laboratory investigations and statistical analysis – Andrey V. Hamaliaka, interpretation of results – Andrey V. Hamaliaka and Irina A. Novikova.

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