NITRIC OXIDE FORMATION IN ACTIVATED MACROPHAGES

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It has now been established that activated macrophages can produce nitric oxide (NO), which determines their cytotoxic and cytostatic action [3-5, 8, 15]. NO formation is monitored by a chemiluminescence method, based on accumulation of oxidation products of NO, namely nitrites and nitrates [3, 5, 8, 13], nitrosomorpholine formation [5], and also the appearance of dinitrosyl complexes of nonheme iron (DNCI), which can be recorded by electron paramagnetic resonance (EPR) [7, 9].

We have developed a new approach to the detection of NO in macrophages, by using an exogenous NO trap, namely complexes of Fe^{2+} with diethyldithiocarbamate (DETC). Binding with NO leads to the formation of paramagnetic mononitrosyl complexes of iron (MNCI) with DETC, that can be recorded by EPR. The appearance of such complexes was recorded previously in the tissues of animals in which NO synthesis was induced in vivo [1, 2].

EXPERIMENTAL METHOD

Macrophages of the J774 line were grown on 100-mm Petri dishes ("Costar," USA) on Eagle's minimal medium ("Flow Laboratories," England), containing 10% fetal calf serum ("Flow Laboratories"). The cells were activated by changing their incubation medium for fresh medium containing 10 μg/ml of lipopolysaccharide (LPS), isolated from *Escherichia coli* ("Sigma," serotype 0128:B12) cells. After incubation for 6-48 h, 1 mg/ml of DETC-Na and 10^{-5} M $FeSO_4$ were added to the medium for 2 h, after which the cells were harvested by scraping them from the dish and centrifuged at 400*g*; the residue was suspended in 1 ml of fresh medium and frozen in liquid nitrogen in ampuls. These specimens, containing 10^6 cells, were used to record EPR signals of MNCI with DETC at 77°K on EPR-V (USSR) and EPR-Radiopan (Poland) radiospectrometers.

After incubation for 16-18 h with LPS, the macrophages were subjected to cytologic analysis. Definite morphological signs of their activation were observed: the cells were enlarged, they formed processes,and they were spread out more densely on the substrate. Multiple inclusions characteristic of the activated lysosomal apparatus appeared in the cytoplasm of the macrophages. After 48-50 h these morphological features were intensified and, in particular, the internal inclusions occupied nearly the whole volume of the cytoplasm.

EXPERIMENTAL RESULTS

In control cells not treated with LPS, but incubated in medium with DETC and $FeSO_4$, an EPR signal of complexes of DETC with endogenous copper (Cu^{2+}) was observed, part of which, with its center at g 2.0, is shown on Fig. 1a. Four components of this signal A, B, D, and E are formed by the superfine structure (SFS) of the copper nuclei, whereas the intensity of the component (C) at g 2.0 is due to an "additional" component of EPR absorption [6]. In cells treated with LPS, DETC, and $FeSO_4$, an EPR signal of MNCI with DETC, reproduced in Fig. 1b, was recorded. It is characterized by triplet SFS at g and by values of the g-factor: g = 2.04, g = 2.02, g_m = 2.03. On the addition of 3 mM L-arginine to the

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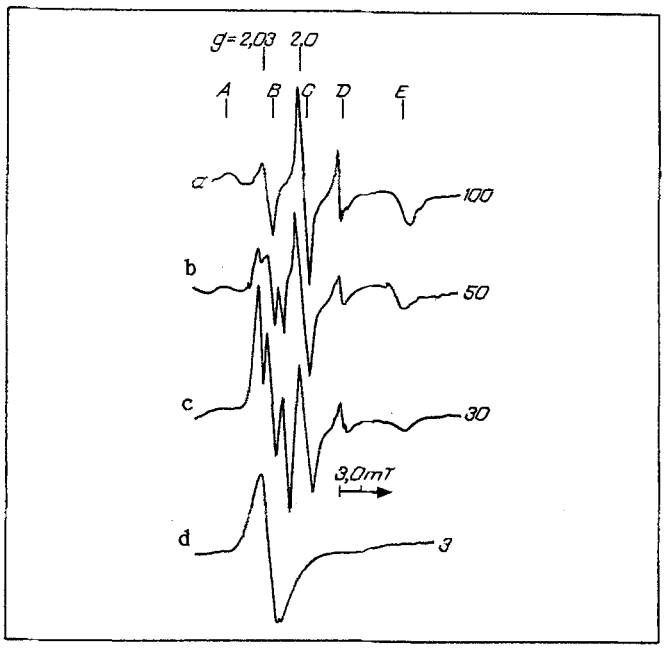

Fig. 1. EPR signals of Cu^{2+}-DETC complexes in control (unactivated) macrophages (a), of the same complexes and MNCI with DETC in activated cells without (b) and with (c) addition of L-arginine (3 mM) to incubation medium, d) EPR signal of specimen (c) after treatment with NO. (Recording made at 77°K, relative amplification of EPR spectrometer indicated on the right).

TABLE 1. Quantity of NO, in ng/10^6 cells, Incorporated into MNCI with DETC in Macrophages, Depending on Incubation Time of Macrophages with LPS and Concentration of LPS in Medium with L-Arginine 3 mM, $FeSO_4$ 10^{-5} M

Experiment	LPS concentration μg/ml	Incubation time of cells with LPS		
		6	20—24	48
1	0,2	—	10	N.d.
	1,0	—	10	N.d.
	10	—	40	N.d.
	50	—	60	N.d.
2	10	0	5	0
3 (n=3)	10	5±2	5±2	40±5
4 (n=3)	10	0	15±2	5±1
5 (n=3)	10	—	100±10	—
	0	—	5±2	—
6 (n=6)	10	—	40±10	—

Legend. n) Number of experiments, N.d.) not determined.

incubation medium the signal was intensified, and predominated over the EPR signal of Cu^{2+}-DETC complexes (Fig. 1c). The quantity of MNCI with DETC and, hence, the quantity of NO in these complexes was estimated by comparing the amplitude of their EPR signal and of the EPR signal of the same complex with a known concentration in a solution of dimethylsulfoxide, synthesized as in [1]. According to our estimates, 10^6 macrophages can synthesize up to 100 ng of NO in

2 h. The possibility cannot be ruled out that this value could be even higher, for not all the NO appearing in the cells may have bound with the trap. Incidentally, in experiments conducted at different times of the year, this amount varied appreciably: here we mention its highest value that we obtained. The quantity of NO incorporated into MNCI with DETC depended on the incubation time of the macrophages with LPS and the concentration of LPS in the medium (Table 1). MNCI with DETC as a rule were found in the cells after 18-24 h of incubation, but sometimes their largest amount was found after 2 days of incubation. With a decrease in the LPS concentration the NO level in the cells fell. The concentration of LPS used in most experiments was 10 μg/ml.

After treatment of macrophages giving the maximal EPR signal of MNCI with DETC with gaseous NO under a pressure of 100 mm Hg for 10 min the quantity of MNCI with DETC in the cells was increased by 10-20 times, and the EPR signal recorded in them had the form of a singlet without SFS (Fig. 1d). After treatment of these preparations with dimethylsulfoxide (1:5) a triplet SFS was again recorded. This result shows that the number of traps of NO—Fe^{2+} DETC complexes in the cells did not limit MNCI formation with the participation of NO arising from endogenous sources. As regards recording of the singlet signal without SFS, this fact is evidence of the high concentration of NO traps in the corresponding cell compartments. They are evidently cell membranes in which Fe^{2+}—DETC complexes are localized by virtue of their hydrophobicity [1]. Their high concentration in the lipid phase maintains strong spin—spin interaction of the MNCI formed with DETC, and this leads to "blurring" of the SFS in the EPR signal. The addition of dimethylsulfoxide, dissolving the membranes and these complexes, weakens their spin—spin interaction, and as a result, triplet SFS is again recorded.

Thus the use of our suggested method of detection of NO in macrophages confirms that it is formed in these cells after their activation. As was shown in [3, 8], nitric oxide appears in them as a result of oxidation of the imino group by the guanidine group of arginine. Addition of the latter to the incubation medium of the macrophages in our experiments potentiated NO formation in the cells, in agreement with conclusions drawn in [3, 4, 7, 9].

In our opinion Fe^{2+}—DETC complexes are more effective traps of NO than complexes of Fe^{2+} with paired thiol groups of proteins, forming paramagnetic DNCI with NO and giving EPR signals with g = 2.037 and g = 2.012 [11]. The appearance of such complexes in activated macrophages also was observed in [7, 9]. The mechanism of formation of DNCI is multimolecular whereas synthesis of MNCI with DETC is a bimolecular reaction of binding of NO with the strong Fe^{2+} DETC complex. Furthermore, as was shown in [1], MNCI with DETC are more stable complexes than DNCI with paired protein thiol groups. The use of Fe^{2+} DETC as NO traps in macrophages and other cells may therefore enable a small quantity of this agent to be detected.

Kinetic studies have shown that the steady-state level of NO, synthesized in macrophages, may in certain cases be at a very low level for 24 h after the beginning of activation of the macrophages, and may then rise sharply toward the end of the 2nd day (Table 1). Meanwhile cytologic analysis has shown that after 18-20 h of incubation with LPS these macrophages were activated: their morphology was characteristic of the activated form of the cells. Turning now to data obtained by other workers estimating NO production in macrophages on the basis of accumulation of its oxidation products (NO_2 and NO_3 anions) in the culture medium [3, 5, 8, 13], it seems that the latter appear gradually in the medium, starting from 6-12 h after contact of the cells with LPS from *E. coli* [12, 13]. This means that NO synthesis in activated macrophages takes place continuously; moreover, judging by the rate of accumulation of NO_2/NO_3 in the medium, NO synthesis is most effective during the 1st day of activation [12]. Why, therefore, could we not find NO in the cells at this time? The reason is evidently the remoteness of the NO—Fe^{2+}—DETC complexes — membranes traps from the site of synthesis of this agent — the cytosol [8, 14]. We know that oxygen and its active forms (O_2^-, $OH^{\cdot}$, H_2O_2) affect NO production in different cells [10, 11]. Contact of NO with them along the path of its transport from cytosol to trap in the membrane could lead to oxidation of NO to NO_2/NO_3, and as a result the steady-state NO concentration close to the traps could fall, and the formation of MNCI with DETC could cease to be significant. It can be tentatively suggested that on the appearance of redox agents in the cells capable of lowering the concentration of oxygen and its active forms, the steady-state NO concentration could rise and, correspondingly, MNCI with DETC could appear in large quantities, for example, after incubation of the cells with LPS for 48 h.

Thus the quantity of NO passing from activated macrophages into their incubation medium may be determined by the intensity of redox conversions of this agent within the macrophages themselves. The possibility cannot be ruled out that in some cases these cells may supply only nitrites and nitrates — oxidation products of NO — to the medium. Hence their cytostatic and cytotoxic action, due to NO, will be greatly weakened and the macrophages, despite their activation, will be

unable to act on target cells. Such a situation may perhaps be observed sometimes in both animals and man, and it may lead to weakening of the immune activity of the body.

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EFFECT OF EXCESS AND DEFICIENCY OF THYROID HORMONES ON BLOOD MELATONIN LEVEL IN MATURE MALE RATS

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The writer showed previously [1-4] that saturation of the body with thyroid hormones has a significant effect on serotonin metabolism in the pineal gland: injection of thyroxine causes activation of the N-acetylation and subsequent O-methylation pathways of serotonin, with the formation of N-acetylserotonin and melatonin, whereas removal of the thyroid gland lowers the pineal concentration of these substances to the trace level. It has been concluded from these findings that thyroid hormones activate pineal function.

Meanwhile, determination of the pineal melatonin concentration alone cannot answer the question of whether, in the case of an increase in the hormone concentration in the gland, biosynthesis in the gland is stimulated and its release into the blood stream is simultaneously increased, or whether this increase takes place without any change in the intensity of biosynthesis and with simultaneous blockade of passage of the hormone into the circulation. If the melatonin concentration in the pineal gland falls, there may be two explanations: either a decrease in the intensity of biosynthesis and, correspondingly, of release of the hormone into the blood stream, or the decrease in the concentration of the hormone in the pineal gland takes place due to the rapid passage of the hormone into the circulation.

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