Role of Pronase E in Ligand Binding to Fc Receptors in the Reaction of Cytotoxicity Mediated by Bovine Blood Neutrophils

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We studied the effects of pronase E on IgG binding to Fcγ receptors on bovine blood neutrophils and kinetic parameters of this reaction (association constant and number of binding sites on neutrophils). During *in vitro* modeling of the cell immune response, we evaluated cytotoxic activity of effector neutrophils against foreign cells.

Key Words: *neutrophil; pronase E; Fcγ receptor; kinetic parameters of binding; cytotoxic activity*

Neutrophils play an important role in the humoral and cell blood systems. Neutrophils are responsible for phagocytosis, maintain immune homeostasis, and directly attack foreign cells. The plasma membrane in neutrophils expresses various receptors, including Fc receptors (FcγR) for IgG. They are similar to receptors on natural killer and K cells [3].

Stimulated neutrophils regulate the immune response realized via cell receptors [2]. The regulation of ligand binding to FcγR and functional activity of blood neutrophils against target cells are poorly studied.

Here we studied the effects of pronase E on kinetic parameters of ligand binding to FcγR and functional activity of bovine blood neutrophils in *in vitro* cytotoxicity reaction mediated by killer cells.

MATERIALS AND METHODS

Heparinized bovine venous blood was centrifuged in a Verografin density gradient (1.077 g/cm^3, Spofa). Erythrocytes obtained after isolation of mononuclear cells were dissolved in 0.84% NH_4Cl. Neutrophils were isolated by centrifugation at 2200 rpm for 20 min [5]. IgG were obtained from bovine blood plasma [4] and labeled with fluorescein 5(6)-isothiocyanate (FITC) (Serva).

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In series I we studied the effects of pronase E from *Streptomyces griseus* (Serva) on ligand binding to FcγR and cytotoxic activity of blood neutrophils. Neutrophils were incubated in 0.25 ml medium 199 (2×10^7 cells/ml) with pronase E (7.8-250 mg/ml) at 37°C for 30 min. Control cells were incubated without pronase E under similar conditions. After incubation these cells were 2 times washed with cold buffered physiological saline (pH 7.2) containing 2% fetal bovine serum (FBS) and 0.1% NaN_3 and resuspended in medium 199. Cell viability was estimated by staining with 0.1% trypan blue (Serva). The count of viable cells was 96-98%. Binding of incubated neutrophils to FITC-labeled IgG (FITC-IgG) at a saturating concentration of 3.9×10^{-5} M was studied as described elsewhere [12].

In series II we studied the effects of pronase E on kinetic parameters of FITC-IgG binding to FcγR on bovine blood neutrophils. Neutrophils were incubated in 0.25 ml medium 199 (2×10^7 cells/ml) with pronase E (15.6 mg/ml) at 37°C for 30 min and treated as described in series I. Quantitative parameters of binding, association constant (*Ka*) and number of binding sites on neutrophils (*n*), were estimated by Scatchard analysis [11]. Fluorescence was measured on an MPF-4 spectrofluorometer (Hitachi) at 493 nm and 520 nm.

The cytotoxic reaction was carried out. Cultured malignized cells of the subcutaneous connective tissue from CH3 mice (long-term culture L) were used as

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target cells in *in vitro* reaction of cell immunity. The cells were cultured in medium 199 with 10% FBS. Bovine blood neutrophils served as the effector cells (effector/target ratio 50:1).

Cytotoxic activity of blood neutrophils was estimated by radiometry of residual radioactivity in target L cells labeled with ^{3}H-thymidine on cover glasses [1].

Cytotoxic activity of effector cells was calculated by the formula:

$$\frac{C\text{-}E}{C} \times 100,$$

where *C* and *O* are counts (cpm) in the control and experimental series, respectively.

The results were analyzed by Student's *t* test.

RESULTS

FcγR expression changed during incubation of neutrophils with pronase E. The maximum ligand binding to FcγR was observed in the absence of pronase E. The increase in pronase E concentration dose-dependently inhibited ligand binding to FcγR (Fig. 1, *a*).

We studied the effects of pronase E on quantitative parameters reflecting binding of FITC-IgG to FcγR on neutrophils. Before and after incubation of blood neutrophils with pronase E, FcγR bound the same number of FITC-IgG molecules ($10.0\pm0.3\times10^5$ and $10.2\pm0.2\times10^5$, respectively, Fig. 2). However, incubation with pronase E 2-fold increased in *Ka* for binding of FITC-IgG to FcγR on neutrophils ($2.6\pm0.4\times10^5$ *vs.* $1.3\pm0.2\times10^5$ M^{-1} before incubation, $p<0.05$).

Previous studies were performed under various experimental conditions, which makes difficult interpretation and comparison of the results. Human neutrophils express 2 types of FcγR, FcγRII and FcγRIII (10^4 and 10^6 on cell, respectively), which depends on the type of stimulation [9]. Our results are consistent with published data that the intensity of FcγRIII expression sharply decreases after treatment with pronase E [8], and affinity of FcγRIII on K562 cells increases by 3 times without changes in the number of binding sites on cells [13]. Other experiments showed that FcγRI on human monocytes are resistant to pronase [10].

We found no data on binding of IgG to FcγR on neutrophils after treatment with proteases of these receptors on bovine blood neutrophils. Taking into account published data, we studied binding of IgG to FcγR on neutrophils after incubation with protease E. In our experiments FcγR were not divided into types. Our results indicate that the effect of protease E on FcγR is realized via various mechanisms. Undoubtedly, bovine blood neutrophils carry several types of FcγR.

The intensity of ligand binding to FcγR was maximum in the absence of pronase E. *In vitro* studies of changes in killer activity of effector cells produced by pronase E showed that inactive bovine blood neutrophils do not display cytotoxicity against xenogenic target L cells. Our results are consistent with published data that inactive human neutrophils exhibit low cytotoxic activity [4]. The efficiency of cytotoxicity depends not only on the expression of receptors involved in its induction, but also on their functional activity [3]. FcγRII and FcγRIII are expressed on the membrane of human blood neutrophils [9]. Cytolysis of target cells depends on activation of FcγRI and FcγRII (but not FcγRIII). FcγRIII do not transfer the activation signal and, therefore, are not involved in

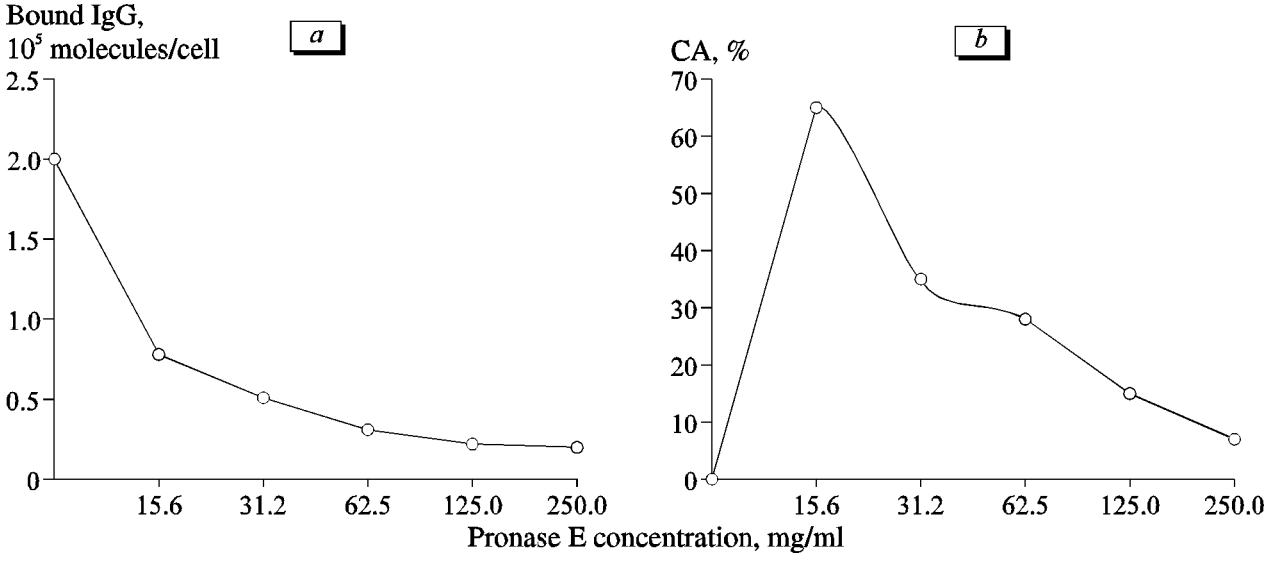

Fig. 1. Binding of FITC-IgG (*a*) and cytotoxic activity (CA, *b*) of bovine blood neutrophils after treatment with pronase E.

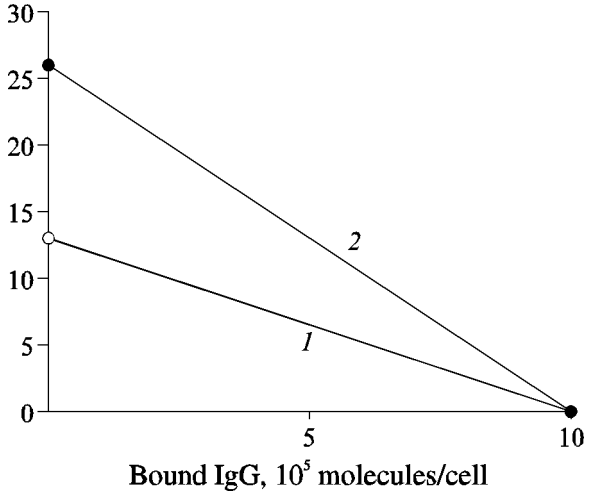

Fig. 2. Scatchard plot for specific binding of FITC-IgG to bovine blood neutrophils before (*1*) and after treatment with pronase E (*2*). Ordinate: ratio between bound and free IgG.

cytotoxicity. Our results confirm published data that after activation with interferon-γ, these cells express FcγRI and induce cytolysis of target cells sensitized with specific antibodies [7].

After incubation of neutrophils with pronase E in a concentration of 15.6 mg/ml and 24-h incubation with target cells cytotoxic activity reached 64.9% (Fig. 1, *b*). Increasing the concentration of pronase E reduced the ability of neutrophils to lyse target cells.

Cytotoxic activity of effector neutrophils was high after treatment with 15.6 mg/ml pronase E. Our findings suggest that this enzyme activates existing receptors or induces expression of new receptors mediating cytotoxic activity of neutrophils and stimulates cytolysis of foreign cells.

Not all proteases can activate FcγR mediating neutrophil cytotoxicity [6]. Activating agents in various concentrations and signals from different receptors produce various reactions. The activating agent usually initiates polyfunctional reactions in neutrophils. These data suggest that pronase E in concentrations of 125 and 250 mg/ml changes topographic properties of the plasma membrane and impair its functions.

Our findings indicate that pronase E dose-dependently inhibits ligand binding to FcγR on blood neutrophils from healthy donors and their cytotoxic activity against foreign target cells *in vitro*. Quantitative parameters reflecting IgG binding to FcγR on neutrophils in the presence of pronase E indicate that binding affinity increases without changes in the number of receptors on cells.

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