# Blood Coagulation Initiates Respiratory Burst in Neutrophils

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Using the *in vitro* reaction of nitroblue tetrazolium reduction we showed that blood coagulation stimulates production of reactive oxygen species by human neutrophils. Heparin and chondroitin sulfate produced by thrombin-activated basophils are good candidates for inductors of these processes. Similar activation probably occurs *in vivo* under the influence of inductors secreted by mast cells.

**Key Words:** *neutrophils; reactive oxygen species; blood coagulability; glycosaminoglycans*

Respiratory burst in neutrophils and production of reactive oxygen species (ROS) during phagocytosis are *in vitro* studied using the reaction of nitroblue tetrazolium (NBT) reduction. Neutrophils can be activated by various inductors, including pyrogenal and prodigiozan. Constituent oligosaccharide fragments of these inductors interact with cell membranes and stimulate generation of ROS. Oligosaccharides and polysaccharides are present in various media and structures of the organism, including the blood. The question arises: whether normal components of biological fluids and tissues whose content varies under physiological conditions can *in situ* stimulate neutrophils? Our previous studies showed that the number of neutrophils reducing NBT increases after thermal preconditioning of the blood for 2-5 h [1]. Despite the presence of anticoagulant (heparin), partial aggregation of platelets with participation of neutrophils cannot be excluded [7-10]. It can be hypothesized that compounds, whose concentration varies during blood coagulation, stimulate ROS production by neutrophils.

Here we studied possible causes of neutrophils activation in the absence of standard inductors.

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## MATERIALS AND METHODS

We examined 14 healthy donors (women, 25-40 years). The blood drawn from the cubital vein was divided into 2 portions. Portion 1 was stabilized with heparin (5 U/ml) at 37°C for 1 h and studied in the reaction of NBT reduction (control) [2]. The reaction mixture contained 0.1 ml blood, 0.05 ml 0.2% NBT in 0.1 M $Na^+/K^+$ phosphate buffer (pH 7.2), and 0.05 ml $Na^+/K^+$ phosphate buffer (spontaneous reaction). Otherwise, an equivalent volume of this mixture included one of the following inductors: 12 mg/ml pyrogenal, serum, or blood clot from portion 2 not containing the anticoagulant and incubated at 37°C for 1 h.

The reaction was performed at 37°C for 1 h. Medium-density smears were stained with 0.1% alcohol solution of neutral red or by the method of Romanowsky and examined under a microscope with an immersion objective. The ratio of activated neutrophils (RAN, %) was estimated by examining 100 cells in 2 parallel samples stained with neutral red. Platelet count in Romanowsky-stained smears was determined 0.5 and 2.5 h after thermostatic treatment. The relative error in measurement of RAN and platelet count did not exceed 8.5 and 5.5%, respectively.

The number of neutrophils remained unchanged over 8-h exposure to temperature-controlled conditions. Therefore, the relative number of platelets cal-

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culated per 20 neutrophils reflects the absolute number of blood platelets.

In the next series the reaction of NBT reduction was performed after blood incubation at 37°C for 0-5 h in the absence of inductor (spontaneous reaction). Heparin (5 U/ml) served the anticoagulant. Otherwise, the reaction was conducted immediately after blood sampling with 5 mg/ml sodium citrate (anticoagulant) and 2.0-7.5 mg/ml heparin or 0.25-2.50 mg/ml chondroitin-4-sulfate (inductor).

The mean and confidence interval were calculated at $p$=0.05.

## RESULTS

In the absence of inductor, the number of activated neutrophils peaked 2.5 h after thermal preconditioning of the blood. RAN increased by 1.79±0.17 times compared to spontaneous reaction conducted 0.5 h after thermal preconditioning of the blood. These data indicate that various factors stimulating ROS production by neutrophils most significantly modulate cell activity over the first 3 h of blood exposure to temperature-controlled conditions.

Pyrogenal stimulated ROS production by neutrophils, this paameter surpassed the control by 3 times ($p$<0.05, Table 1). Stimulation of neutrophils with the serum or blood clot preexposed to temperature-controlled conditions without anticoagulant 2-fold increased RAN ($p$<0.05). In experiments with blood clot as the inductor, RAN was much lower compared to that observed during stimulation with pyrogenal ($p$<0.05). These data suggest that products present in the serum and to a lesser extent in the clot stimulate the respiratory burst in neutrophils. Non-heparinized blood added to the reaction mixture several minutes after sampling had no effect on RAN (as compared to spontaneous reduction of NBT). Our findings show that blood coagulation is accompanied by the formation of products stimulating ROS production by neutrophils. The degree of stimulation did not differ from that observed during pyrogenal-induced respiratory burst. The concentration of pyrogenal was selected so that it produced the most significant effect.

It should be emphasized that platelet count was constant and did not depend on the presence or type of the inductor (Table 1). Platelets cannot be counted in the clot and are absent in the serum. Platelet count in the blood exposed to temperature-controlled conditions for 2.5 h was 74.0±3.9% of the level observed 0.5 h after the start of incubation ($p$<0.05). The number of cells did not depend on the type of anticoagulant (heparin or sodium citrate). Both anticoagulants prevent blood coagulation over a longer period compared to the length of our study. Therefore, the decrease in platelet count in the reaction mixture is probably associated with aggregation.

*In vivo* platelet aggregation is accompanied by the release of thrombin, which stimulates tissue basophils (mast cells) [6]. It results in secretion of heparin and chondroitin sulfate [5,6,11]. These glycosaminoglycans (GAG) *in vivo* initiate respiratory burst and ROS generation by neutrophils. Basophils play a role of mast cells during *in vitro* activation of neutrophils. These cells secrete GAG into the blood under temperature-controlled conditions. We showed that neutrophils can be activated in the presence of heparin or chondroitin-4-sulfate. RAN in samples containing GAG from the circulating blood was 4%. In these experiments, sodium citrate served as an anticoagulant. Heparin (2.0-7.5 mg/ml) and chondroitin sulfate (0.25-2.5 mg/ml) increased RNA to 6-15 and 36-50%, respectively. It can be hypothesized that GAG *in vivo* maintain spontaneous activity of neutrophils. *In vitro* and *in vivo* heparins differ by chemical composition, degree of polymerization, branching, and ability to stimulate neutrophils. Moreover, NBT reduction reaction suggests dilution of the blood, which can modulate neutrophil activation. GAG stimulate ROS production by neutrophils, which has serious physiological consequences. For example, stress is accompanied by activation of the blood coagulation system. Under these conditions GAG-induced stimulation of the respiratory burst in neutrophils, increased production of ROS by cells, and their release into the blood can cause genetic mutations, osteoporotic damage to the connective tissue, and miscarriage [3]. It should be noted that pregnancy is associated with initiation of thrombic episodes [4].

Our results indicate that products of *in vitro* and, probably, *in vivo* blood coagulation stimulate ROS generation and respiratory burst in neutrophils. A possible mechanism for these changes is the release of thrombin into the blood, degranulation of basophils and mast cells, secretion of heparin and chondroitin sulfate, and stimulation of neutrophils. This hypothesis requires further investigations.

**TABLE 1.** Effect of Inductors on Neutrophil Activation and Platelet Count ($M$±$m$)

| Inductor | RAN, % | Platelets, 1 cell per 20 neutrophils |
|---|---|---|
| Without inductor | 22.0±6.6 | 210±20 |
| Pyrogenal | 63±18* | 210±24 |
| Serum | 41±11* | 220±28 |
| Clot | 35.0±4.8* | 200±29 |

**Note.** *$p$<0.05 compared to the control.

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