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In vitro effects of hormones and autacoids on the hydrogen peroxide production and the morphology of endotoxin-activated rat peritoneal macrophages

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Summary. Peritoneal macrophages activated in vitro by endotoxin exhibit alterations of their capability to produce hydrogen peroxide after phorbol ester stimulation when certain hormones or autacoids are present in the culture medium. They also show morphological changes, mainly concerning cell size and nuclear appearance. Agents known to increase the intracellular levels of cyclic AMP, e.g. adrenalin and PGE2 reduce the hydrogen peroxide production. Insulin, which is known to decrease cyclic AMP levels, produces opposite results. Agents postulated to act via phospholipase C, e.g. serotonin, augment the production of hydrogen peroxide. We assume that this form of modulation may represent a regulatory mechanism of macrophage activation.

Key words: Activated macrophages, Endotoxin, Cyclic AMP, Hydrogen peroxide

Introduction

Macrophages are cells of the primary defense system that may exist in three functional states: quiescent (responsive), inflammatory (primed) and activated (Adams and Hamilton, 1992). In each of these states macrophages are characterized by different biological capabilities, e.g. capability to produce reactive oxygen and nitrogen species or to phagocytose. The capability of activated macrophages to produce reactive oxygen intermediates such as superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radical is long known and its significance is well established (Johnston, 1978; Johnston et al., 1978; Nathan, 1982; Adams and Hamilton, 1984). The production of these intermediates has been proposed as a criterion of macrophage activation (Karnovsky and Lazdins, 1978). Such an activation can be induced in vitro by incubation with bacterial endotoxin - lipopolysaccharide (Cooper et al., 1984). Lipopolysaccharide initiates second messenger cascades that ultimately cause activation of the multicomponent membrane enzyme NADPH-oxidase. This enzyme is responsible for the production of superoxide anion by catalyzing the one-electron reduction of oxygen. Hydrogen peroxide is mainly formed by the action of superoxide dismutase on the superoxide anion.

Macrophage morphology also varies with the functional status (Adams and Hamilton, 1984, 1992). Changes in macrophage morphology, i.e. cell shape and cytoplasm spreading are visible by light or electron microscopy (Brederoo and Daems, 1972; Williams and Mayhew, 1973; Daems and Brederoo, 1973; Mayhew and Williams, 1974a,b; Morland and Kaplan, 1977; Cooper et al., 1984).

The classification of three distinct states of macrophage activation combined with the study of second messenger systems mediating this activation, raised the possibility that the macrophages are subject to regulation of their functions. Several reports have been published concerning the second messenger systems that were connected to macrophages stimulated by various pharmacological agents. Certain publications pertained to the elevation of cyclic AMP after incubation of macrophages with adrenergic agonists or prostaglandins (Remold-O'Donnel, 1974; Ikegami, 1977). The cyclic AMP-protein kinase A pathway has been considered a suppressor of macrophage function, as well as of other cells that participate in inflammatory and immune reactions (Kammer, 1988).

The complex network of second messengers involved in macrophage activation has not yet been completely unraveled. Activation of monocytes by LPS requires activation of mitogen-activated protein kinases (p42/p44 ERK) and a calcium-independent isoform of protein kinase C, presumably PKC-ζ (Liu et al., 1994). The activation of this isoform could be mediated by phosphatidylinositol-3-kinase that in turn may be activated by src-tyrosine kinases (Herrera-Velit and Reiner, 1996; Herrera-Velit et al., 1997). It has been

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further elucidated in alveolar macrophages that the signaling cascade caused by LPS also involves the activation of a phosphatidylcholine-specific phospholipase C and phosphatidylinositol 3-kinase and subsequent phosphorylation of protein kinase C-ζ. This leads to the downstream activation of mitogen-activated protein/ERK kinase (MEK) by PKC-ζ (Monick et al., 1999, 2000). Moreover, in a macrophage-like cell line, evidence has been presented that activation of MEK and ERK by lipopolysaccharide requires upstream activation of phosphatidylinositol 3-kinase, phospholipase D and a diacylglycerol-dependent protein kinase C isoenzyme (Procyk et al., 1999).

Effects of hormones and autacoids on hydrogen peroxide production may be caused by modulations in the NADPH-oxidase activation. This could be mediated by effects on the signaling pathways that control phosphorylation of the different subunits of this oxidase. It has been previously found that NADPH-oxidase activation requires serine phosphorylation of the $p47^{PHOX}$ and $p67^{PHOX}$ subunits by protein kinase C, and possibly other kinases (El Benna et al., 1996, 1997; Park et al., 1997; Dewas et al., 2000). Protein kinase A negatively regulates the phosphorylation of $p47^{PHOX}$ and may diminish the activation of NADPH-oxidase (Babior, 1999). It has been reported that predominantly PKC-ß isotypes contribute to NADPH-oxidase activation in PMA-stimulated neutrophils (Dekker et al., 2000). The availability of arachidonic acid is also a possible regulator of NADPH-oxidase activity, since there is evidence that arachidonate facilitates the interaction of $p47^{PHOX}$ with $p22^{PHOX}$ (Shiose and Sumimoto, 2000). NADPH-oxidase may also be modulated by the pathway leading to $p22^{PHOX}$ phosphorylation. This subunit could be phosphorylated via two different pathways, either through phospholipase D, phosphatidic acid and a phosphatidic acid-activated protein kinase or through phospholipase C, diacylglycerol and a classical protein kinase C isoform (Regier et al., 2000).

In this study we present biochemical and morphological observations which imply that hormones and autacoids such as adrenalin, prostaglandin E2 or insulin affect the intensity of hydrogen peroxide production and the morphology of the macrophage. This may represent a form of regulation of the state of macrophage activation.

Materials and methods

Chemicals

Dopamine Hydrochloride (H-8502), Ethylene-Diamine-Tetraacetic Acid Tetrasodium Salt (E-6511), Glucagon (G-9154), D(+)-Glucose (G-7021), Histamine (H-7125), Insulin (I-6634), Horseradish Peroxidase Type II (P-8250), Phorbol-12-Myristate-13-Acetate (P-8139), Prostaglandin E2 (P-5640), Prostaglandin D2 (P-5172) and Serotonin Hydrochloride (H-9523) were obtained from Sigma. The lipopolysaccharide (LPS) from E.coli, strain O55:B5, was also obtained from Sigma, either Phenol-extracted LPS (L-2880) or TCA-extracted LPS (L-4005). Adrenaline Hydrochloride (1 mg/ml solution, Lot. 98008) was purchased from DEMO Pharmaceuticals. Dexamethasone 21-Phosphate (Decadron) (4 mg/ml solution, Lot.4532) was purchased from Merck & Co. Inc. Phenol Red Sodium salt was a product of Serva (32097). For cell culture, sterile 6-well culture plates manufactured by Corning were used (25810). RPMI-1640 medium (F-1235), L-Glutamine (K-0282) and fetal calf serum (S-0113) were provided by Seromed. Concentrated Giemsa stain was from Merck (9204).

Animals and isolation of macrophages

The experiments were conducted in primary cultures of macrophages obtained from pathogen-free adult Wistar rats of both sexes, aged 3 months and weighing approximately 180-210g each. These animals were housed in pairs in cages and allowed free access to water and standard chow in pellets. Rats were sacrificed by light ether anesthesia, followed by rapid cervical dislocation. For each experiment three to four rats were used, all of the same litter. Once sacrificed, animals were immediately swabbed twice with 70% ethanol and isolation of peritoneal macrophages was performed aseptically with modifications of standard methods (Cohn and Weiner, 1963; Cohn and Benson, 1965; McCarron et al, 1984).

Peritoneal cell isolation was performed by inserting 25 ml of ice-cold PBS-0.1% EDTA into the peritoneal cavity through a 14-gauge catheter, gently massaging the belly of the animals and immediately aspirating the fluid into a syringe. The aspirate was centrifuged at 210 g for 10 minutes at 4 °C and the cell pellet was washed twice in cold PBS and suspended in PBS so that the cell concentration and viability could be estimated by using Trypan blue dye and a hemacytometer slide. The cells were centrifuged again and the cell pellet was resuspended in warm RPMI-1640 medium and plated into 6-well tissue culture plates at a concentration of $4x10^6$ cells/well.

Cell culture

After plating the cell suspension, each well was filled with 2 ml of RPMI-1640 medium containing 10% FCS and supplemented with 20 mM HEPES, 100,000 U/L penicillin, 100 mg/L streptomycin, 5.6 mg/L amphotericin-B and 0.3 g/L L-glutamine. Macrophages were allowed to adhere to the plates during a 2 hour incubation at 37 °C in a 5% carbon dioxide-95% air environment. Then the wells were extensively washed with warm PBS and replenished with medium. Approximately 50% of the cells plated adhered to the plate (approx. $2x10^6$ cells/well). Phase-contrast observation with an inverted microscope revealed less than 2% contamination of the cell population with non-

macrophage cells.

Hydrogen peroxide assay

The capability of macrophages to produce hydrogen peroxide after stimulation with phorbol-12-myristate-13 acetate (PMA) was assayed colorimetrically as described previously (Pick and Keisari, 1980). Macrophages were incubated with medium containing 0.1 μg/ml PMA for 30 minutes. Then the cell wells were washed three times with warm PBS and filled with 2 ml of phenol red solution (10 mM potassium phosphate buffer pH 7.0, 140 mM NaCl, 5.5 mM D(+)-glucose, 8.5 units/ml type II peroxidase and 0.1 g/L phenol red disodium salt) and further incubated for 45 minutes at 37 °C in a CO_2-incubator. The supernatant was aspirated and the reaction terminated by using 1N NaOH. After 10 minutes at room temperature, absorbance was read at 610 nm. Results were expressed as micromoles (μmoles) of hydrogen peroxide produced per $2x10^6$ cells per hour ± standard deviation. Hydrogen peroxide production was determined in pairs. Each pair consisted of the supernatant of one control well and the supernatant of one experimental well. The "n" value signified the number of control-experimental pairs included in the results. Differences between controls and experimentals were evaluated by the Student's t-test. P values smaller than 0.05 were considered as significant.

Experimental design

The cells used in each experiment were obtained from rats of the same litter. Pooled cells from 3-4 rats were plated in 6-well cell culture plates. Each two adjacent rows of wells were used as "controls" and "experimentals". Macrophages in control wells were incubated with lipopolysaccharide only, whereas macrophages in experimental wells were incubated with lipopolysaccharide and the effector agent (hormone or autacoid). Lipopolysaccharide (LPS) concentration in the culture medium was 2 micrograms/ml (μg/ml). The concentrations of adrenalin, serotonin, dopamine, histamine and glucagon were 10^{-4} M, $2x10^{-5}$ M, $2.5x10^{-5}$ M, 10^{-6} M and 10^{-7} M, respectively. The concentrations of prostaglandins E2 (PGE2) and D2 (PGD2) were 10^{-5} M. In the experiments with dexamethasone two concentrations were used, 10^{-4} M (high dose) and $5x10^{-6}$ M (low dose). Insulin concentration was 25 micrograms/ml (0.625 units/ml). These incubations lasted 4 hours, unless otherwise specified. Usually, each experiment involved 5 pairs of control-experimental. The experiment was repeated two to five times, on different days. The results of the biochemical determinations were pooled for statistical analysis.

Light microscopy

The morphology of macrophages was evaluated by culturing the cells directly on acid-treated and sterile glass microscopy slides placed inside Petri dishes. After incubation with the medium containing the agents under investigation, slides were washed in warm PBS and allowed to air dry at 4 °C in a dessicator. The cells were then fixed in cold absolute methanol (4 °C) for 10 minutes, washed with Sörensen's buffer, pH 7.25, and stained for 45 minutes with dilute Giemsa stain in the dark. After washing for 15 seconds in Sörensen's buffer, slides were air-dried and mounted with Entallan and a clear glass coverslip. The specimens were studied with a Leitz Ortholux-II microscope by two independent observers. Photographs were taken at a magnification of x 1000, with a Leica DMLS microscope, connected to a Sony Hi-Resolution CCD-IRIS Color Video Camera.

Results

Biochemical results

Our initial experiments involved short-duration incubations with lipopolysaccharide (LPS). Rat peritoneal macrophages incubated with 2 micrograms/ml of LPS for two hours, produced 26.83% more hydrogen peroxide than cells incubated for the same time period without LPS (controls: 8.894±0.387 μmoles $H_2O_2/2x10^6$ cells, experimentals: 11.281±0.533 μmoles $H_2O_2/ 2x10^6$ cells, n=18, p<0.001).

Table 1. The effects of adrenalin, prostaglandin E2, dopamine, insulin, glucagon or serotonin on hydrogen peroxide production, by lipopolysaccharide-stimulated peritoneal macrophages.

	AGENT TESTED					
	ADRENALIN (4-hour incubation)	PGE2 (4-hour incubation)	DOPAMINE (14-hour incubation)	INSULIN (14-hour incubation)	GLUCAGON (14-hour incubation)	SEROTONIN (4-hour incubation)
Control	11.060±0.477 (15)	14.387±1.049 (12)	5.485±0.692 (23)	4.692±0.390 (26)	4.381±0.520 (27)	4.017±0.216 (15)
Experimental	9.593±0.552 (15)	13.063±1.060 (12)	4.796±0.794 (23)	5.877±0.849 (26)	6.533±0.534 (27)	4.657±0.157 (15)
p	<0.001	<0.01	<0.005	<0.001	<0.001	<0.001

Results are means±standard deviations and are expressed as micromoles of hydrogen peroxide produced per $2x10^6$ cells per hour. Numbers in parentheses indicate the number of observations included in the results.

A 4-hour incubation of rat peritoneal macrophages with 2 μg/ml LPS plus 10^{-4} M adrenalin caused a significant decrease in hydrogen peroxide production (13.26%) compared to cells treated only with LPS (controls: 11.060±0.477 moles $H_2O_2/2x10^6$ cells, experimentals: 9.593±0.552 μmoles $H_2O_2/2x10^6$ cells, n=15, p<0.001). Similarly, peritoneal macrophages incubated for four hours with 2 μg/ml LPS plus 10^{-5} M Prostaglandin E2 (PGE2) caused a smaller but still statistically significant decrease in hydrogen peroxide production (9.20%) compared to control cells treated only with LPS (controls: 14.387±1.049 μmoles $H_2O_2/2x10^6$ cells, experimentals: 13.063±1.060 μmoles $H_2O_2/2x10^6$ cells, n=12, p<0.01) (Table 1). Adrenalin and PGE2 are known to activate the macrophage adenylate cyclase and elevate intracellular levels of cyclic AMP. Regarding PGE2, additional experiments were also conducted to further study the mechanism involved. When PGE2 was added during both the 4-hour incubation with LPS and the 30-minute incubation with phorbol-12-myristate-13-acetate the effect was pronounced. The hydrogen peroxide production was inhibited by 14.08% as compared to control cells treated only with LPS (controls: 8.970±0.509 μmoles $H_2O_2/2x10^6$ cells, experimentals: 7.707±0.627 μmoles $H_2O_2/2x10^6$ cells, n=15, p<0.001). But when PGE2 was added only during the 30-minute incubation with the phorbol ester, no significant alteration of hydrogen peroxide production was observed. In contrast to PGE2, incubation of peritoneal macrophages with LPS and prostaglandin D2 did not produce statistically significant results, even when the incubation period was extended to 14 hours (controls: 4.075±0.454 μmoles $H_2O/2x10^6$ cells, experimentals: 4.100±0.849 μmoles $H_2O_2/2x10^6$ cells, n=20, p>0.05).

The action of dopamine, a catecholamine known to produce certain effects through adenylate cyclase activation, was investigated. Peritoneal cells were treated for 14 hours with lipopolysaccharide and $2.5x10^{-5}$ M dopamine. This caused a 12.56% inhibition of hydrogen

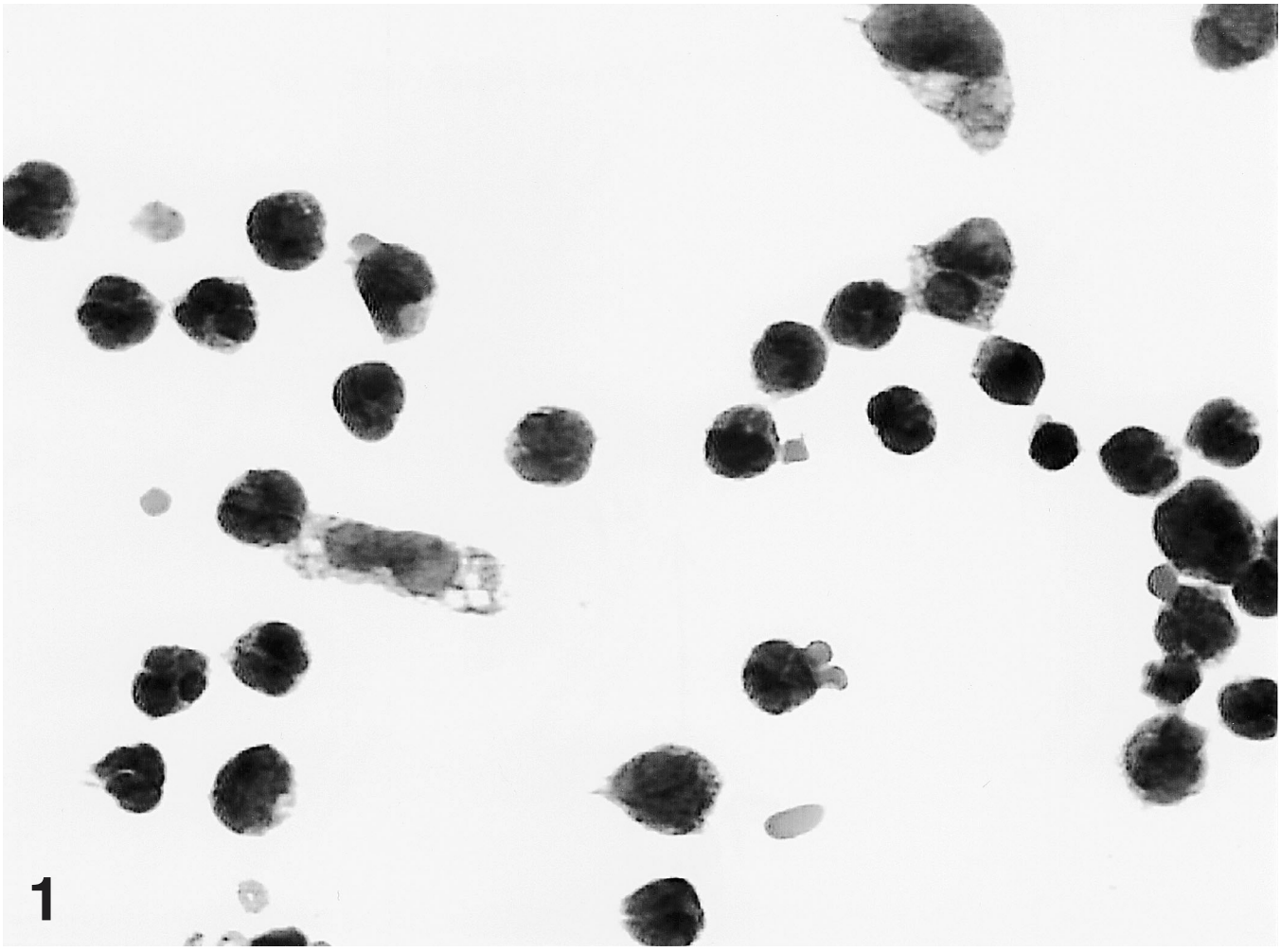

Fig. 1. Peritoneal macrophages incubated for 4 hours with lipopolysaccharide. The cells often show abundant cytoplasm. Giemsa, x 1000

peroxide production as compared to the control cells (controls: 5.485±0.692 μmoles $H_2O_2/2x10^6$ cells, experimentals 4.496±0.794 μmoles $H_2O_2/2x10^6$ cells, n=23, p<0.005).

The effects of insulin and glucagon were also determined on macrophages. Peritoneal macrophages were treated for 14 hours with 2 μg/ml LPS (controls) or 2 μg/ml LPS plus 25 μg/ml insulin (experimentals). Insulin caused a 25.25% increase in hydrogen peroxide production (4.692±0.390 μmoles $H_2O_2/2x10^6$ cells vs. 5.877±0.849 μmoles $H_2O_2/2x10^6$ cells, n=26, p<0.001). (Table 1). Glucagon also caused a stimulatory effect. Peritoneal cells incubated with lipopolysaccharide plus 10^{-7} M glucagon for 14 hours produced 49.12% more hydrogen peroxide than their controls (controls: 4.381±0.520 μmoles $H_2O_2/2x10^6$ cells, experimentals: 6.533±0.534 μmoles $H_2O_2/2x10^6$ cells, n=27, p<0.001).

The effects of dexamethasone, were also investigated. Peritoneal macrophages incubated with lipopolysaccharide plus a high dose of dexamethasone (10^{-4} M) for 12 hours produced 30.42% less hydrogen peroxide than their controls whereas macrophages incubated with lipopolysaccharide plus a low dose of dexamethasone ($5x10^{-6}$ M) for 12 hours exhibited a 23.73% increase in hydrogen peroxide production, as compared to their controls.

Two autacoids, serotonin and histamine, were tested for their effects on peritoneal macrophages. In experiments using 4-hour incubations, the addition of serotonin ($2x10^{-5}$ M) to lipopolysaccharide-activated macrophages caused a 15.93% increase in hydrogen peroxide production as compared to control cells (controls: 4.017±0.216 μmoles $H_2O_2/2x10^6$ cells, experimentals: 4.657±0.157 μmoles $H_2O_2/2x10^6$ cells, n=15, p<0.001). Histamine, at a 10^{-6} M concentration, caused no significant alteration of hydrogen peroxide production during a 14-hour incubation (controls: 3.916±0.724 μmoles $H_2O_2/2x10^6$ cells, experimentals:

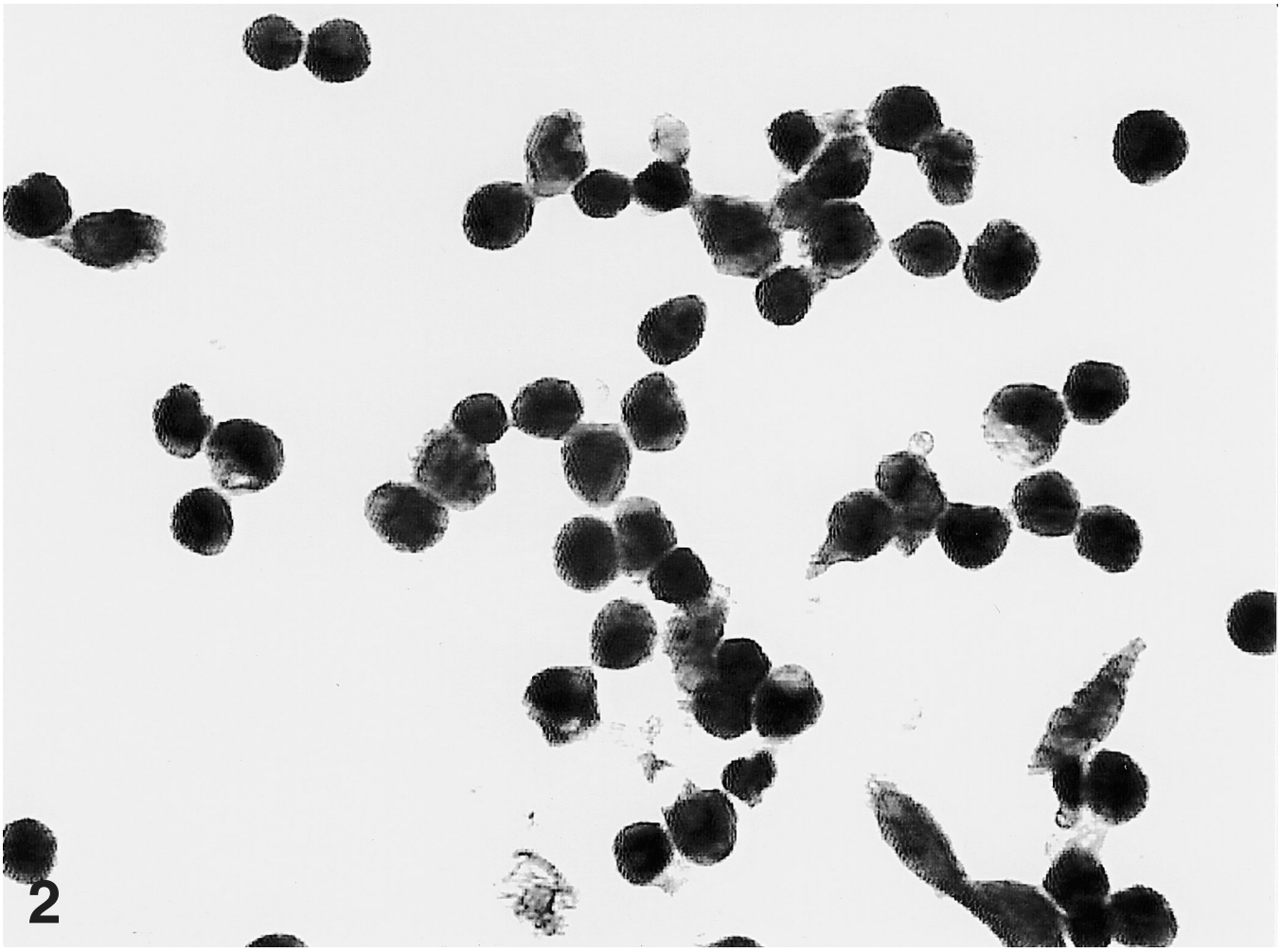

Fig. 2. Peritoneal macrophages incubated for 4 hours with lipopolysaccharide plus adrenalin. The cells are usually small and show scarce cytoplasm and dark round or lobulated nucleus. Giemsa, x 1000

3.832±0.770 μmoles H_2O_2/2x10^6 cells, n=25, p>0.05).

Morphological results

Morphological changes produced by adrenalin in the LPS-activated peritoneal macrophages cultured on acid-treated glass slides, are described.

Peritoneal macrophages incubated in culture medium for 4 hours usually displayed a typical morphology. These cells were round with pink cytoplasm and a dark red or purple nucleus. The nucleus-to-cytoplasm ratio was roughly 1:1. The nucleus was usually kidney-shaped or oval with an indentation. A few macrophages had an irregularly-contoured nucleus and abundant vacuolar or blebbed cytoplasm. Some cells showed a dark and discontinuous rim around their nucleus. Elongated macrophages were very rare. Cells cultured for 14 hours had a similar appearance. Only a few macrophages appeared with abundant foamy cytoplasm and an ellipsoid nucleus that stained lightly.

When the peritoneal macrophages were activated for 4 hours with 2 μg/ml lipopolysaccharide, cells with typical morphology became rare. (Fig. 1) Many macrophages appeared quite large and displayed abundant vacuolar or blebbed cytoplasm and a large, lightly stained, irregularly contoured nucleus. The nucleus-to-cytoplasm ratio was usually less than 1:1. Some macrophages were small and had scarce pink cytoplasm and a dark polygonal or round nucleus. Elongated cells with lightly stained cytoplasm and nucleus were not uncommon. Cells cultured for 14 hours had a similar appearance.

Peritoneal macrophages incubated for 4 hours with lipopolysaccharide plus adrenalin showed characteristic changes. (Fig. 2) Most of the cells were small, with scarce pink cytoplasm and a round or lobulated nucleus. Some of these cells stained intensely while others were pale. The nucleus-to-cytoplasm ratio varied over a wide range but usually was close to 1:1. Many macrophages were very small and round, with scarce cytoplasm and a

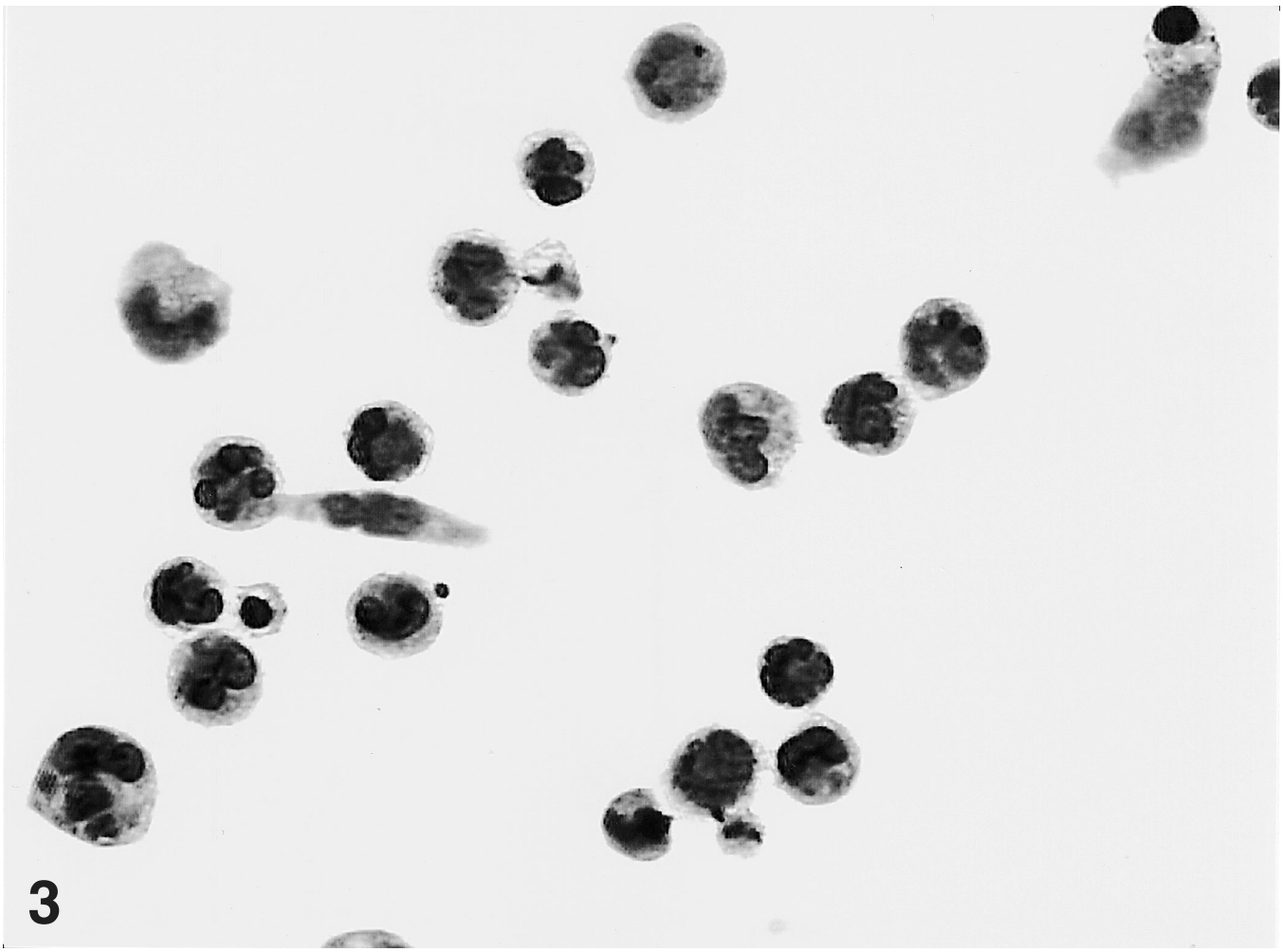

Fig. 3. Peritoneal macrophages incubated for 14 hours with lipopolysaccharide plus adrenalin. Lobulation of the nucleus is excessive. Giemsa, x 1000

dense round nucleus. Large macrophages were rare. Some elongated cells appeared. Macrophages cultured for 14 hours with lipopolysaccharide plus adrenalin had a similar appearance. Lobulation of the macrophage nuclei was excessive, often assuming the appearance of separate lobes (Fig. 3).

Discussion

The data presented in this study raise the possibility that hormones and autacoids, e.g. adrenalin, prostaglandin E2 and insulin may regulate the production of hydrogen peroxide in activated macrophages. Our results indicate that agents known to increase the cyclic AMP levels in macrophages, such as adrenalin, prostaglandin E2 or dopamine (Koopman et al., 1973; Remold-O'Donnel, 1974; Ikegami, 1977), reduce hydrogen peroxide production. Agents that activate the adenylate cyclase-protein kinase A-CREB pathway moderate pro-inflammatory functions of macrophages or lead to the production of anti-inflammatory cytokines that diminish other arms of the immune response (Guirao et al., 1997; Delgado et al., 1998, 1999). Cyclic AMP is known to inhibit the respiratory burst (Smith et al., 1980). The cyclic AMP-dependent protein kinase negatively regulates the phosphorylation of $p47^{PHOX}$. This leads to the inactivation of NADPH-oxidase (Babior, 1999). Although modulation of lipopolysaccharide-induced TNF-α and nitric oxide production by using specific D1- and D2- dopamine receptor agonists and antagonists has been reported (Hasko et al., 1996), it is not certain that our results with dopamine were due to the occupation of such receptors. It is possible that the effects of dopamine on hydrogen peroxide production are also mediated through adrenergic receptors, as has been described for IL-12 p40 (Hasko et al, 2002).

Catecholamines are neurotransmitters and stress hormones that are known to affect macrophage functions (Miles et al., 1996; Hasko et al., 1998; Kohut et al., 1998; Ortega et al., 2000). Since macrophages accumulate catecholamines into functional pools and lymphocytes themselves produce catecholamines, (Spengler et al., 1994; Josefsson et al., 1996; Musso et al., 1996) an autocrine or paracrine regulatory loop may exist that would attenuate the functions of macrophages, e.g. hydrogen peroxide production, according to the conditions and requirements of the inflammatory site. Such an autoregulatory mechanism would allow maximal macrophage activity while avoiding the detrimental effects of excessive hydrogen peroxide production, since it would be controlled by the cells that participate in the local reaction. Another self-regulatory mechanism would involve prostaglandin E2 produced by macrophages at the inflammatory site; PGE2 acting in an autocrine and paracrine fashion would moderate hydrogen peroxide production. Prostaglandin E2, acting via the cAMP-PKA pathway (Lim et al., 1983; Yamamoto and Suzuki, 1987) has been shown to diminish the production of free oxygen radicals via cyclic AMP, an effect reproduced by cAMP agonists, e.g. isoproterenol and cholera toxin and by cAMP analogues, e.g.dibutyryl-cAMP (Metzger et al., 1981).

Glucagon, a hormone known to elevate cAMP in a variety of cells, has been reported not to activate adenylate cyclase or elevate cAMP in macrophages (Smith et al., 1980). It is possible that the increase in hydrogen peroxide production encountered in our experiments was due to an alternative signal transduction pathway involving phospholipase C, as has been described in other cells (Wakelam et al, 1986; Murphy et al., 1987).

Insulin increased hydrogen peroxide production. The presence of insulin receptors on macrophages has been known since the mid-1970's (Schwartz et al., 1975; Bar et al., 1976). Insulin has been reported to inhibit certain macrophage functions (Rhodes, 1975; Bar et al., 1977; Muschel et al., 1977; Abrass and Hori, 1984; Bermudez et al., 1990) while potentiating others (Iida et al., 2001). Its stimulatory effect on hydrogen peroxide production may be explained on the basis that this hormone antagonizes the effects of cyclic AMP (Walkenbach et al., 1978; Heyworth and Houslay, 1983; Larner, 1990). Moreover, insulin activates phosphatidylinositol-3-kinase and MAP-kinases (Davis and Granner, 1996). Phosphatidylinositol 3-kinase has been reported to mediate Fc-receptor-mediated phagocytosis (Araki et al., 1996). Also, insulin inhibits autophagocytosis in other cells (Kotoulas, 1981; Kalamidas and Kotoulas, 2000). The effect of insulin on hydrogen peroxide production by macrophages is very important when considering two parameters: insulin regulates the entrance of glucose into cells, therefore it participates in stressful conditions and debilitating diseases in which macrophages may also play an important role; also, in the hyperinsulinemic diabetic patient, macrophages may present an altered function that would participate in the pathophysiology of the complications of diabetes.

Serotonin caused an increase in hydrogen peroxide production. Such an effect may be mediated by 5-HT2 serotoninergic receptors which are known to activate phospholipase C (Sanders-Bush and Mayer, 1996a,b). Ionized calcium plays an important role in the generation of the respiratory burst in phagocytes (Scully et al., 1986). The stimulatory effect on hydrogen peroxide produced by certain agents, e.g. serotonin, may be mediated by a facilitation of the cytoplasmic free calcium increment. By activation of phospholipase C serotonin could cause an increase in the cellular diacylglycerol (a known protein kinase C stimulator) and ionized calcium that potentiate the respiratory burst. Modulatory effects on interferon-gamma-activated macrophages by serotonin, that were mediated by 5-HT2 receptors have been reported by others (Sternberg et al., 1986, 1987). Serotonin is not only a neurotransmitter of the central nervous system, but also an autacoid released by platelets in sites of thrombosis (as for example in inflammatory lesions). In rodents it is also found in mast

cells. Our observation that serotonin augments hydrogen peroxide production is in accordance with the following role in the immune response: serotonin is required for optimal macrophage accessory cell function and is considered an enhancer of macrophage-mediated natural immunity (phagocytosis and superoxide anion production) while altogether augmenting the immune response (Mossner and Lesch, 1998).

Dexamethasone, a potent synthetic analogue of glucocorticoid hormones, is known to produce many biological effects (Schimmer and Parker, 1996). Dexamethasone inhibited hydrogen peroxide production in high doses but increased this production in low doses. These are similar to the results presented by others concerning Reactive Nitrogen Intermediates (Broug-Holub and Kraal, 1996) and should be explained by the same mechanism, i.e. differential occupation of positive or negative glucocorticoid responsive elements according to the concentration of the glucocorticoid. It is still not clear how these gene-expression regulating elements affect NADPH-oxidase. It is possible that the effects of dexamethasone may be further mediated by indirect mechanisms, e.g. moderated availability of arachidonate, due to the modulated expression of genes encoding lipocortin (Errasfa et al, 1988; Maridonneau-Parini et al., 1989) or COX-2 (Lee et al., 1992).

As to the morphological findings, the lipopolysaccharide-activated peritoneal macrophages presented certain features of macrophage activation, i.e. increased size, abundant cytoplasm and a large lightly stained nucleus (Morland and Kaplan, 1977; Cooper et al., 1984). Membrane ruffling and pseudopodia extension were not readily visible. Adrenalin abolished the increase in cell size and cytoplasm observed in LPS-treated cells and caused a characteristic change in the shape of the nucleus which became quite lobular. These changes may be due to the inhibition of the LPS-induced activation. Characteristics resembling apoptotic processes (nuclear condensation or cell fragmentation) were not apparent.

Observations have accumulated implying the bidirectional functional interrelation between the neuroendocrine and immune systems (Besedovsky et al, 1986; Chrousos and Gold, 1992; Reichlin, 1993; Chrousos, 1995; Husband, 1995). The neural and endocrine systems affect and modulate the function of the immune system, e.g. through the hypothalamic-pituitary-adrenal axis. Immune cells also possess both receptors for chemical mediators of stress and the capability to secrete other mediators as well as cytokines that affect the function of the central nervous system. Primary and secondary organs of the immune system receive innervation by postganglionic adrenergic fibers and by fibers secreting other neurotransmitters (Van Epps and Saland, 1984; Bernton et al., 1987; Gordon et al., 1988; Reichlin, 1993; Husband, 1995). Severe emotional (Syvalahti, 1987; Stark et al., 2001) or physical stress, such as sepsis, burns, trauma, strenuous exercise, metabolic disturbances, etc. (Fitzgerald, 1988; Green and Faist, 1988; Dantzer and Kelley, 1989, Zellweger et al, 1995; Engelich et al, 2001) lead to depressed immune responses. The study of the effects of stress-related hormones and autacoids on macrophage functions (Pavlidis and Chirigos, 1980; Koff and Dunegan, 1985, 1986; Bermudez et al, 1990; Huynh et al., 2000), may elucidate the neuroendocrine mechanisms that regulate macrophage function in acute inflammatory disorders, immune diseases, sepsis, allergy, trauma or shock and help to comprehend how old age and chronic diseases (e.g. diabetes, alcoholism, neoplasia, depression, malnutrition, etc) lead to macrophage malfunction (Ortega et al., 1992, 2002; Bermudez, 1994; Hunt et al, 2001), in an attempt to elaborate the pathophysiology of the complications of these conditions. Also, to design new strategies for therapeutic intervention and to understand the natural history of diseases in the pathogenesis of which the macrophages are involved, e.g. atherosclerosis, asthma, inflammatory bowel disease and autoimmune (rheumatic) diseases.

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