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EFFECT OF DEFENSIN HNP-1 OF HUMAN NEUTROPHILS ON PRODUCTION OF TUMOR NECROSIS FACTOR α BY HUMAN BLOOD MONOCYTES IN VITRO

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Intercellular interactions, including those between macrophages and granulocytes play an important role in the immune response. It has been shown that granulocytes can be activated by macrophagal cytokines: IL-8, IL-1, tumor necrosis factor a (TNF-α) [4, 13, 15]. Meanwhile, little is known of the effect of granulocytic soluble factors on monocytes/macrophages. One of the bactericidal mechanisms used by granulocytes is the system of the defensins and certain other proteins contained in granules of neutrophils [1, 9]. The defensins are a family of cationic peptides with mol, wt. of 3-4 kV, which account for 5-7% of the total cell protein in human neutrophils [8].

We have suggested that defensins, released from granulocytes into the extracellular median, can act on synthesis of macrophagal cytokines. The aim of our investigations was to study the effect of the defensin HNP-1 of human neutrophils on production of TNF-α in a culture of human monocytes.

EXPERIMENTAL METHOD

Mononuclear leukocytes (MNL) were isolated from fresh heparinized donated blood by the standard method [3]. Monocytes were obtained by fractionating MNL on preformed continuous Percoll density gradient ("Pharmacia") [10] at 4°C. The fraction of monocytes contained about 80% of cells giving a positive reaction for α-naphthyl acetate esterase (kit from "Sigma"). Monocytes ($2 \cdot 10^6$/ml) were cultured in medium RPMI 1640 ("Gibco") with antibiotics and L-glutamine in plastic Petri dishes ("Flow Laboratories"). Heat-inactivated fetal calf serum (FCS) (0.1%) was added to the monocyte cultures 30 min after the beginning of incubation. To induce TNF-α, a bacterial suspension of *Staph. aureus* Cowan 1 (SAC), prepared by the method in [11] was used, in a final concentration of 0.00025-0.001% (by volume) in the culture medium together with phorbol myristate acetate (PMA) ("Calblochem")

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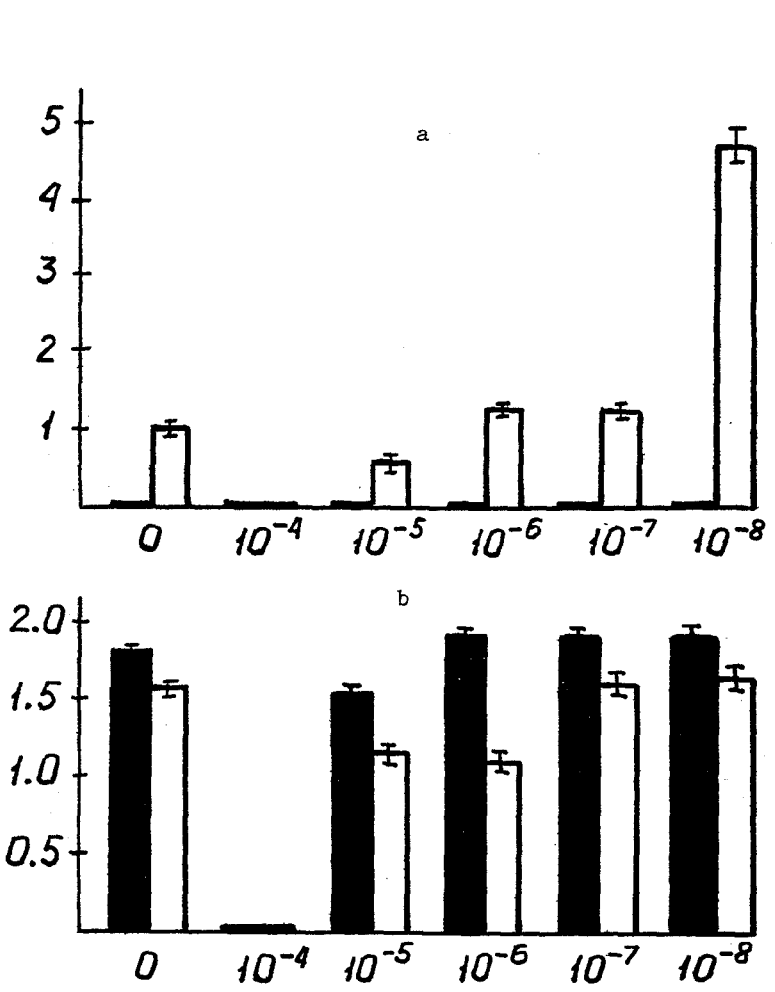

Fig. 1

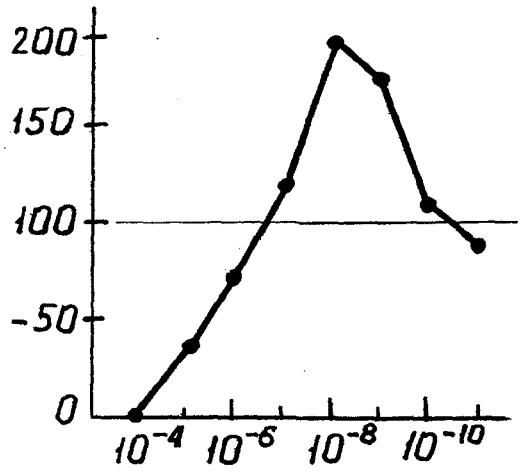

Fig. 2

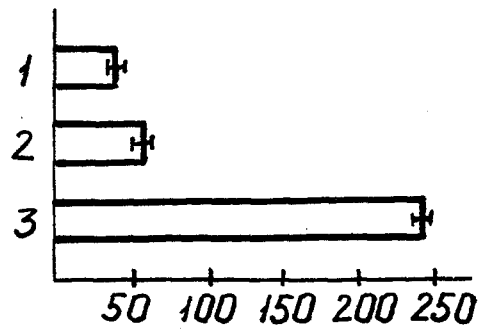

Fig. 3

Fig. 1. Effect of defensin HNP-1 on production of TNF-α by monocytes (a) and on viability of monocytes (b). Abscissa, HNP-1 concentration in medium (in M). Ordinate a) activity of TNF-α (U/ml, $\times 10^2$), b) number of viable cells (in millions/ml). Black columns denote unstimulated monocytes, white columns – monocytes stimulated by SAC.

Fig. 2. Dependence of TNF-α production by monocytes, stimulated by SAC, on HNP-1 concentration in medium. Abscissa, HNP-1 concentration in medium (in M). Ordinate, TNF-α production (in % of level induced by SAC). Results of seven experiments are shown.

Fig. 3. Intensification of TNF-α production by monocytes activated by PMA, under the influence of defensin HNP-1. 1) Monocytes + PMA, 2) monocytes + PMA + HNP-1 (10^{-7} M), 3) monocytes + PMA + HNP-1 (10^{-8} M). Abscissa, TNF-α activity (in U/ml).

in a concentration of 1 mg/ml. Defensin HNP-1, isolated from human blood neutrophils and purified as described in [8], was used in the experiments. The content of endotoxin in the preparation, determined by the Limulus Amebocyte Lysate test ("Sigma"), was 2.6 ng/mg protein.

TNF-α activity in supernatants from monocyte cultures was determined in the cytotoxic test on mouse fibroblastoid cells, strain L-929, in the presence of actinomycin D (1 μg/ml, "Serva") [15]. To prove the association between cytotoxicity and TNF-α, the test supernatants, in different dilutions, were incubated with neutralizing monoclonal antibodies (MCA) against human recombinant TNF-α [1] for 30 min at 37°C. Activity of TNF-α in cytotoxicity units was calculated by determining the dilution of supernatant at which 50% lysis of sensitive cells was ob-

TABLE 1. Dependence of Effect of Defensin HNP-1 on TNF-α Production by Monocytes, Stimulated by SAC, on Concentration of FCS and Time of Its Addition to Culture Medium

Monocyte culture	TNF-α production, % of level induced by SAC							
	serum-free		with 0.1% FCS from beginning of culture		with 0.1% FCS after culture for 30 min		with 5% FCS after culture for 30 min	
	experiment number							
	1	2	1	2	1	2	1	2
SAC +HNP=1 10^{-7}M	160	90	110	90	150	190	110	110
SAC +HNP=1 10^{-8}M	—	90	130	90	170	210	190	100
SAC +HNP=1 10^{-9}M	150	90	110	80	230	130	190	80

served [5] relative to activity of a standard for biological activity of TNF-α (1 U = 25 pg rTNF-α), suggested by the National Institute of Biological Standards and Controls (England).

EXPERIMENTAL RESULTS

We studied the effect of defensin HNP-1 on the number of viable cells in a culture of fresh and SAC-stimulated monocytes. For this purpose the monocytes were cultured for 18 h in the presence of HNP-1 in a concentration of 10^{-4}-10^{-8} M. As Fig. 1 shows, defensin in a high concentration (10^{-4} M) caused total death of the monocytes in the culture. When monocytes were cultured with HNP-1 in a concentration of 10^{-5}-10^{-6} M some decrease was observed in the number of living cells compared with cultures not treated with defensin, and more marked in the case of monocytes stimulated by SAC. Addition of HNP-1 to the culture medium in lower concentrations (10^{-7}-10^{-8} M) had no effect on the number of viable cells in the monocyte culture. Thus defensin HNP-1 in a high concentration (10^{-4} M) has a cytotoxic action on monocytes cultured in vitro. A similar effect of some polypeptides of the defensin family on mammalian cell cultures was described previously by other workers [12]. On this basis it can be postulated that the cytotoxic action of neutrophils, mediated by defensins, against monocytes/macrophages may take place in vivo if a high concentration of defensins is present in a zone of inflammation.

To study the ability of HNP-1 to induce TNF-α production by monocytes, the cells were cultured for 18 h in the presence of different concentrations of defensin in medium containing 0.1% FCS. As Fig. 1 shows, production of TNF-α was not observed in monocyte cultures treated with HMP-1. Similar results were obtained on culture of monocytes in serum-free medium (data not given). Consequently, in the absence of additional stimulation, HNP-1 cannot induce TNF-α production by human monocytes in vitro.

The effect of HNP-1 in a concentration of 10^{-5}-10^{-11} M on TNF-α production by monocytes, stimulated by SAC, was studied. As Fig. 2 shows, defensin in concentrations of 10^{-5}-10^{-6} M had an inhibitory action on TNF-α production, induced by SAC. On the addition of HNP-1 in lower concentrations (10^{-8}-10^{-9} M) to monocyte cultures the ability of the monocytes to produce TNF-α in response to stimulation by SAC was enhanced. The effect observed was expressed differently in monocyte cultures obtained from blood from different donors, and the amplification factor varied from 1.1 to 4.3. The cytotoxic activity of the culture medium of monocytes stimulated by SAC in the presence of HMP-1 was completely neutralized by MCA against TNF-α. Thus it can be concluded from the results of these investigations that deiensins, released from granules of neutrophils, can potentiate local TNF-α production by monocytes in a zone of inflammation. It has also been shown that in a concentration of 10^{-8}-10^{-9} M defensins HNP-1 and HNP-2 possess maximal chemotaxic activity relative to macrophages [16].

We know that defensins can bind with the components of serum: with serum albumin [9], with α_2-macroglobulin [14]. In our experiments the FCS concentration in the culture medium and the order in which the serum, defensin, and SAC were added had an effect on the degree of potentiation of TNF-α production by monocytes under tho influence of HNP-1. As Table 1 shows, the maximal increase in TNF-α production induced ny SAC was observed after the addition of 0.1% FCS to the culture medium after 30 min of serum-free incubation of the monocytes with SAC and HNP-1. However, if the reagents were added to the medium in the same order, but with a higher serum concentration (5%), the potentiating effect of defensin on TNF-α production was weaker or was not observed at all. TNF-α production by monocytes, stimulated by SAC, was unchanged or was increased but not significantly after

addition of FCS from the beginning of culture of the cells. Serum proteins, binding with HNP-1, may probably weaken its action on monocytes. It can also be tentatively suggested that the presence of a certain serum factor is essential for stimulation of TNF-α production by defensin.

It was shown previously that defensins can bind with bacterial cells and facilitate their ingestion by phagocytes, i.e., they can behave as opsonins [6]. Accordingly in the next experiments we used a soluble activator to stimulate the monocytes, namely phorbol myristate acetate (PMA). The results obtained by culturing monocytes in the presence of PMA and HNP-1 (10^{-7}-10^{-8} M) in medium containing 0.1% FCS, added 30 min after the beginning of culture, are shown in Fig. 3. Just as with stimulation of the monocytes by SAC, TNF-α production in a culture of monocytes activated by PMA (1 mg/ml) was intensified by the presence of HNP-1. The cytotoxic activity of supernatants from monocyte cultures stimulated by PMA was totally neutralized by MCA against TNF-α.

Thus the action of defensin HNP-1, isolated from human neutrophils, on human blood monocytes in vitro depends on its concentration. In high concentration HNP-1 has a cytotoxic effect, causing death of the cells in culture, in average concentrations it inhibits TNF-α production and, finally, in lower concentrations, when acting together with an activating signal, potentiates TNF-α production by monocytes isolated from human blood. The results of this investigation, and also those obtained by other workers [16], suggest that defensins can participate not only in regulation of the number of mononuclear phagocytes in the region of inflammation, but can also regulate their production of one of the most important of the inflammatory cytokines, namely TNF-α and, consequently, they can act as mediators of the inflammatory response.

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