COMBINED ASSESSMENT OF PRODUCTION OF THREE TYPES OF INTERLEUKIN-1 BY HUMAN PERIPHERAL BLOOD MONOCYTES

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The interleukins are very important mediators responsible for interaction between cells of the immune system. Their closer study is of both theoretical and clinical diagnostic importance [4, 5]. Many aspects of interleukin-1 (IL-1) have now been studied and its production in various immunopathological diseases has been assessed [2, 3, 8]. Nearly all these studies have been devoted to the secreted type of IL-1. Recent investigations have demonstrated the existence of another two pools of this monokine, namely membrane-associated and intracellular [11, 13]. These three types of IL-1 differ in their physicochemical characteristics [6], the kinetics of their production by monocytes in culture in vitro [10], and their role in the regulation of immune processes [12]. If we regard the intracellular, membrane-associated, and secreted forms of IL-1 as successive stages in the formation of the monokine, the study of simultaneous assessment of activity of the above-mentioned types of human IL-1 is an important task in modern clinical immunology. The aim of this investigation was to develop and use a simple and widely available method of combined determination of production of IL-1 pools by human monocytes.

EXPERIMENTAL METHOD

Monocytes were isolated from human peripheral blood by two methods. In the first method the usual technique of isolation of monocytes in a Ficoll—Verografin density gradient according to Böyum [7] followed by adhesion of the monocytes on glass [1-3] was used. The second method involved isolation of monocytes by Recalde's method [14], which is based on centrifugation of the cells in a hypertonic Ficoll—Verografin gradient (d = 1.080 g/cm^3). The purity of the isolated monocytes was verified by morphological analysis of films stained by the Romanovsky—Giemsa method. The cells were cultured in flat-bottomed 96-well plates for cell culture in medium RPMI-1640 containing 10% fetal calf serum (Serva, West Germany), 1% L-glutamine, and 40 μg/ml gentamicin, at 37°C in an atmosphere with 5% CO_2. Activity of the intracellular, membrane-associated, and secreted IL-1 thus produced was estimated in different parts of the monocyte culture. Secreted IL-1 was determined in the culture fluid. Intracellular IL-1 was estimated in the cell lysate after freezing and thawing of the monocytes followed by centrifugation at 10,000*g* for 60 min at 1°C. In some experiments, to remove possible contaminating high-molecular-weight compounds the cell lysates were filtered through anisotropic CP-30 membranes (Amicon, USA) with permeability of 30 kD. Activity of the membrane-associated IL-1 pool was estimated on a monolayer of monocytes. For this purpose, at the end of culture the cells were fixed with a 1% solution of paraformaldehyde (PFA, Sigma, USA) [6, 12]. After treatment with PFA for 15 min at room temperature the monocyte monolayer was washed 3 times with medium, which was replaced by fresh after every additional 4-8 h of incubation in order to remove completely the soluble fractions of IL-1. Activity of all IL-1 pools was estimated in the test of costimulation of C3H/HeJ

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TABLE 1. Coefficients of Activation of Proliferation of C3H/HeJ Mouse Thymocytes in Response to Three Types of IL-1 in the Presence of Lipopolysaccharide (LPS) and (or) Indomethacin (IM) (M ± m)

Stimulation	Type of IL-1			
	intracellular		membrane-associated	secreted
	with filtration of supernatant (n=48)	without filtration	(n=48)	(n=48)
Spontaneous	2,52±0,23	2,16±0,40	1,51±0,06	2,06±0,12
LPS (25)	2,59±0,29	2,10±0,40	1,69±0,16	2,08±0,13
LPS (25)+ IM (10)	3,05±0,47	—	1,73±0,45	2,09±0,19
LPS (25)+ IM (5)	2,87±0,23	—	1,83±0,22	2,80±0,17
LPS (25)+ IM (1)	3,87±0,47	—	3,08±0,56	3,27±0,28
IM (1)	2,69±0,67	—	1,75±0,14	2,07±0,34

Legend. *p < 0.05 compared with control, n) number of experiments. Dose of stimulator (in μg/ml) given between parentheses.

mouse thymocytes with a suboptimal dose (10 μg/ml) of phytohemagglutinin (Difco-P, USA) [9]. Supernatants containing intracellular or secreted IL-1 were added to a suspension of thymocytes. Activity of membrane-associated IL-1 was estimated by applying a suspension of thymocytes to the monolayer of PFA-fixed monocytes. The level of thymocyte proliferation was determined as incorporation of ^{3}H-thymidine and expressed in coefficients of activation (CA), calculated as the number of pulses in samples with the test specimen and the number of pulses in samples not containing the test specimen.

EXPERIMENTAL RESULTS

In the initial experiments monocytes were isolated by the two methods, based on adhesion of the adherent cells and on centrifugation of leukocytes in a hypertonic density gradient. The purity of isolation of monocytes obtained by the two methods was not less than 90% and did not differ significantly Monocytes isolated by the different methods have about equal ability to produce IL-1 spontaneously. The fact that differences in CA, characterizing the activity of secreted IL-1 in monocytes obtained by adhesion (CA = 2.12 ± 0.14) and by Recalde's method (CA = 2.06 ± 0.12) are not significant confirms that either of these methods may be used. In subsequent experiments we used isolation of monocytes in a hypertonic density gradient has been less laborious and time consuming.

The optimal number of monocytes needed to produce IL-1 in culture in vitro was estimated. No single recommendations on this matter could be found in the familiar literature. We found that 10^5 cells per well is sufficient to give marked production of secreted IL-1 Cultivation of $1.5 \cdot 10^5$ and $2.0 \cdot 10^5$ monocytes per well did not lead to any significant change in IL-1 production, and in addition, it required the use of a large volume of blood from one patient, which is extremely undesirable if a complete immunologic investigation is to be undertaken.

The study of the time course of IL-1 production by monocytes showed that the optimal duration of cell culture is 24 h, for during that time maximal activity of both membrane-associated IL-1 (CA_{24} = 1.51 ± 0.06) and secreted IL-1 (CA_{24} = 2.06 ± 0.12) is manifested. Reduction of levels of activity of membrane-associated and secreted IL-1 pools in monocyte cultures after 48 and 72 h (for the first CA_{48} = 1.24 ± 0.05, CA_{72} = 1.20 ± 0.05, and for the second, CA_{48} = 2.01 ± 0.17, CA_{72} = 1.80 ± 0.18) is probably due to the influence of prostaglandins [1, 3].

Estimation of the activity of secreted IL-1 in supernatants of monocyte cultures is not difficult and has been described sufficiently well [2, 8, 9]. To determine intracellular IL-1, different authors used different approaches [6, 11, 13]. We decided on a relatively simple method of lysis of monocytes by freezing and thawing, followed by removal of the cell fragments by centrifugation. The study of the stimulating activity of supernatants containing intracellular IL-1, before and after their filtration through anisotropic membranes, retaining high-molecular-weight compounds, showed that possible contamination with lysosomal enzymes did not affect the estimation of activity of this monokine pool (Table 1). For clinical tests we therefore recommend using the shortened scheme of obtaining intracellular IL-1, excluding the stage of ultrafiltration through expensive anisotropic membranes. The stimulating activity of membrane-associated IL-1 was found to be significantly lower than that of the intracellular and secreted pools and, consequently, its determination in some immunopathological states could be associated with some degree of difficulty. Attention is drawn to the need to replace the culture

medium after every treatment of the monocytes with PFA, so as to rule out the possibility that intracellular and secreted IL-1 may be present in the culture fluid. Table 1 gives normal values of CA for the three monokine pools in a group of healthy blood donors.

IL-1 production induced by lipopolysaccharide (LPS) from *E. coli* ("Sigma," USA) was studied, although it did not differ from the spontaneous level (Table 1). Considering the ability of LPS to stimulate production not only of IL-1 by monocytes, but also products of arachidonic acid metabolism [11, 13], the suggestion had to be put forward that the presence of indomethacin (IM), a cyclooxygenase inhibitor, changes the level of the parameters studied. The combined use of LPS and IM led to a marked increase in activity of all three monokine pools, and makes it possible to determine activation of IL-1 synthesis by monocytes.

On the basis of these results we suggest the following scheme for combined assessment of production of the three types of IL-1 by human monocytes: stage 1 — isolation of monocytes from peripheral blood. Preference is given to the use of Recalde's method — isolation of the cells in a hypertonic density gradient; stage 2 — preparation of three IL-1 pools in monocyte culture. The micromethod of cultivation of monocytes in wells of 96-well flat-bottomed plates is used. The culture time was 24 h. The cell concentration 10^5 per well. The conditions of monocyte culture for obtaining the three parallel IL-1 pools are identical. Secreted IL-1 is determined in the culture medium, intracellular in the cell lysate, and membrane-associated on a monolayer of monocytes, as described above; stage 3 — estimation of activity of the three types of IL-1 by studying their effect on the proliferative ability of thymocytes.

The basic distinguishing features of the suggested method of estimating the three IL-1 pools are as follows: 1) the use of a micromethod reduces to a minimum (10-15 ml) the volume of blood taken from the donor, 2) simultaneous estimation of all types of IL-1 is carried out under identical conditions of culture of monocytes from the same person, 3) maximal production of three IL-1 pools is observed in response to combined stimulation of the monocytes by LPS and IM.

In general we can recommend the method we have developed, which can characterize the production of three IL-1 pools by the monocytes of each individual, for use in clinical immunology and for scientific research.

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