THE ENSYMATIC ACTIVITY OF RIBOSOME INACTIVATING PROTEINS II TYPE AND MODULATION OF NEUTROPHIL CYTOTOXICYTY

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There were investigated the action of viscum, ricin, agglutinin ricin and their complexes with lactose or galactose on the fMLF induced respiratory burst in the neutrophils. The peritoneal zymosan evoked neutrophils of mice NMRI strain were used. Production of the reactive oxygen species was estimated by luminol-dependent chemiluminescence. It was shown that investigated RIPs II could exhibit properties of chemoattractant receptor antagonist (fMLP receptor) and modulate fMLP-induced respiratory burst. Ricin is more effective modulator of fMLP-induced respiratory burst than agglutinin ricin. This effect could be related to interaction of RIPs II with galactose but not mannose receptors. It was shown that modulating action of RIPs increased as the amount of lactose or galactose binded to molecular of RIPs was added. We assume that affinity of RIPs to galactose receptor increased as the amount of saccharides binded with molecular of RIPs increased. Structure peculiarities of RIPs II lead to character of their modulating effects.

Ribosome-inactivating proteins of type II (RIP II) hold a special position among lectins since they provide basis for devising of antitumor drugs. The plant toxins both dimer and tetramer consist of two types of subunits, i.e. catalytic A-subunit and binding B-subunits. It is known that A-subunit inhibits protein synthesis by depurinating an adenosine residue in a highly conserved RNA loop of the 28 S ribosomal subunits [1].

The modified ribosome can no longer synthesize protein and are apoptosis takes place. The A-subunit active site consists of a number of conserved residues, their point mutagenesis results in considerable or even loss of catalytic activity. Such structure stability of the A-subunit active site takes place in the absolute majority of RIP. Most likely, a substantial difference in catalytic activity between different RIP II are related not as much to the peculiarities of ribosome interaction as to the character and specific nature of previous receptor interactions. Of great importance is the first step of RIP II interaction with the surface of a target cell. At present it is shown that RIP II are taken up by two routes into the cells [2]: by interacting with the cell surface glycoproteins and glycolipides having galactose or by interacting with the cell receptors (mannose and galactose) that can bind RIP sugar (galactose of B-subunite and mannose of A-subunite). Another way of RIP penetration into the cell is also possible. It was shown by us that viscum (representative of RIP II) can demonstrate the properties of the antagonist of FMLP-receptor [3]. This property of viscum and other RIP II can probably result from their interaction with FMLP-receptor [3]. The aim of this paper is to investigate the action of ricin, agglutinin ricin and their complexes with lactose or galactose on the fMLF induced respiratory burst in the neutrophils.

Methods of Investigations

Materials. Dry environment HENKS, N-formyl-methionyl-leucyl-phenylalanin (fMLP), luminol, zymosan, cycloheximide, HEPES (all of Sigma, USA), viscum, ricin, agglutinin ricin, lactose, galactose, mannose (Serva, Germany).

Biological object. The work is performed on peritoneal caused neutrophils of mice of NMRI strain. The average weight of an experimental animal was 20–25 g. Isolation of the peritoneal neutrophils was carried out following a slightly modified technique [4]. The density of the cells was counted up in the Goryaev's chamber with colouring by tripan dark blue. The living cells content was not less than 95%. Approximately 80% of cells in suspension were neutrophils.

Measurement of chemiluminescence. Production of the reactive oxygen species (ROS) of neutrophils was estimated from luminol-dependent chemiluminescence (ChL), measured by a traditional technique [5]. Spontaneous ChL (not activated neutrophil) was registered within the first 3 min at 37°C, then proteins (viscum, ricin, and agglutinin ricin) was added at different concentrations and incubated with cells during 1 min. After that neutrophils was activated by fMLP. To inhibit galactose or mannose receptor we added 25 nM galactose or mannose to normal medium.

The data analysis. To estimate a total amount of ROS which was produced by the cells for a certain period of time and entered into reaction with luminol, we integrated the curve of the ChL intensity dependence from the time, accepting for a zero the level of average spontaneous ChL intensity in the control cells. The moment of fMLF addition was a beginning of read out. Modulating action of

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RIP was estimated on the ratio of ROS production of RIP treatment cells to ROS production of control cells. The statistical analysis of the data was carried out with use of Student's t-criterion. Reliability of results estimated on borders of a confidential interval with probability $p > 0.95$.

Results

To answer the question of whether neutrophils have specific receptors for RIP II, as it was shown for cancer cells [6, 7] we checked whether the proteins, namely, viscum, ricin, and agglutinin can induce respiratory burst alone. The character of changes of Chl in a wide concentration range does not give ground to think that protein act via a specific receptor. It is known that activation of respiratory burst occurring through specific cytoplasmic membrane receptor is characterised by a threshold value, monotonic increase and saturation but not by a variety directed action. Based on these data we can suggest that the action of investigated proteins on the respiratory burst of the neutrophils are mediated by nonspecific receptor of studied proteins.

Vicum modification of the receptor response. Modification of neutrophil activity can occur on the receptor level (the change of receptor affinity to ligand as a result of membrane modification) and on the level of intracellular signalling system. In the following experimental set we investigated the change in the Chl intensity of neutrophils activated by fMLP depending on the viscum incubation time. Different effects were observed depending on the incubation time: in the case of small incubation time (1 min)—the neutrophil response was decreased; at longer incubation time (60 min)—there was not any noticeable difference between the responses of intact and viscum treated cells; when the preliminary incubation of cells was 90 min and more—the neutrophil response was enhanced as compared to control. Thus, the viscum action on the neutrophils depends on the incubation time with cells. Similar data were obtained for ricin and agglutinin ricin. It is possible at the short incubation time, we observed effects associated with action of viscum on fMLF receptor binding and starting the relevant intracellular signal systems responsible for the response of a cell. The observed enhancement of Chl response at longer incubation time with viscum can be associated with A-subunits activity. To test this assumption we have compared the effects of viscum and inhibitor of protein synthesis—an antibiotic cycloheximide inactivating catalytic 80S ribosome subunit. The effects of that cycloheximide and viscum are significant and have a similar direction. It has been shown that cycloheximide and viscum provoke significant activation as compared with the control. This result suggests that the observed increase in Chl response to viscum action, most likely, is associated with termination of protein biosynthesis. Thus, in the case of long incubation time we have registered the effects caused the action of A - subunits. The effects obtained for 1-min incubation are related with RIP action on the fMLP receptor binding.

General description of the effects of RIP and their complexes with sugar on fMLP induced respiratory burst. All examined proteins act on the ribosome practically similarly but it is very important to reveal via which receptors RIP II are taken up into the cells. We tested the RIP (ricin, agglutinin ricin and their complexes) influence on the fMLP induced respiratory burst. Ricin more effectively modulated fMLP induced respiratory burst as compared with agglutinin ricin (Fig. 1).

Addition of galactose to protein molecule makes impossible the interaction of investigated proteins with galactose-contaning cell receptor but may enhance their affinity to cell receptor binding galactose (galactose receptor). An increase in the amount of galactose bound by agglutinin ricin may change the modulating properties of ricin and agglutinin ricin. The modulating properties of ricin and agglutinin ricin become more pronounced at increased the amount of galactose bound by agglutinin ricin (Fig. 1*b*, curves *1* and *2*). Increased amount of galactose bound by ricin does not change the effect in the investigated concentrations range, since there are no differences between

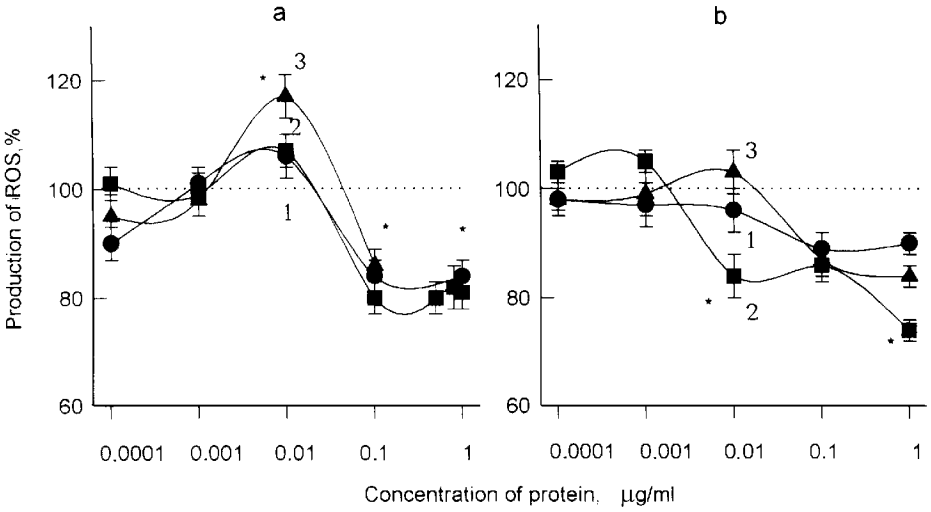

Fig. 1. Dependence of the modulating action of ricin (a), agglutinin ricin (b) and its complexes with sugar on the ROI production of the neutrophils in responce of 10 μM fMLP. ●—ricin (a), agglutinin ricin (b); ■—"ricin–galactose" (a), "agglutinin ricin–galactose" (b); ▲—"ricin–lactose" (a), "agglutinin ricin–lactose" (b). The response of intact cells is 100%. It has been shown mean mean square error, mean results of 10–12 independent experiments.

Table 1

The galactose binding sites and fMLP similar sites of RIP II

Concentration of protein, μg/ml	agglutinin ricin, normal medium, %	agglutinin ricin, medium with 25 nM galactose, %	"agglutinin ricin–galactose", medium, %	"agglutinin ricin–galactose", medium with 25 nM galactose, %
0.0001	98 ± 2	96 ± 6	103 ± 6	91 ± 2
0.001	96 ± 6	92 ± 2	105 ± 6	91 ± 2
0.01	$97 \pm 2^*$	$79 \pm 6*$	$84 \pm 5^*$	$108 \pm 4^*$
0.1	87 ± 3	86 ± 6	86 ± 6	92 ± 4
1	$84 \pm 2^*$	$54 \pm 7^*$	74 ± 7	86 ± 4
	ricin, normal medium, %	ricin, medium with 25 nM galactose, %	"ricin–galactose", normal medium, %	"ricin–galactose", medium with 25 nM galactose, %
0.0001	90 ± 4		101 ± 3	
0.001	$101 \pm 3^*$	$130 \pm 10^*$	$99 \pm 4^*$	$80 \pm 5^*$
0.01	106 ± 4	100 ± 10	107 ± 3	97 ± 5
0.1	$84 \pm 3^*$	$93 \pm 10^*$	80 ± 3	156 ± 12
1	$84 \pm 3^*$	$110 \pm 10^*$	$81 \pm 3^*$	$104 \pm 5^*$

* Modulating action of ricin, agglutinin ricin and its complexes with sugar on the ROI production of the neutrophils in responce of 10 μM fMLP in the normal medium and in the medium with 25 nM galactose. The response of intact cells in normal medium is 100% for results in normal medium. The response of intact cells in medium with 25 nM galactose is 100% for results in medium with 25 nM galactose. It has been shown mean square error, mean results of 10–12 independent experiments.

action the studied proteins and their complexes on ROS production (Fig. 1*a*, *b*, curves *1* and *2*). Modulating properties on fMLP induced respiratory burst of agglutinin ricin do not change with to increase binding lactose by protein but modulated properties of ricin are amplified in this case (Fig. 1*a*, curves *3*). Note that, the modulating properties of each protein were changed in the range 0,001–0,1 μg/ml. These concentrations are in agreement with the order of RIP binding constant on its specific interaction and with order of lectin binding constants with galactose and mannose receptor [1]. It is possible that the observed effects are related with changes in the protein affinity to mannose and galactose receptor, promoting phagocytosis.

The role of mannose and galactose receptor in the modulation of fMLP induced respiratory burst. It is known that RIP II penetrate into the cell binding to mannose and galactose receptors [2]. We have tested whether the modulating action of proteins on the respiratory burst of the neutrophils is due to their interaction with mannose or galactose receptors. It has been shown that incubation of different cell types with 50 mM of mannose or galactose leads to inhibition of these receptors [1]. At the same time it is known that mannose or galactose at concentrations of 50 and 100 mM inhibit the respiratory burst of the neutrophils on the level of hexosomonophosphate shunt but not on the receptor level [8]. Therefore, we used mannose and galactose at concentration 25 nM. This is minimum concentration inhibiting the respiratory burst. At the same time it has known for macrophages that the binding constant of mannose with reseptor is equal to 1.67 nM [9]. Incubation of neutrophils with 25 nM galactose during 3 min result in 25±5% decreased of ROS production upon addition of fMLP. While incubation of neutrophils with 25 nM mannose during 3 min results in 15 ± 5% decreased ROS production upon addition of fMLP.

Mannose. There are any changes of effects of proteins and its complexes with sugar on the fMLP induced respiratory burst.

Galactose. The influence of different concentrations of the studied proteins on ROS production upon addition of fMLP in normal medium and after preliminary incubation of the cells in the medium with 25 nM galactose is shown in Table 1. It is revealed differences in the action of ricin and agglutinin ricin after incubation in the medium with 25 nM galactose: modulating action of ricin on the respiratory burst is absent after incubation in the medium with 25 nM galactose, but modulating action of agglutinin ricin is increased (Table 1). This can be result from, first, different quarter structure of proteins: ricin is dimer with molecular weight 60 kDa, consisting of A and B subunits. While aggluinin ricin is tetramer with molecular weight 120 kDa, consisting of two A and B subunits [10]. Possibly, therefore ricin slightly penetrates into the cell. This assumption suggests experiments showing that tetramers are less toxic for the cells [1]. This fact may explain differences in the action of complexes "ricin–galactose" and "agglutinin ricin–galactose" on the fMLP induced respiratory burst after incubation in the medium with 25 nM galactose. Differences in the action of studied proteins RIP are assotiated with different carbohydrate specificity, for example, agglutinin ricin at contrast to ricin is impossible to bind with N-acetylgalactoseamin [10]. Disparity in modulating action of studied RIP more likely is due to different structure of galactose binding sites as well as fMLP similar sites [3]. Thus, studied RIP II at short incubation time in the certain concentration range can reveal the property of fMLP receptor antagonist. Possibly, cytotoxic properties of neutrophils may change with activity of A-subunits at longer incubation time. Ricin is more effective modulator of fMLP-induced respiratory burst than agglutinin ricin. This effect could be related to interaction of RIPs II with galactose but not mannose receptors. It was shown that modulating action of RIPs increased as the amount of lactose or galactose binded to molecular of RIPs was added. We assume that affinity of RIPs to galactose receptor increased as the amount of saccharides binded with molecular

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