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ORNITHINE DECARBOXYLASE, TRANSGLUTAMINASE, AND ACID PHOSPHATASE ACTIVITIES IN THE ACTIVATED MACROPHAGE

by

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ABSTRACT

Studies were undertaken to determine if the expression of transglutaminase (TGase) and ornithine decarboxylase (ODC) enzymes, coupled with expression of the lysosomal enzyme acid phosphatase (AcP), operates in the activation of macrophages. Murine monocytoma cell line P388D1, and Swiss Webster elicited peritoneal exudate cells (PEC) were stimulated in vitro with lipopolysaccharide (LPS).

In P388D1 cells, ODC activity was elevated, TGase activity was not significantly elevated, and AcP activity did not change upon LPS addition. Protein and RNA synthesis rates did not differ from control values. AcP activity was not affected by ODC inhibition.

PEC exhibited elevated TGase activity, but ODC activity was not detected. Protein synthesis proceeded as in controls, but RNA synthesis was reduced following LPS stimulation. AcP activity was decreased below controls at 4 h, and subsequently increased by 6 h.

Proliferating P388D1 cells and non-proliferating elicited PEC responded differently to LPS stimulus. P388D1 AcP and ODC activity are not linked.

INTRODUCTION

Peripheral blood monocytes as well as resident peritoneal macrophages are characteristically regarded as being cell types that are undifferentiated. This distinction is based upon morphologic and functional criteria. As immature cells differentiate in response to age and to environmental stimuli, they undergo changes and become morphologically more complex and functionally more active. Morphologic changes that have been recorded include increased cytoplasmic to nuclear ratio (Fedorko and Hirsch 1970), increased cell size (Cohn and Benson 1965a), increased membrane ruffling, expression of surface markers such as Fc receptors (Arend and Mannik 1973) and Ia antigens (Cowing, Schwartz and Dickler 1978), and increased number of intra-cellular organelles such as lysosomes and mitochrondria (Cohn and Benson 1965a). Functional changes include alterations in pathways of energy provision for endocytosis in response to environmental oxygen availability (Axline 1970), increased cytotoxicity against specific target tissues (Evans and Alexander 1976), increased activity of lysosomal enzymes (Cohn and Benson 1965a) and increased microbicidal activity (Mackaness 1970). Cohn and Benson (1965a) conducted classic studies in this field showing that differentiation of monocytes could be duplicated <u>in vitro</u> permitting detailed morophologic and biochemical investigations using homogeneous cell populations. Freshly explanted mouse peritoneal macrophages were

shown to resemble circulating blood monocytes. With prolonged incubation striking morphologic changes occurred which included increased number of mitochrondria, increased number of lipid droplets and progressive elevation of lysosomal enzyme activity such as in acid phosphatase (AcP), β-glucuronidase and cathepsin D. These data and autoradiographic evidence (Cohn, Fedorko and Hirsch 1966) of accumulation of newly formed protein in lysosomes suggested that synthesis of hydrolytic enzymes accompanied differentiation.

Mechanisms that regulate lysosome formation and replenishment are not well understood. What is known, however, is that lysosomes make up a complex intracellular organelle system in the macrophage that performs a number of functions including digestion (deDuve 1969) and microbicidal activity (Allen 1969). They are responsive to intracellular as well as extracellular environment. Pinocytosis and phagocytosis induce lysosomal enzyme synthesis (North 1970). Additionally, it is known that elicited macrophages have an enriched complement of lysosomes which contain acid hydrolases. These cells show increased ability to kill microorganisms relative to unstimulated macrophages. Little is known, however about basic mechanisms which control the induction of lysosomal enzyme production that accompanies differentiation of the macrophage.

Studies by Axline and Cohn (1970) have established a clear functional link between endocytosis and lysosome formation that accompanies differentiation. In the lysosome, acid phosphatase (EC 3.1.3.2)

is one of several hydrolases that have been used to identify the regulatory controls that promote enzyme induction (Cohn and Benson 1965b) and to follow the events of cellular differentiation (Cohn and Benson 1965a). The majority of AcP activity is reported to reside in the lysosomal fraction (Axline 1968) of macrophages. Since the lysosomal fraction contains an enriched source of AcP activity, AcP is classically used as a marker for these organelles. Recently, McCarthy, Musson and Henson (1982) have reported that during the early period of stimulation, selected macrophage poplulations were able to mobilize preformed intracellular enzymes, among them AcP, to the external environment. A key question, therefore, relevant to macrophage differentiation is which intracellular signals regulate lysosome formation.

This study evaluates the possibility that intracellular enzymes, ornithine decarboxylase (ODC) and transglutaminase (TGase) operate as regulatory enzymes in the steps leading to the differentiation of macrophages. These two intracellular enzymes, reported to function in concert with one another in growth and differentiation (Russell and Manen 1982), were used as markers to determine if correlations exist among LPS stimualation, changes in ribonucleic acid (RNA), and cellular protein. Changes in ODC and TGase activities, and activities of lysosomal enzymes in the macrophage were also studied. Cole and Axline (1983) report that purified lysosomal AcP from P388D1, a murine monocytoma cell line with macrophage-like characteristics (Koren 1975), and normal mouse peritoneal exudate cells (PEC) from the outbred Swiss Webster murine strain exhibit similiar physiochemical properties

and share common immunodeterminants, thus serving as satisfactory models. Additionally, it was important to determine if inhibition of either ODC or TGase would have an effect on lysosomal AcP, and thus, determine if any of these enzymes are critical to macrophage differentiation.

When exposed to a variety of inflammatory agents resting macrophages undergo a range of changes (North 1978) and are termed activated due to their enhanced metabolically active state. Studies with peritoneal mononuclear phagocytic cells have shown that environmental stimuli can potentiate differentiation. Morphologic, functional, and biochemical changes, many of which are reversible, are achieved in response to the stimuli (Axline 1970). Bacterial endotoxin, lipopolysaccharide (LPS), is reported to activate normal mouse peritoneal macrophages (Nozawa, Yanaki and Yokota, 1980), and to potentiate the differentiation process. A more recent report (Haefner-Cavaillon et al. 1982) suggests the presence of a lectin-like receptor for LPS on the surface of certain macrophages. Evidence of an LPS receptor implies an attachment phase in the pinocytic process (Bona 1973) that has been postulated to activate the macrophage via an increase in cellular cyclic adenosine-3'- 5'-monophosphate (Kikutani et al. 1981), a major route of induction of ornithine decarboxylase (Russell and Haddox 1979).

Ornithine decarboxylase (EC 4.1.1.17) is the rate limiting enzyme in polyamine biosynthesis and catalyzes the first step in the biosynthesis of putrescine by decarboxylation of ornithine utilizing

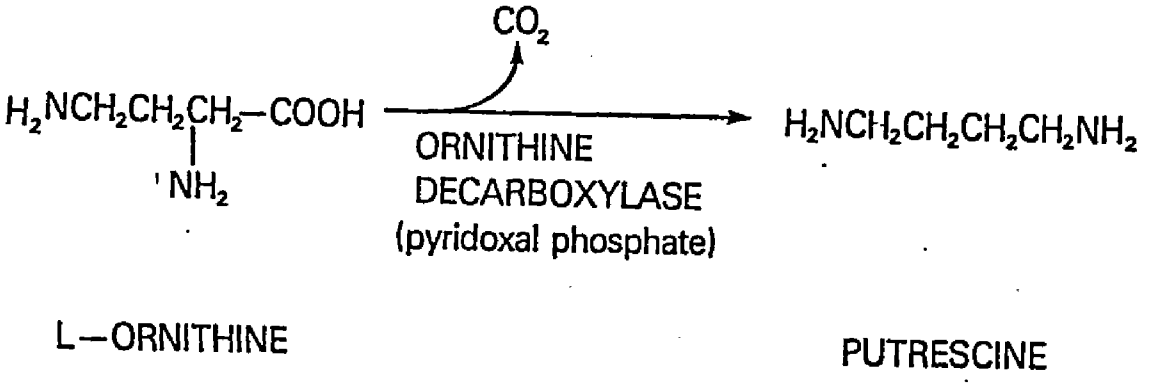

Figure 1: Schematic involvement of ODC in the metabolism of ornithine resulting in the production of putrescine and $^{14}CO_2$.

pyridoxal-5'-phosphate as a cofactor (Fig. 1). The mechanism by which regulation of ODC activity is brought about is not well understood (Canellakis et al. 1979). Increased levels of ODC are correlated with periods of rapid growth such as regeneration of rat liver, neoplastic proliferation of rat sarcoma, development of chick embryo (Russell and Synder 1968), chinese hamster ovary cells (CHO) (Haddox, Scott and Russell 1979), mouse lymphoma cells (Coffino 1981) and mouse fibroblasts (Weiss, Lembach and Boucek 1981). When specific inhibitors of ODC activity have been examined in proliferating systems such as CHO cells (Choi and Scheffler 1981), and Ehrlich ascites tumor cells (Oredsson, Anehus and Heby 1980) inhibition of ODC activity was associated with inhibition of cell replication.

The accumulation of both spermidine and ribosomal RNA are parallel in a variety of growth-stimulated tissues that have been studied (Russell 1980). Manen and Russell (1977) have correlated ODC increases with increases in RNA synthesis and have recently documented posttranslational modification of ODC activity to occur <u>in vitro</u> through transamidation via a putrescine moiety by a calcium-dependent enzyme, transglutaminase. This modified ODC-putrescine conjugate, when added to isolated rat liver nuclei, stoichiometrically increased RNA polymerase I activity (Fig. 2) (Russell and Manen 1982). An alternative method of ODC activity regulation has been documented by Kuehn and Atmar (1982). A polyamine-dependent protein kinase is thought to phosphorylate ODC which has been shown to bind to purified ribosomal deoxyribonucleic acid (rDNA) isolated from nucleoli. Phosphorylated ODC

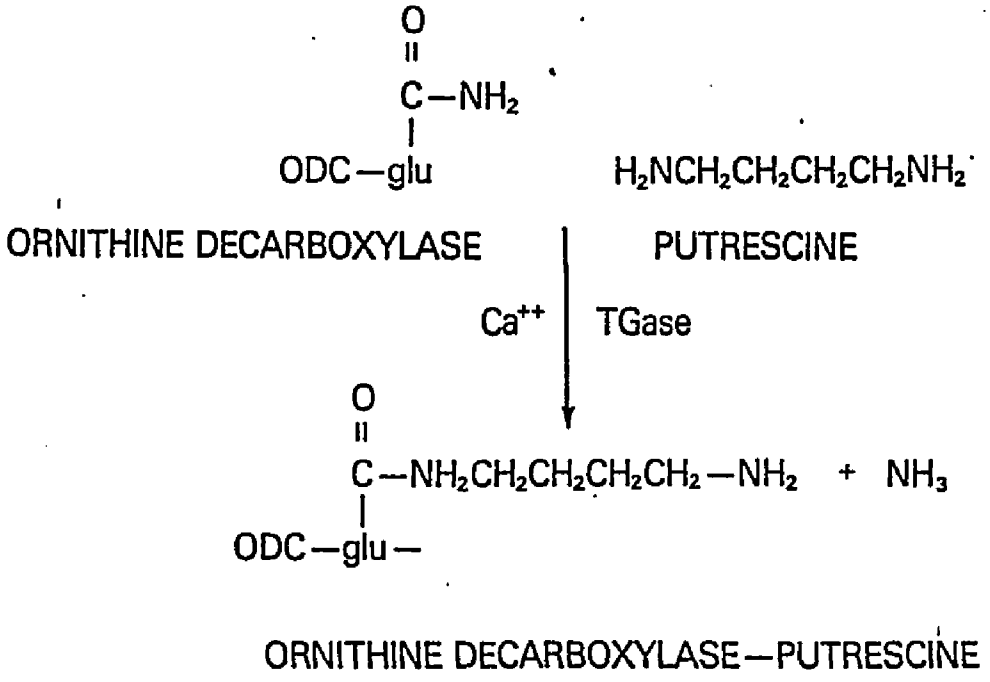

Figure 2: Posttranslational modification of ODC by TGase with putrescine.

then stimulates transcription of the ribosomal genes by RNA polymerase I in a chromatin form of rDNA.

A role for ODC in the macrophage has been postulated but not established conclusively. Nichols and Prosser (1980) have suggested that induction of ODC activity with LPS and immunoadjuvants directly stimulates some macrophage functions such as protein synthesis. They concluded that ODC induction promoted by adjuvants is one of the early biochemical changes in the activated macrophage. Studies were not included, however, to measure changes in AcP, RNA, and protein upon stimulation.

Transglutaminase (EC 2.3.3.13) is a calcium dependent enzyme reported to catalyze the acyl-transfer reaction in which γ - carboxamide groups of peptide-bound glutamine residues and primary amines serve as acyl doners and acceptors, respectively (glutaminyl-peptide γ - glutamyl transferases). Typical acyl-transfer reactions catalyzed by TGase are shown in Fig. 3. There are several types of TGases reported to be active <u>in vivo</u> (Folk 1980). One of these TGases catalyzes the production of covalent polymerization of fibrin during hemostatis and another TGase catalyzes formation of a vaginal plug as a result of post ejaculatory clotting of rodent seminal plasma. All act solely on peptide-bound glutamine residues. In contrast to their limited donor substrate specificity, differences among TGases are expressed in their broad specificity for acceptor substrates (Folk 1980). Shrode and Folk (1978) and later Folk et al. (1980) proposed that the polyamines spermidine and spermine serve as substrates for

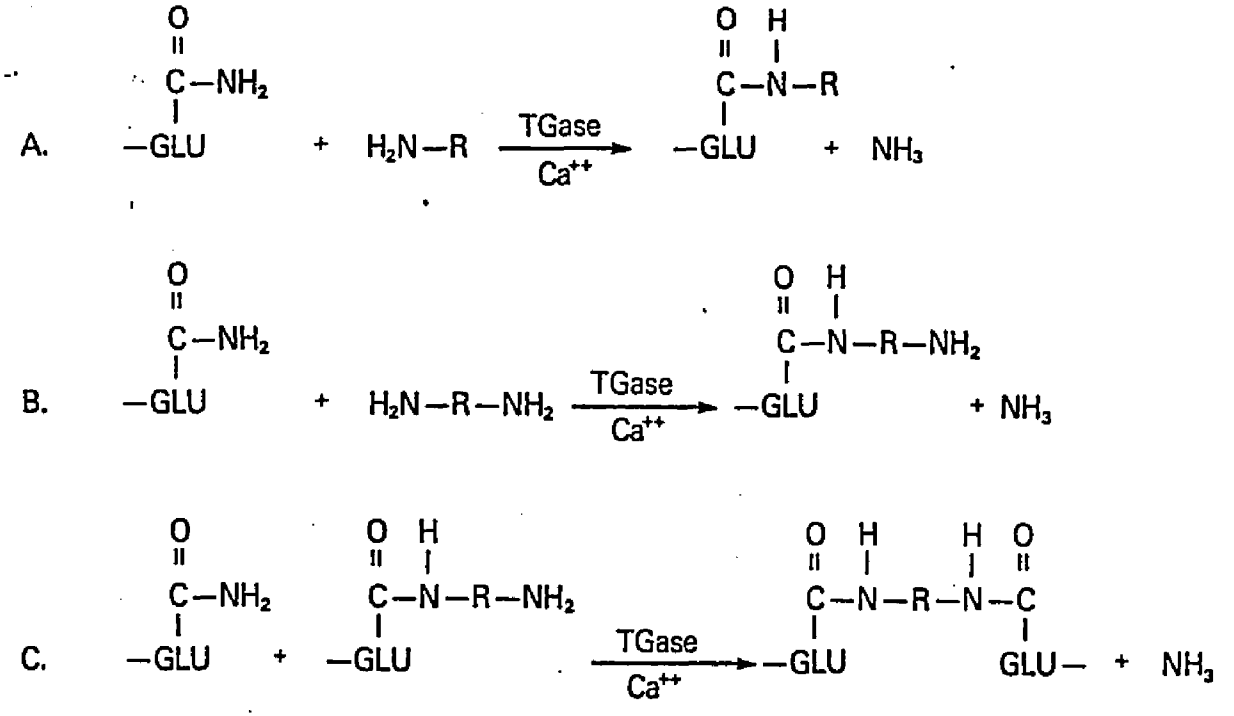

Figure 3: Schematic involvement of TGase in the formation of ε-(γ-glutamyl)-lysine between protein molecules and primary amines. Primary amines (a.), primary diamines (b.) and bifunctional diamines (c.) may participate in the coupling process to the γ-carboxyl residue of glutamine.

TGases in both stimulated human lymphocytes and extracellular secretions (fluid from the glandular lumens of rat seminal vesicles). They observed pronounced differences in the two systems. Few proteins of lymphocytes were conjugated with the polyamines while many of those of seminal secretions appeared to be involved in covalent attachment between protein and polyamine through γ-carboxamide linkage. They concluded that these differences between the cellular and extracellular substrate features of polyamines probably reflect separate physiological roles in the two systems for both the polyamines and their protein conjugates. Additionally, there could be many physiological events in which changes in TGase activity are associated but in which crosslinking through polyamines has not been measured. Among these events are lymphoctye blastogenesis (Novogrodsky et al. 1978) and receptor-mediated endocytosis in CHO cells (Davies et al. 1980; Levitski et al. 1980). It is possible that TGase may have additional roles in cells such as transmembrane signaling involving crosslinked receptor stimulation, or as a connection to cellular functional alterations as a result of the endocytic process.

TGase may play a role in macrophage function. Fésüs et al (1981) report that binding of soluble immune complexes or hemolysin-sensitized erythrocytes to the Fc receptor is followed by a rapid increase of TGase activity. Leu et al. (1982) report that inhibition of macrophage phagocytosis by TGase inhibitors may be due to effects on Fc receptor expression. Schroff, Neuman and Sorg (1981) have reported that normal murine peritoneal lavage cells and macrophage PEC exhibit

TGase activity which was assayed in single cells by fluorescence microscopy using dansylcadaverine as the substrate. Macrophage cell lines IC21, J774.2, and P388D1 were included in the study and all exhibited some degree of TGase activity. Kannagi et al. (1982) report transglutaminase activity in murine leukemia M1 cells induced to differentiate into mature macrophage-like M1+ cells upon addition of LPS. An enhancement of TGase activity was detected 12 hours after addition of LPS and the activity increased linearly for 4 days.

Evaluation of control mechanisms which operate in the proliferation and differentiation of macrophages offers a major challenge in understanding cellular regulatory processes. It would seem likely that enzymes such as ODC and TGase, which have been reported to be involved in the differentiation and proliferation of other cells such as fibroblasts and lymphocytes, would be active in the macrophage. This report is concerned with studies that were undertaken to measure induction of ODC and TGase activities following LPS stimulation at hourly intervals (0-6). Measurements of RNA and protein synthesis, required events prior to proliferation and differentiation in the P388D1 cell line and in PEC, were also addressed at hourly intervals following LPS stimulation. AcP activity was measured from 0-6 hourly intervals to observe expression of lysosomal enzyme activity relative to ODC and TGase changes. Finally, the use of α-difluoromethylornithine, a suicide inhibitor of ODC activity was used to determine if inhibition of ODC activity had an effect on expression of AcP activity.

MATERIALS AND METHODS

Macrophages

The continuous cell line, P388D1 has several macrophage characteristics and is a rich source of lysosomal AcP (Snyderman et al. 1977). Cells were grown as adherent monolayers in 75 cm^2 tissue culture flasks (Falcon Labware Div., Oxnard, CA) in a 5% CO_2 -95% air atmosphere at 37°C with Eagles minimum essential medium (MEM) (Irving Scientific, Santa Ana, CA) containing 10% heat inactivated fetal calf serum (FCS) (GIBCO, Grand Island, NY) and 200 units/ml penicillin G (Parke-Davis, Detroit, MI). Cultures were fed three times a week and passed weekly.

Cells from the peritoneal cavity of thioglycollate (Difco Laboratories, Detroit, MI) stimulated (Dy et al. 1978) male Swiss Webster mice, 28-30g (Simonson, Gilroy, CA) were harvested in heparinized phosphate-buffered saline (PBS), pH 7.4, according to the method of Cohn and Benson (1965a) with minor modifications. Cells were aliquoted in MEM containg 10% FCS (MEM-FCS) to each 60 mm tissue culture dish (Falcon) and incubated for 2 h at 37°C. The dishes were then washed twice in MEM to remove nonadherent cells and replenished with MEM-FCS.

Both P388D1 and PEC were challenged with nontoxic levels (25 ng/ml) of lipopolysaccharide (LPS) E. coli endotoxin strain 0127:B8; TCA extract, lot #91F-4015, Sigma, St. Louis, MO), reincubated, and harvested at sequential time intervals. Replicate cultures without LPS treatment were harvested as controls. Cell numbers were determined using a Coulter model Z_f (Coulter Electronics, Hialeah, FL).

Ornithine Decarboxylase Activity Assay

ODC activity in P388D1 cells and in PEC cultures was assayed using the method of Scott and Russell (1982). P388D1 cells were removed from tissue culture flasks by gentle scraping and aliquots ($4x10^6$ in 6 mls MEM-FCS) were distributed into 60 mm tissue culture dishes. Following incubation for 16-18 h, LPS (final concentration 25 ng/ml) in Hanks Balanced Salt Solution (HBSS) was added to the existing medium. PEC were harvested, resuspended, and aliquots ($20x10^6$ in 5 mls of MEM-FCS) were distributed into 60 mm tissue culture dishes and incubated for 2 h at which time monolayers were washed to remove non-adherent cells and LPS-containing media (final concentration 25 ng/ml) was added to the cells. Two plates of adherent PEC were combined to assay ODC activity.

At the time of harvest, the LPS containing tissue culture media was removed and discarded, and the macrophage monolayers were rinsed 3 times with PBS. LPS-treated and control cells were removed from the dishes by gentle scraping in 0.6 ml, 50 mM, $NaKPO_4$, pH 7.2, containing in final concentration 0.1 mM ethylenediaminetetracetic acid (EDTA), 2

mM dithiothreitol, 5 mM NaF, 0.04 mM phenylmethylsulfonylfluoride, and 0.01 mM pyridoxal phosphate, transferred to 1.5 ml polypropylene tubes and stored at -20°C until assayed.

A 200 µl aliquot of cell lysate (freeze-thawed) supernatant (10,000 x g, Beckman Microfuge B, Beckman Instruments, Fullerton, CA) from each sample was incubated for 30 min at 37°C in the presence of 5 mM L-[^{14}C] ornithine (0.05 mCi/ml, 54 mCi/mmol) (New England Nuclear, Boston, MA). The reaction was terminated by addition of 0.5 ml of 1 M citric acid, and evolved $^{14}CO_2$ was trapped on a Whatman No. 3mm filter paper prespotted with 20 µl 2 N NaOH suspended above the reaction in a center well (Kontes Co., Vineland, NJ). The filter paper was placed in 5 ml of Betaphase scintillant (West Chem Products, San Diego, CA) and radioactivity measured in a liquid scintillation counter (Beckman). Background radioactivity was determined in blank assays which did not contain the cell lysate and subtracted from the assays. A rat liver homogenate supernatant (10,000 x g) was included in all assays as a positive control. ODC activity was expressed as the amount of $^{14}CO_2$ released from 0.5 µCi ^{14}C-ornithine/min/mg supernatant cell protein.

Inhibition of ODC Activity

ODC activity was inhibited in P388D1 cells ($4x10^6$ in 6 mls of MEM-FCS) in 60 mm tissue culture dishes by the addition of 1 mM (final concentration) α-difluoromethylornithine (DFMO) (RMI 71,782,

Merrell Dow Pharmaceuticals Inc., Cincinnati, OH) to cells at the time LPS was added.

Transglutaminase Activity Assay

TGase activity in P388D1 and in PEC cultures was assayed by the method of Harris, Pabst and Johnston (1983), using a modified filter paper assay adapted from that outlined by Lorand, Campbell-Wilkes and Cooperstein (1972). P388D1 cells were removed from tissue culture flasks by gentle scraping. Cells ($4x10^6$ in 6 ml of MEM-FCS) were aliquoted into 60 mm tissue culture dishes. Following incubation for 16-18 h, LPS was added as described. PEC were harvested, resuspended, and aliquoted ($6.0x10^6$ in 5 ml MEM-FCS) to 60 mm dishes, incubated for 2 h, washed to remove nonadherent cells, and LPS in fresh media was added as described for ODC activity assays.

At the time of harvest, LPS-containing tissue culture medium was removed, and the macrophage monolayers were rinsed 3 times with PBS. LPS-treated and control cells were then removed from the dishes by gentle scraping in 0.6 ml, 60 mM Tris-HCl, pH 7.5, containing in final concentration 28 mM NaCl, 4.6 mM $CaCl_2$, 9.2 mM dithiothreitol, and 1.8 mg/ml dimethylcasein, transferred to polypropylene tubes and stored at -20°C until assayed. Replicate cultures were harvested in 0.6 mM Tris-HCl buffer that excluded the dimethylcasein so that cell lysate protein could be assayed.

A 200 µl freeze thawed aliquot consisting of 60 mM Tris-HCl, pH 7.5 buffer, 10 µM 1,4 [^{14}C]-putrescine (0.5 µCi, 107.1 mCi/

mmol) New England Nuclear), (Kannagi et al. 1982) and 40-50 µg cell protein was incubated for 60 min at 37°C, and terminated by the addition of an equal volume 10% trichloroacetic acid (TCA). A 25 µl aliquot was then spotted onto GF/A filters (Whatman Laboratory Products, Clifton, NJ) presoaked with 5% TCA. Filters were supported on a Hoefer Microanalysis Filter manifold (Hoefer Scientific Instruments, San Francisco, CA). Filter papers were washed with 5% TCA, dried, and radioactivity counted in 5 ml Betaphase scintillant as described previously. Background radioactivity was determined in blank assays and subtracted as described previously. Positive controls consisting of a 10,000 x g guinea pig liver homogenate supernatant fraction were included in all assays. TGase activity was expressed as the calcium dependent incorporation of ^{14}C-putrescine/min/mg acid precipitable protein.

Acid Phosphatase Activity Assay

AcP activity in P388D1 cells and in PEC cultures was assayed according to the method of Cole and Axline (1983). P388D1 cells ($4x10^6$ in 6 ml MEM-FCS) and PEC ($4x10^6$ in 5 ml MEM-FCS) were added to 60 mm culture dishes as described for the previous enzyme assays. Experiments were initiated upon addition of LPS (final concentration 25 ng/ml) and harvested at sequential time periods. After washing control and LPS-treated cultures free of medium, 0.6 ml of 0.1 M Citrate buffer, pH 5.3, containing 0.1% Triton X-100 (Bio-Rad, Richmond, CA) (Axline, 1968) was added to each tissue culture dish to lyse the cells.

Cells were removed by gentle scraping and transferred to polypropylene tubes. Cells were further subjected to 3 freeze-thaw cycles at -20°C and a 10,000 x g supernatant was assayed with 4-methyl-umbelliferyl-phosphate (Sigma) as the AcP enzyme substrate (Peters, Müller and deDuve, 1972). The enzyme is reported to hydrolyse the substrate to the products Pi and the fluorogen 4-methyl-umbelliferone (MUF). A 0.1 ml aliquot of cell lysate supernatant was incubated for 15 min with 0.1 ml of the 10 mM substrate prepared in 0.1 M Acetate buffer, pH 5.3. The reaction was stopped by the addition of 0.2 ml, 0.1 N NaOH-glycine buffer, pH 10.5. The liberated MUF was measured in a Varian SF-330 Spectrofluorometer (Varian Assoc. Instruments, Palo Alto, CA) equipped with a micro cell adapter with excitation wavelength at 358 nm and emission at 447 nm. Appropriate enzyme and substrate blank assays were performed. Quinine bisulfate (4 µg/ml) in 0.1 N H_2SO_4 was used as the calibrating primary standard (Mead, Smith and Williams 1955). A standard curve was prepared by aliquoting 0.01-0.2 µg/ml MUF in the appropriate amount of 0.1 M glycine buffer and measuring the amount of fluorescence. The readings were corrected for the primary standard and the value by the equation:

$$E_n = E_s - E_b \; / \; E_q$$

where E_s is the emission of the fluorescent sample, E_b is the blank and E_q is the quinine bisulfate. E_n was plotted verses µg MUF.

The standard curve was evaluated by linear regression. AcP enzyme activity is expressed as AcP/15 min/ml/mg cell protein.

Cellular Protein Determination

Protein quantitation for ODC, TGase, and AcP activities was determined by the method of Cole and Axline (1983), a fluorometric microassay adapted from that outlined by Böhlen et al. (1973). Fluorescamine interacts with the primary amino groups of proteins to yield highly fluorescent derivatives. Sample volumes contained approximately 5-50 µg of protein in a final volume of 0.5 ml, 0.05 M phosphate buffer, pH 8.0. Fluorescamine (Sigma) was dissolved in dioxane at a concentration of 0.3 mg/ml and composed 1/4 of the sample volume. Bovine serum albumin (BSA) (Pentax, Miles Laboratories, Inc., Kankakee, IL) (5-50 µg/ml) in 0.1 M PO_4 buffer, pH 8.0 was used in the standard curve. Quinine bisulfate was used as the primary standard. Samples were read in a Varian spectrofluorometer SF-330 at an excitation and emission wavelengths of 390 nm and 475 nm respectively. The readings were corrected as in the AcP enzyme assays. E_n was plotted versus µg protein, and evaluated by linear regression analysis.

RNA and Protein Synthesis Determinations

Cellular RNA was measured according to the method of Varesio et al. (1981). A 200 µl aliquot of P388D1 cells ($5x10^5$/ml) or PEC ($1.6x10^6$/ml) in MEM-5% PBS-dialyzed FCS was allowed to adhere in 96 well flat bottom tissue culture clusters (Costar, Cambridge, MA) for 2 h at 37°C. The medium was then aspirated from the wells, and the

monolayers were washed to remove nonadherent cells. Fresh medium was then added to the microcultures in a final volume of 0.2 ml. Cells were pulsed in triplicates for two hour intervals with ^{3}H-uridine (1 µCi/µl, 5.0 Ci/mmol) (New England Nuclear). At each 2 h time point, control and LPS-containing media were aspirated, and 25 µl of 7 M guanidine-HCl (Sigma) was added. The next group of triplicates was aspirated and fresh labeled medium added for 2 h. Plates were reincubated until all sequential time points were harvested. To measure the radioactivity, 50 µl of 1 µg/ml BSA and 0.1 ml 20% TCA at room temperature (RT) were added to each well. The plates were incubated for 10 min at RT and at 4°C overnight. TCA-insoluble material was collected on glass fiber filter paper (M.A. Bioproducts, Walkerville, MD) using a mini-MASH (Multiple Automated Sample Harvester, M.A. Bioproducts) cell culture harvester, washed with 5% TCA for 30 sec, and rinsed with 95% ethanol. The filters were dried and counted in Betaphase scintillant as described previously.

Cellular protein synthesis was measured as described for cellular RNA determinations with minor modifications. P388D1 ($5x10^{6}$/ml) cells or PEC ($1.6x10^{6}$/ml) were suspended in leucine deficient MEM (GIBCO)-5% PBS dialyzed FCS and aliquoted to the tissue cluster plates. Cells were pulsed with ^{3}H-leucine (1 µCi/µl, 25.5 Ci/mmol) (New England Nuclear), and harvested at 2 h time intervals as described earlier. Cell lysates were harvested as in the RNA determinations and radioactivity was measured.

Statistical Analysis

All enzyme activity determinations were reported as the mean activity/min/mg supernatant cell protein ± standard error (SEM) or standard deviation (SD) of duplicate samples per experiment. Experiments were performed at least 3 times, and one or more representative experiments are reported. Straight lines were generated for given sets of data points with a linear regression equation by the least mean squares method which provides values for the intercept, slope, and correlation coefficient. Statistical significance of differences was calculated by Student's t test. Statistical and linear evaluations were performed using an HP-97 (Hewlett Packard, Palo Alto, CA) programmable calculator.

RESULTS

LPS Effects on P388D1 Macrophages

Ornithine Decarboxylase Activity

ODC activity was measured in P388D1 cells and the results are shown in Fig. 4. The cells were incubated 16-18 h prior to addition of LPS to allow ODC activity increases resulting from addition of fresh serum to subside (Nichols and Prosser, 1980). The amount of ODC activity detected following LPS stimulation was contingent to a large extent upon the condition of the cells on a particular day. ODC activity above control levels fluctuated form experiment to experiment and is expressed as picomoles $^{14}CO_2$/min/mg protein. A representative experiment is shown in Fig. 4. ODC activity was observed to increase after LPS addition from 146 (0 h) up to a maximum 276 (4 h), (126 $^{14}CO_2$/min/mg protein above control at 4 h, $p < 0.01$). The increase in activity was observed to fluctuate upon continuous presence of LPS. A decrease to 189 (4.5 h), (60 $^{14}CO_2$/min/mg protein above control at 4.5 h, $p < 0.05$) in activity was observed, followed by an increase to 261 (6 h), (66 $^{14}CO_2$/min mg protein above control at 6 h, $p < 0.05$). ODC activity again subsequently decreased to control levels of 137 (7.5 h). To normalize the ODC activity, experimental activity was divided by control activity from 2 separate experiments

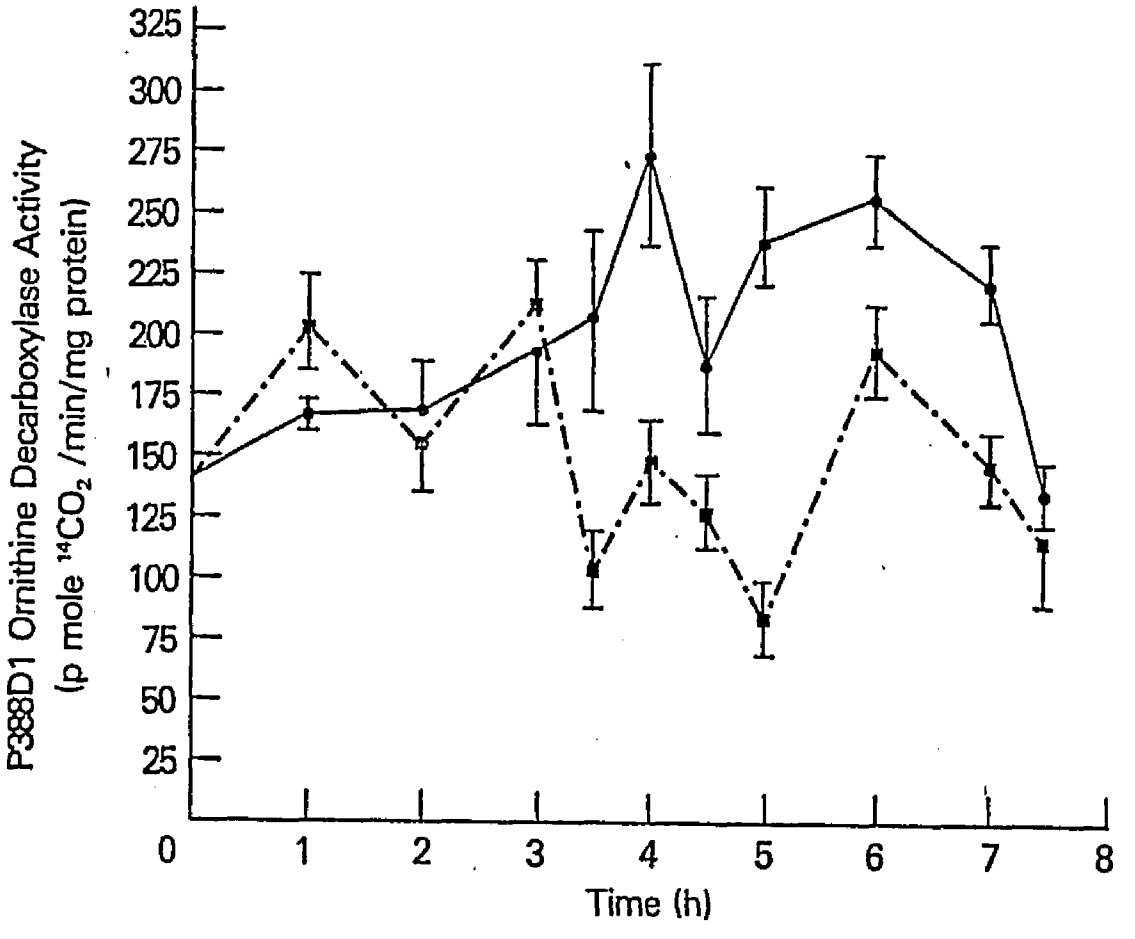

Figure 4: Stimulation of ODC activity in P388D1 macrophages by LPS. P388D1 cells ($4x10^6$ cells/60 mm tissue culture dish) were preincubated for 16-18 h prior to the addition of LPS (25 ng/ml). Control (■) and treated (•) cells were harvested at the times indicated and assayed for ODC activity. ODC enzyme activity is expressed as picomoles of $^{14}CO_2$/min/mg protein ± SD from a representative experiment performed in duplicate.

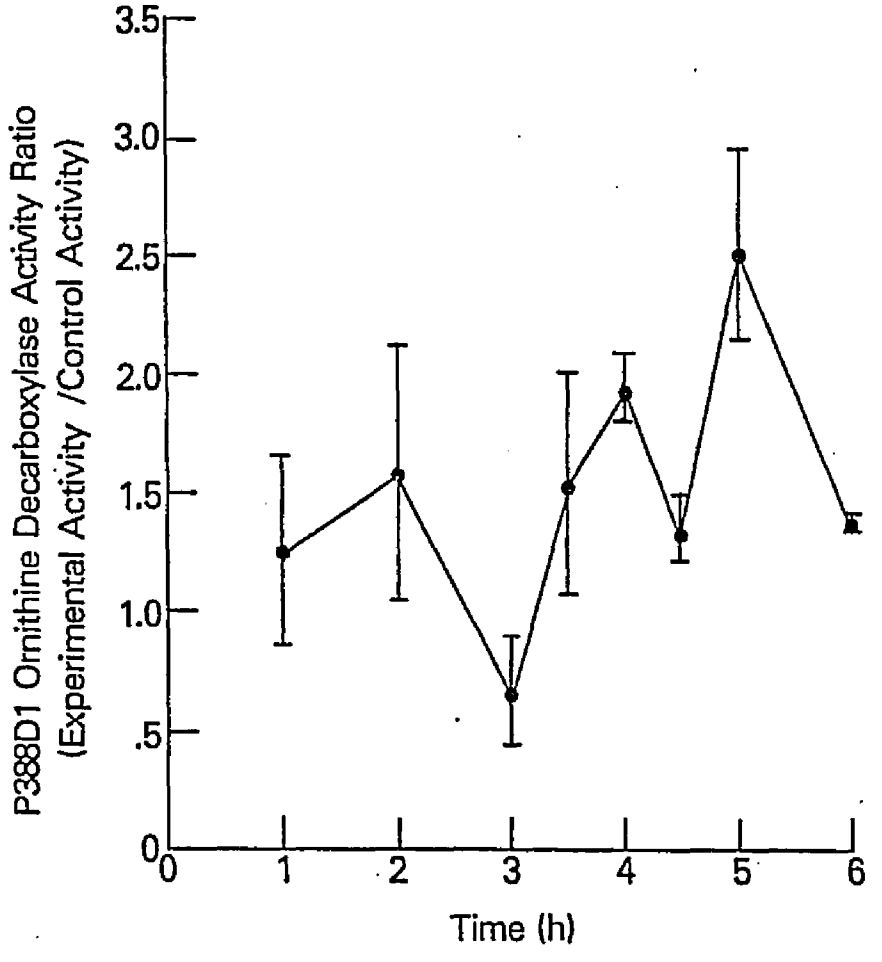

Figure 5: Normalized stimulation of ODC activity in P388D1 macrophages by LPS. P388D1 cells ($4x10^6$ cells/60 mm tissue culture dish) were preincubated 16-18 h prior to the addition of LPS (25 ng/ml). Control and treated cells were harvested at the times indicated and assayed for ODC activity. ODC enzyme activity is expressed as activity ratio ± SEM to normalize two separate experiments performed in duplicate.

and the results are shown in Fig. 5. ODC activity was observed to decrease after LPS addition from 1.26 (1 h) to 0.67 (3 h). ODC activity subsequently increased to 1.96 (4 h). Maximal increase occurred at 2.5 (5 h).

Transglutaminase Activity

TGase activity was measured in P388D1 cells and the results are shown in Fig. 6. Cells were incubated 16-18 h prior to addition of LPS. With continuous presence of LPS, the enzyme activity remained at or below control levels (900 picomoles ^{14}C-putrescine incorporated/ min/mg protein). Although a slight increase to 1.500 picomoles ^{14}C-putrescine incorporated/min/mg protein above control levels was observed at 6 h, these results suggest tht LPS concentration at nanogram levels may not be sufficient to stimulate existing levels of TGase activity.

Acid Phosphatase Activity

AcP activity was measured in P388D1 cells and the results are shown in Fig. 7. The cells were incubated 16-18 h prior to addition of LPS or LPS-DFMO. Control cells contained neither agent. With continuous presence of LPS or LPS-DFMO, enzyme activity remained at control leveles (27 AcP specific activity; units/mg protein). The enzyme was not inducible over the 6 h period. The continuous presence of the ODC inhibitor, DFMO, did not affect AcP activity.

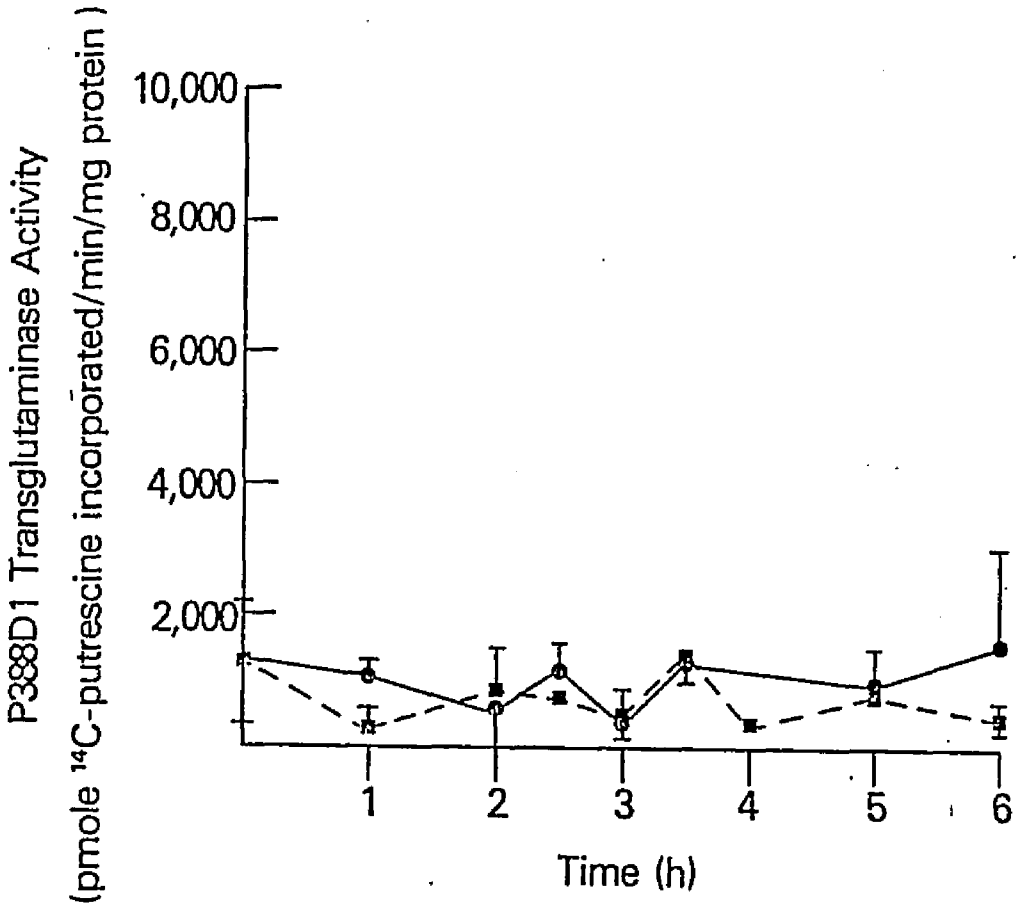

Figure 6: Effect of LPS on TGase activity in P388D1 macrophages. P388D1 cells ($6x10^6$ cells/60 mm tissue culture dish) were preincubated for 16-18 h prior to the addition of LPS (25 ng/ml). Control (■) and treated (●) cells were harvested at the times indicated and assayed for TGase activity. TGase activity is expressed as picomoles of ^{14}C-putrescine incorporated/min/mg protein ± SD from a representative experiment.

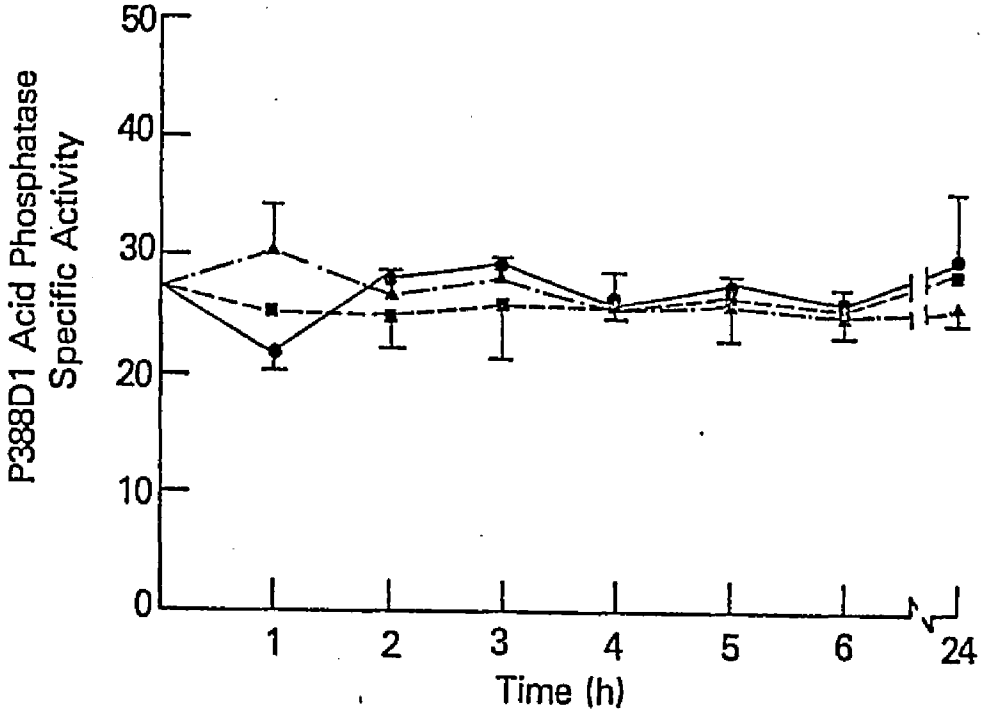

Figure 7: Effect of LPS on AcP activity in P388D1 macrophages. P388D1 cells ($4x10^6$/60 mm dish) were preincubated for 16-18 h prior to the addition of LPS (25 ng/ml) or LPS and 1 mM DFMO. Control (■), LPS treated (●), and LPS/ DFMO (▲) treated cells were harvested at the times indicated and assayed for AcP activity. AcP activity is expressed as specific activity ± SEM from three separate experiments.

Protein Synthesis

Protein synthesis was measured in P388D1 cells after a 2 h adherence period in leucine deficient medium and the results are shown in Fig. 8. Cells were pulsed with ^{3}H-leucine to measure protein synthesis at 2 h intervals over an 8 h period. LPS, 25 ng/ml, did not significantly affect protein synthesis. For example, upon addition and continued presence of LPS, protein synthesis continued to proceed at an active rate over the 8 h time period. Using the two hour pulse protocol, an almost constant rate of protein synthesis (3,300 cpm of TCA precipitable protein) occurred from 4-8 h. The lack in differences observed between control and LPS-treated cells is similiar to what has been reported by Varesio and Eva (1980).

Ribonucleic Acid Synthesis

RNA synthesis was measured in P388D1 cells, and the results are shown in Fig. 9. After a 2 h adherence period in MEM-FCS, cells were pulsed at 2 h intervals with ^{3}H-uridine. RNA synthesis was measured over an 8 h period. When compared to controls, the rate of synthesis was that of control levels (200 cpm). LPS induced effects were minimal during the observed time intervals.

LPS Effects on Peritoneal Elicited Macrophages

Ornithine Decarboxylase Activity

ODC was measured in Swiss Webster PEC. Thioglycollate was injected into mice in order to elicit a greater number of cells into

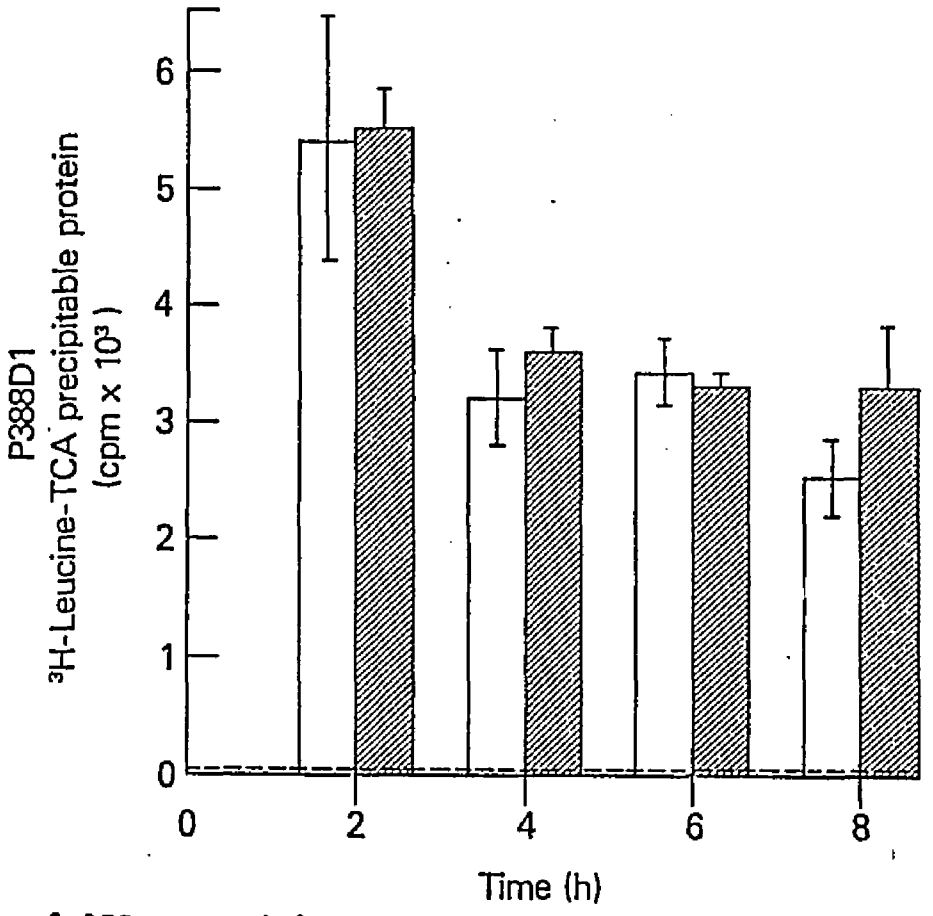

Figure 8: Effects of LPS on Protein synthesis in P388D1 macrophages. P388D1 cells were scraped and aliquoted into 96 well microtiter tissue culture plates ($1x10^5$ cells/well) and allowed to adhere 2 h in leucine deficient medium prior to addition of ^{3}H-leucine supplemented medium. The cells were lysed after 2 h pulse intervals by guanidineTCA treatment. The results are expressed as CPM x 10^3 ± SD of TCA insoluable material over an 8 h period from a represented experiment performed in triplicate. Control cells are indicated by open bars an LPS (25 ng/ml) treated cells by crosshatched bars. Dashed lines indicate background activity.

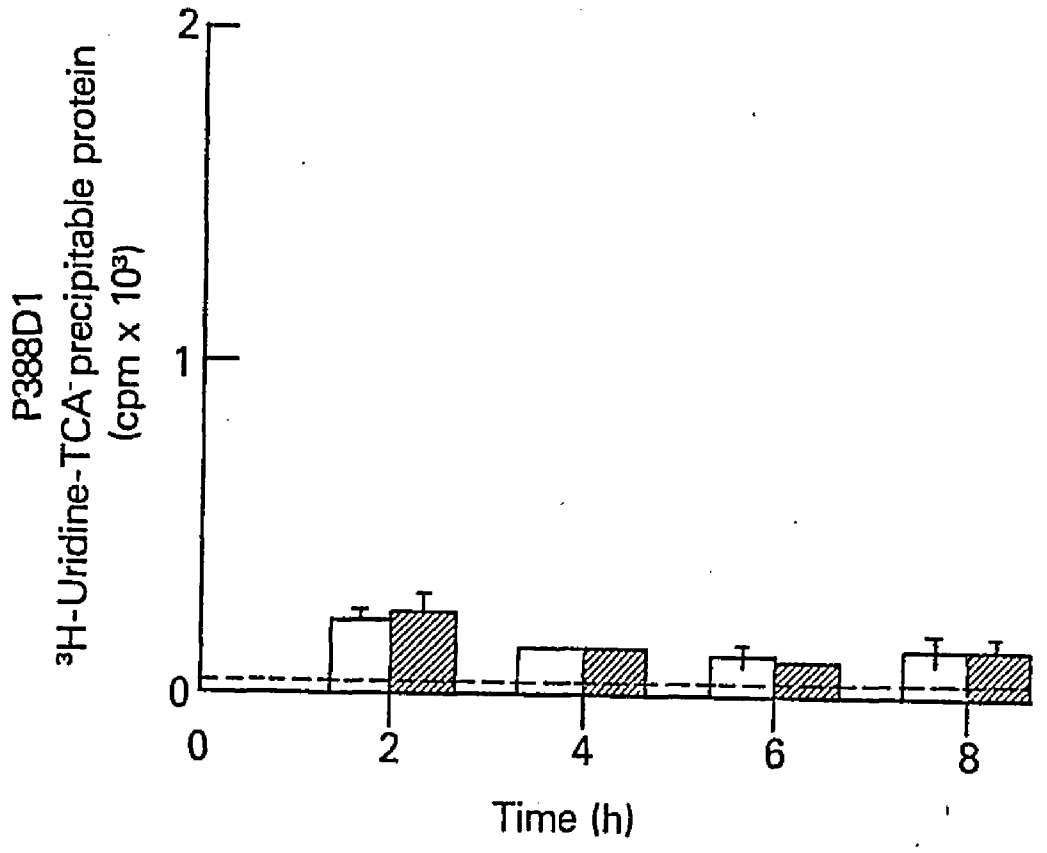

Figure 9: Effects of LPS on RNA synthesis in P388D1 macrophages. P388D1 cells were scraped and aliquoted into 96 well microtiter tissue culture plates ($1x10^5$ cells/well) and allowed to adhere 2 h prior to addition of ^{3}H-uridine supplemented medium. The cells lysed after 2 h pulse intervals by guanidine-TCA treatment. The results are expressed as CPM x 10^3 ± SD to TCA insoluble material over an 8 h period from a represented experiment performed in triplicate. Control cells are indicated by open bars and LPS (25 ng/ml) treated cells by crosshatched bars. Error bars are not inclued at points were SD < 10 CPM. Dashed lines indicate background activity.

the peritoneal cavity (Dy et al. 1978). Additionally, peritoneal cells are reported to have higher levels of AcP when inducing agents are used (Goodrum and Spitznagel 1982). PEC were harvested and allowed to adhere 2 h prior to addition of LPS. Despite numerous attempts, ODC activity was not measurable under the conditions used in the study. It is possible that a more sensitive assay is necessary to measure ODC activity in cells from Swiss Webster mice. Increasing the cell density from $2x10^7$/60 mm dish to $4x10^7$/35 mm dish was not sufficient to detect ODC activity.

Transglutaminase Activity

TGase activity was measured in Swiss Webster PEC and the results are shown in Fig. 10. Thioglycollate was injected into the peritoneal cavity to elicit a greater number of cells prior to harvest. PEC were harvested and allowed to adhere 2 h prior to addition of LPS. TGase was observed to increase 2.3 fold above control levels at 3 h. Both control and LPS-stimulated TGase activity continued to increase during the remaining time intervals. Maximum induction occurred at the 6 h time point when LPS-stimulated TGase activity was 1.6 fold above control levels (3,100 picomoles ^{14}C-putrescine incorporated/min/mg protein above control at 6 h, $p < 0.05$).

Acid Phosphatase Activity

AcP activity was measured in Swiss Webster PEC and the results are shown in Fig. 11. Thioglycollate was injected into the peritoneal cavity 3 days prior to cell harvest. It is also known to influence

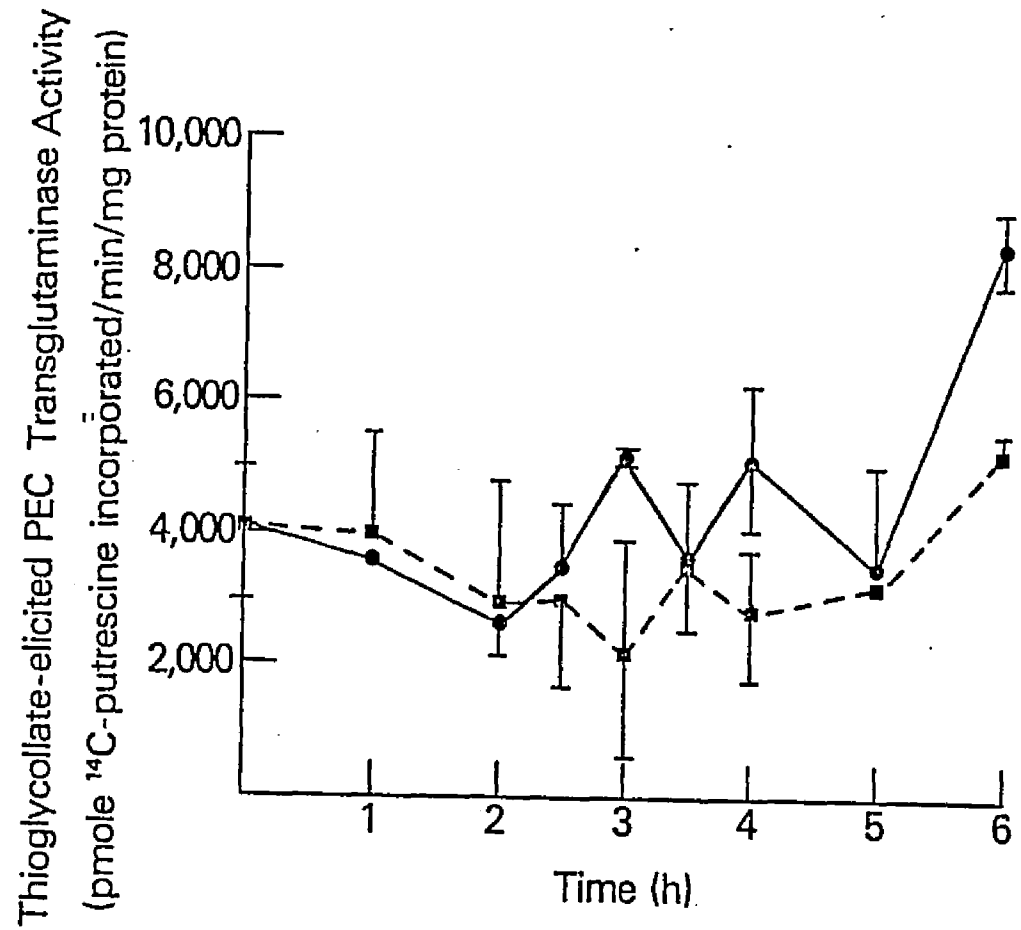

Figure 10: Effect of LPS on TGase activity in PEC macrophages. PEC were harvested from Swiss Webster male mice 3 days after an intraperitoneal injecton of 3% thioglycollate. Cells were washed, resuspended and allowed to adhere ($6x10^6$ cells/60 mm dish) 2 h prior to the addition of LPS (25 ng/ml). Control (■) and treated (●) cells were harvested and assayed for TGase activity. TGase activity is expressed as picomoles of ^{14}C-putrescine incorporated/ min/mg protein ± SD from a representative experiment.

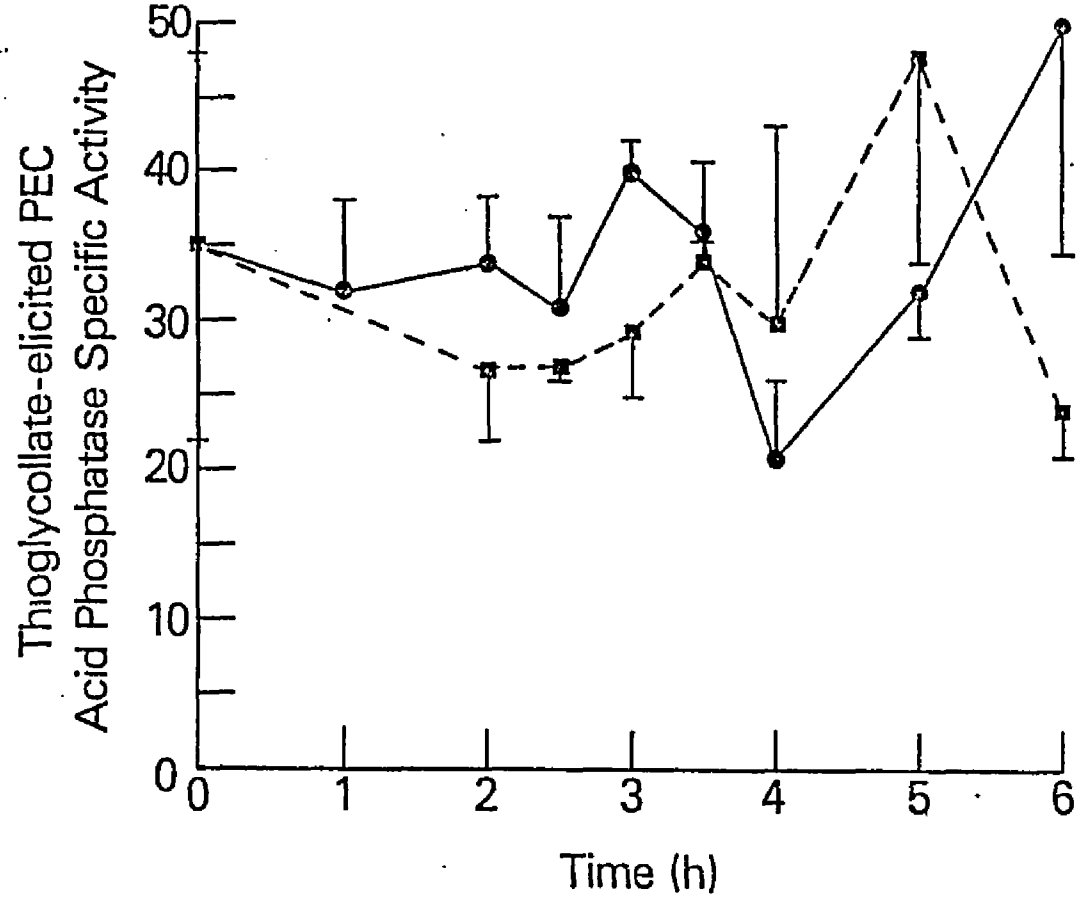

Figure 11: Effect of LPS on AcP activity in PEC macrophages. PEC were harvested from Swiss Webster male mice 3 days after an intraperitoneal injection of 3% thioglycollate. Cells were washed, resuspended and allowed to adhere ($4x10^6$ cells/60 mm dish) 2 h prior to the addition of LPS (25 ng/ml). Control (■) and treated (●) cells were harvested and assayed for AcP activity. AcP activity is expressed as specific activity ± SEM from two separate experiments.

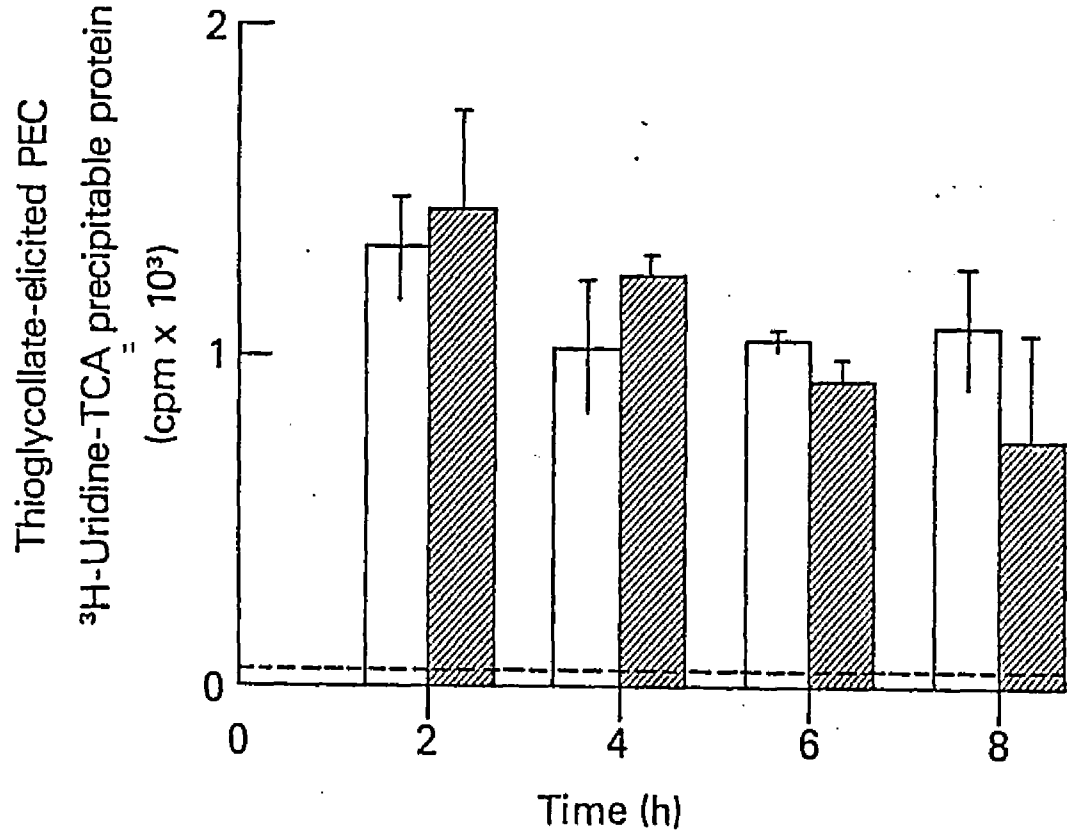

Figure 12: Effects of LPS on Protein synthesis in PEC macrophages. PEC cells were scraped and aliquoted into 96 well microtiter tissue culture plates ($3.2x10^5$ cells/well) and allowed to adhere 2 h in leucine deficient medium prior to addition of ^{3}H-leucine supplemented medium. The cells were lysed after 2 h pulse intervals by guanidine-TCA insoluble material over an 8 h period from a represented experiment performed in triplicate. Control cells are indicated by open bars and LPS (25 ng/ml) treated cells by crosshatched bars. Dashed lines indicate background activity.

existing levels of AcP activity. Upon continuous presence of LPS, the enzyme activity remained at control levels until 4 h when it was observed to decrease below control levels. AcP activity subsequently increased 2 fold above control levels by th 6 h time point.

Protein Synthesis

Protein synthesis was measured in PEC after a 2 h adherence period in leucine deficient medium as shown in Fig. 12. Cells were pulsed with ^{3}H-leucine to measure protein synthesis at 2 h intervals over an 8 h period. LPS did not significantly affect protein synthesis. When compared to controls, protein synthesis in LPS-treated cells continued to proceed at an active rate over the 8 h time period. Using the 2 h pulse protocol, a small decrease (7%) from that of control levels was observed at 4, 6, and 8 h. control and LPS-treated cells did not differ in amount of ^{3}H-leucine incorporation into acid precipitable protein similar to what has been reported by Varesio, Thurman and Taramelli (1981).

Ribonucleic Acid Synthesis

RNA synthesis was measured in PEC after a 2 h adherence period and the results are shown in Fig. 13. Cells were pulsed at 2 h intervals with ^{3}H-uridine to measure RNA synthesis over an 8 h time period with continuous presence of LPS, control and treated cells together exhibited declining rates of RNA synthesis. At 6 h, RNA synthesis was reduced 11% ($p < 0.05$). At the 8 h time point, RNA synthesis was

reduced 30% ($p < 0.05$) compared to controls. Varesio, Thurman and Taramelli (1981) have reported similiar findings.

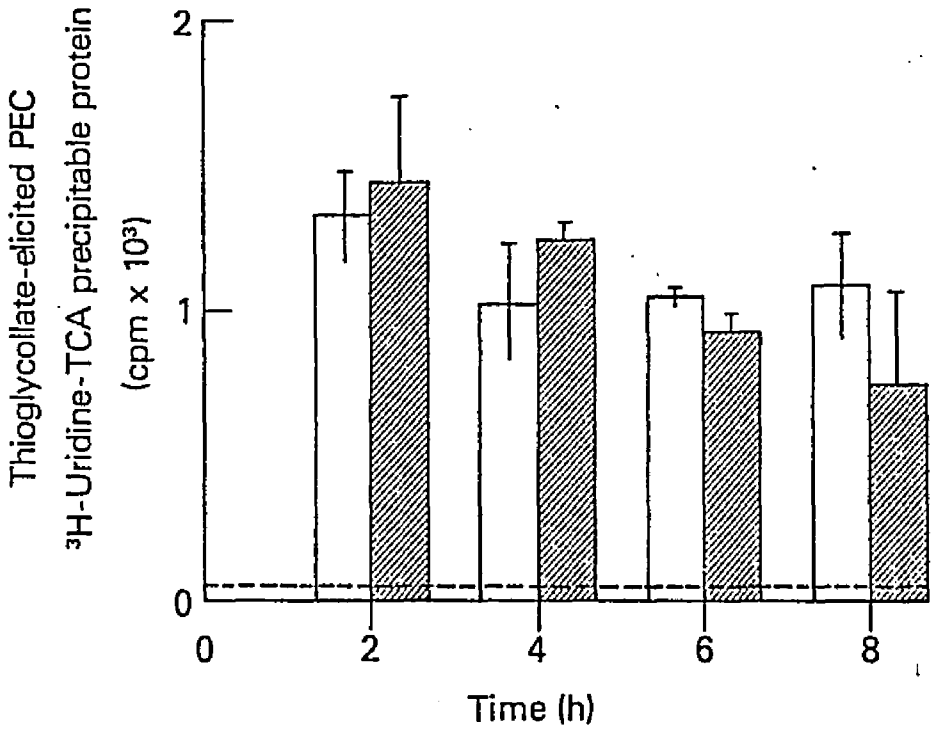

Figure 13: Effects of LPS on RNA synthesis in PEC macrophages. PEC cells were scraped and aliquoted into 96 well microtiter tissue culture plates ($3.2x10^5$ cells/well) and allowed to adhere 2 h prior to addition of ^{3}H-uridine supplemented medium. The cells were lysed after 2 h pulse intervals by guanidine-TCA treatment. The results are expressed as CPM x 10^3 ± SD of TCA insoluble material over an 8 h period from a represented experiment performed in triplicate. Control cells are indicated by open bars and LPS (25 ng/ml) treated cells by corsshatched bars. Dashed lines indicate background activity.

DISCUSSION

The activities of ODC and TGase in the macrophage have not been previously measured together in response to environmental stimuli. AcP activity, protein synthesis, and RNA have been extensively studied in the macrophage (Cohn and Benson 1965a, b, c; Morland 1979; Varesio and Eva 1980; Varesio et al. 1981) and reported to increase with activation and cellular differentiation (Axline and Cohn 1970; Axline 1970; Morland and Kapland 1977). In this study, macrophage activation, as defined by increased ODC and TGase activities, was achieved with LPS stimulus. LPS has been reported by Nozawa, Yanaki and Yokota (1980) to induce marked morphological changes in murine macrophages. The cells assume a flat shaped appearance with a ruffled plasma membrane. The authors also report that LPS-treated (10 µg/ml) murine macrophages have an enhanced antifungal activity. In addition, murine macrophages can be activated to develop cytotoxicity against P815 mastocytoma tumor cells with 10 µg/ml LPS as has been reported by Schultz (1982). It is likely that the transient ODC and TGase activity changes differ in response to the normal and transformed (tumor) state of the macrophage as they do in other cell systems (Cress and Gerner 1980; Weiss, Lembach and Boucek 1981; Birckbichler et al. 1977). The presence of these two additional enzymes following endocytosis, therefore, may be a necessary

signal either required for or indicative of early activation in the marcophage.

It is evident from the results of this study that macrophage continuous cell line, P388D1, and thioglycollate-elicited PEC respond differently to LPS stimulus. In the P388D1 cells, ODC activity was detected within 2 h after addition of LPS. TGase activity, on the other hand, was only silghtly elevated by 6 h. No discernable increase above control levels in AcP activity was observed even at 24 h after LPS addition. It may be that higher concentrations of LPS such as those used by Schultz (1982) in the 10 μg/ml range are necessary to induce AcP activity. However, LPS then becomes toxic for cell growth (Nozawa, Yanaki and Yokota 1980). Upon addition of LPS, AcP activity in the P388D1 cells did not seem affected by the continuous presence of the ODC inhibitor. Protein synthesis was quite active and unaffected by LPS. With continuous presence of LPS, RNA synthesis was the same as control at 8 h.

ODC activity in the P388D1 cells did not exhibit the continuous increase and gradual decline reported by Nichols and Prosser (1980) for the J774.1 macrophage tumor cells even though the assay procedures were identical. The response observed in two separate studies was reproducible. It could have been due to the fact that ODC activity was assayed at half hour intervals at which activity was observed to decrease. Nichols and Prosser report only hour intervals. Ralph et al (1978) report that the macrophage cell lines J774.1 and P388D1 differ in sensitivity to inducing agents, presumably because of differences in

quantity or affinity of specific receptors for each kind of activating molecule. Ralph and Nokoinz (1977) have reported that LPS at 100 ng/ml inhibits the growth of the monocyte-macrophage line J774.1 but not P388D1. It is also possible that the observed decreases in ODC activity is attributable to posttranslational modificaton of ODC through the action of TGase as has been reported by Russell and Manen (1982). Such a role in the macrophage could be plausible and requires further study.

The presence of 1 mM DFMO, the reported irreversible inhibitor of ODC (Merrell Dow Pharmaceuticals Inc., Investigational Brochure 1983), did not affect lysosomal AcP activity upon addition of LPS. therefore, inhibition of ODC is not related to AcP activity, which remains constant for 24 h.

TGase activity was not significantly inducible in the P388D1 cell line. The existing low levels detected by the macrophage assay protocol provided by Harris, Pabst and Johnston (1983) (personal communication) possibly play some physiological role in these cells which remains to be elucidated.

Under the conditions employed in this study, thioglycollate-elicited PEC did not exhibit measurable ODC activity. TGase activity was elevated at 3 h after LPS stimulation. AcP lysosomal enzyme decreased at 4 h followed by a gradual increase above control levels at 6 h. As in the P388D1 cells, protein synthesis was not significantly affected by the LPS addition to the medium. RNA synthesis was observed to be depressed at 8 h in the continuous presence of LPS.

The lack of detectable ODC activity in the thioglycollate-elicited PEC might be explained from the differences in either mouse strain or LPS type compared with those used in previous reports. Nichols and Prosser (1980) reported ODC activity in PEC obtained from C3H/HeN mice using the identical assay. It was observed to be 30 fold less than that observed in the J774.1 continuous cell line. In addition, Lee, Wong and McIntyre (1981) report that within the cells obtained from a stimulated murine peritoneal cavity, PEC, there are subpopulations which can be activated by LPS. Their results indicate that the capacity to respond to activation increases with cell size which reflects the state of maturation or activation. Since elicited PEC exist in a continuum of activated states, it may be that in this study there were not sufficient subpopulations capable of responding to the LPS stimulation in vitro in the Swiss Webster strain, thus accounting for the lack of measurable ODC activity. It is also possible that since elicited macrophages have elevated total cell protein as compared to non-elicited cells (Goodrum and Spitznagel 1982), ODC enzyme levels may have been transiently induced in the cells prior to removal from the peritoneal cavity.

LPS-stimulated thioglycollate-elicited PEC exhibited higher initial levels of TGase activity. The activity was observed to increase over time upon LPS stimulation. Birckbichler et al. (1977) report that TGase activity increases only slightly during the rapid proliferative phase in WI-38 embryonic lung cells. However, enzyme activity increases and continues to increase as the majority of the

cells enter a non-proliferative state. It may be that the higher TGase activity measured in PEC is due to their non-proliferative state. An alternative explanation can be deduced from the data from Leu et al. (1982) that reveal that resident guinea pig peritoneal macrophages have very low levels of TGase activity, whereas oil-induced guinea pig macrophages have higher levels of activity. Furthermore, they report a 3-fold increase in TGase activity when guinea pig PEC are cultured for 18 h with LPS. Time points between 0 h to 18 h, however, were not reported as in this study. They conclude that TGase may have a role in macrophage activation. Additionally, since TGase activation is involved in the Fc-mediated binding event as reported by Fésüs et al. (1981) and Leu et al. (1982) in guinea pig oil-elicited macrophages, it was detectable in this study in thioglycollate-elicited PEC following an LPS-pinocytic event.

In PEC, lysosomal AcP decreased to a low at 4 h and began to increase at 5 h following LPS stimulation. The decrease in activity at 4 h could possibly be attributed to release into the medium of pre-formed granule constituents (McCarthy, Musson and Henson 1982). Maximum increases of AcP activity are reported to occur in 24-72 h as reported by Cohn and Benson, (1965a) when the enzyme, stimulated with 10 mg/ml LPS, continues to increase dramatically at a rapid rate. It is known that lysosomes develop with progressive increases in cell size and protein content during activation (Axline and Cohn 1970).

In PEC, protein synthesis proceeded at the same rate as controls upon LPS addition. Using the 2 h pulse protocol, the cells

exhibited a slight lag in the protein synthesis rate at 4 h, but reached control rates again at 8 h as described by Varesio, Thurman and Taramelli (1981).

In contrast to that observed with protein synthesis, RNA synthesis was depressed upon addition of LPS. LPS-induced effects in PEC were observed within 6 h after LPS addition, and were decreased further at 8 h. Varesio and Kowall (1982) report that endotoxin-activated PEC macrophages exhibit depressed RNA synthesis due to inhibition of the 28s ribosomal RNA, which may account for the observed results.

The present study which deals with examining the changes in ODC and TGase activities with respect to lysosomal AcP enzyme activity adds to information available in that it provides evidence of ODC and TGase activities participating in an early activation process of the macrophage. P388D1 cells and thioglycollate-elicited PEC were observed to respond differently in ODC and TGase activities. It is possible that these two enzymes have different roles in proliferating cells (P388D1) than in non-proliferating cells (PEC) as has been described for ODC in other cell systems (Luk et al. 1982).

To date, many questions remain unanswered regarding the mechanisms and stimuli which evoke biochemical and morphological phenomena in the macrophage differentiation process. The data in this study indicate that levels of macrophage enzymes are governed by LPS stimulus. It cannot be assumed however, that the presence of ODC and TGase activities automatically indicate that other manifestations of macrophage activation are present. It is possible that activation may

occur in a series of steps, and that ODC and TGase activities are part of the early activation process. Thus, there would have to be an interaction between media constituents (LPS), pinocytosis, cytoplasmic, and lysosomal constituents (represented by AcP). A clear answer is, as yet, not available, but it can be assumed that the sequential development of ODC, TGase, and AcP enzymes concomittant with protein and RNA synthesis leads to a mature or activated macrophage. The conversion of a monocyte-like cell to a mature macrophage, therefore, is accompanied by the activation of either ODC or TGase enzymes prior to lysosome formation. Further studies would be necessary to define whether these two enzymes make up a small part of the many complex biochemical signals communicating from the external to the internal environment that may be required for further acquisition of metabolic and functional changes which result in a fully differentiated or activated macrophage.

In summary, it is evident from this study that both cytoplasmic enzmes, ODC and TGase, have some connection with the process of macrophage maturation. In the proliferating P388D1 cells, ODC activity was observed to be quite active early after LPS addition. TGase, although present, does not seem to be significantly elevated in order for them to work in concert with one another. Non-proliferating thioglycollate-elicited PEC, on the other hand, exhibited elevated TGase activity after LPS addition, but measurable levels of ODC activity were not detected. In both cell types protein and RNA syntesis was typical as previously described (Varesio et al. 1981; Varesio, Thurman and Taramelli 1981). Lysosomal enzyme activity did not significantly

increase after stimulation for 6 h in the P388D1 cells, yet it had begun a gradual increase above control levels at 6 h in PEC. In the P388D1 cells, AcP activity was not affected by ODC inhibition, thus the two events do not seem connected. Finally, since the two cell types differed in response to LPS stimulation, each cell type, the proliferating P388D1 cell line and the non-proliferating PEC, have provided information that ODC and TGase behave independently of each other in an inverse relationship. Both cell types have served to expand the current knowledge by which two more controlling factors connect with macrophage differentiation into its activated state.

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