

**REACTIVE OXYGEN PRODUCTION BY CULTURED RAT
GLOMERULAR MESANGIAL CELLS DURING
PHAGOCYTOSIS IS ASSOCIATED WITH STIMULATION OF
LIPOXYGENASE ACTIVITY***

BY LAURENT BAUD, JACQUELINE HAGEGE, JOSÉE SRAER,
ERIC RONDEAU, JOELLE PÉREZ, AND RAYMOND ARDAILLOU

From the INSERM Research Unit 64, Hôpital Tenon, Paris, France

The glomerular mesangium is a supporting structure that includes the mesangial cells and an intercellular substance, the mesangial matrix. Mesangial cells are stellate in shape and resemble morphologically smooth muscle cells (1, 2). They contract in response to binding of angiotensin II (3, 4) and vasopressin (4). They also produce prostaglandins (PG)¹ (5) and synthesize the mesangial matrix (6). In addition to these contractile and biosynthetic properties, phagocytic function has been ascribed to mesangial cells, but the data regarding this point are controversial. Following their intravenous administration to rats, macromolecules such as thorium dioxide, colloidal gold, and ferritin (7) are found very rapidly within the mesangial cells. However, other materials, particularly antigen-antibody complexes, have been observed only in the mesangial matrix and very rarely within the cells (8), and Striker et al. (9) demonstrated that only the marrow-derived monocytes present within the mesangium were able to phagocytize the immune complexes. Recently, using heat-aggregated antiperoxidase immunoglobulins (Ig), Mancilla-Jimenez et al. (10) showed that mesangial cells could incorporate this material following its *in vivo* administration. They concluded that mesangial cells possess a vacuolar apparatus capable of ingesting heat-aggregated Ig. It was also shown in *in vitro* studies that cultured mesangial cells did not phagocytize latex particles (11), and did not possess C3 and IgG Fc-binding sites (12). These results contradict the previous findings of Camazine et al. (13) who, 18–24 h after isolation, obtained a population of glomerular cells, probably comprising mainly mesangial cells, which were highly phagocytic for opsonized zymosan particles and had the potential to develop Fc and C3 receptors. Schreiner et al. (14) have concluded that the phagocytic ability belongs to a well-defined fraction of the mesangial cells bearing Ia determinants. Only these cells, which represented a very small percentage of the total glomerular cell population, were able to phagocytose latex beads *in vitro* or heat-aggregated

* Supported by grants from the Institut National de la Santé et de la Recherche Médicale and from the Faculté de Médecine Saint Antoine.

¹ Abbreviations used in this paper: ETYA, eicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroxyperoxy intermediates of arachidonic acid; HPLC, high performance liquid chromatography; Ig, immunoglobulin; KRP, Krebs-Ringer phosphate; NDGA, nordihydroguaiaretic acid; PAS, periodic acid-Schiff reagent; PG, prostaglandins; STZ, serum-treated zymosan.

gamma globulins injected in vivo.

During the process of phagocytosis, neutrophils, monocytes, and macrophages release oxygen metabolites, PG, via the cyclooxygenase pathway and hydroxyeicosatetraenoic acids (HETE) via the lipoxygenase pathway (15). The present study was undertaken to determine whether well-characterized mesangial cells in culture have phagocytic capability, and, if so, whether such phagocytosis is associated with an oxidative burst and an alteration in arachidonic acid metabolism. Our results indicate that well-characterized cultured mesangial cells can phagocytose opsonized particles and that such phagocytic activity results in an oxidative burst with generation of superoxide anion and hydrogen peroxide, and in the stimulation of lipoxygenase activity.

Materials and Methods

Cell Cultures. Cultured glomerular mesangial and epithelial cells were obtained from primary cultures of glomeruli isolated from rat renal cortex according to the method of Foidart et al. (12). Glomeruli were prepared from the renal cortices of Sprague-Dawley rats weighing 150–220 g by sieving techniques and differential centrifugations (16). The final preparation was checked for purity by light microscopy. It contained decapsulated glomeruli with virtually no afferent or efferent arterioles. Isolated glomeruli were then cultured at 37°C in plastic flasks containing 5 ml of RPMI medium (Flow Laboratories, Irvine, UK) supplemented with 10% decomplexed fetal bovine serum and buffered with 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2 ethanesulphonic acid (Hepes), pH 7.2. Under such conditions, epithelial cells grow rapidly with a peak of cell division on day 4, whereas mesangial cells grow at a slower rate with a maximum of cell division on day 21. Studies utilizing epithelial cells were performed on day 4 at which time mesangial cells are present in negligible amount. Studies with mesangial cells were performed on day 21 or 22, at which time epithelial cells can no longer be detected. The phagocytic activity and the associated biochemical events were studied mainly with mesangial cells. In some cases, epithelial cells served as a control. To confirm that mesangial cells were not contaminated by macrophages or monocytes of extra-renal origin, the nonspecific esterase stain (17) was used with dissociated mesangial cells and with rat peritoneal macrophages obtained for control either immediately after collection, or after 3 wk of culture under the same conditions.

Characterization of Mesangial Cells. Cells with the morphologic features of either endothelial or epithelial cells were not seen. Mesangial cells appeared large and stellate, whereas epithelial cells are small and polyhedral (12). Immunofluorescence staining was negative with a specific antifactor VIII antibody. Mesangial cells were destroyed after a 24-h incubation with 10 µg/ml mitomycin C but were resistant to similar concentrations of aminonucleoside of puromycin which are toxic for epithelial cells (18). Macrophage contamination was excluded on the basis of the following evidence: (a) Cultured mesangial cells studied after dissociation were not esterase-positive, whereas rat peritoneal macrophages studied either immediately after collection or after 3 wk of culture under the same conditions as those used for mesangial cells stained markedly in the presence of the reagents specific for esterase. Moreover it has been shown that glomeruli of normal rats contain fewer than 0.1% esterase-positive cells (19). (b) Rat cultured peritoneal macrophages did not proliferate in vitro and were fewer after 3 wk of culture than they were initially. (c) Cultured mesangial cells in contrast with macrophages (10) contained large bundles of microfilaments similar to those observed in renal cortical sections under electron microscopy (20). These microfilaments stained in the presence of a specific antimyosin antiserum using indirect immunofluorescence technique. (d) Even after 3 wk of culture, mesangial cells contained very few lysosome-like structures, whereas the cytosol of the macrophages included many of these organelles. Their number increased with the time of culture in accord with the results of Cohn and Benson (21). (e) Cultured mesangial cells synthesized in vitro type I and III collagens, type IV procollagen and fibronectin in

1838 REACTIVE OXYGEN PRODUCTION DURING PHAGOCYTOSIS

agreement with the results of Foidart et al. (6). They also produced PG, mainly PGE₂, and in a decreasing order of abundance, PGF_{2 α} , TXB₂, and 6-keto-PGF_{1 α} as observed previously (5) whereas peritoneal macrophages from mouse (22) or rat (23) origin synthesize, for the most part, 6 keto-PGF_{1 α} . (f) Finally, only the mesangial cells in accord with our previous results (3) exhibited a specific and reversible contractile activity in the presence of angiotensin II.

Preparation of Opsonized Particles. Zymosan A (Sigma Chemical Co., St. Louis, MO) was boiled for 30 min, washed twice with 0.16 M NaCl, and incubated at a concentration of 10 mg/ml with fresh rat serum for 30 min at 37°C. After centrifugation and washing, the resulting preparation of serum-treated zymosan (STZ) was resuspended in Krebs-Ringer phosphate (KRP), pH 7.4, containing 0.8 mM CaCl₂ and used at final concentrations between 125 and 2,000 μ g/ml. Zymosan treated with heated serum (preincubation for 30 min at 56°C) was used as a control.

Incubation of Mesangial Cells. Phagocytosis was studied with dissociated mesangial cells scraped off their flasks. The flasks were washed twice with 3 ml of Dulbecco's solution, and then exposed for 2 min at room temperature to the same solution containing 2.5 mM EDTA (disodium salt). This medium was removed, and the flasks were kept for 15 min at 37°C. 3 ml of Dulbecco's solution was then added to the flasks and the cells were scraped away using a rubber policeman. The suspension obtained was filtered through a 50- μ m sieve and centrifuged at 120 g for 10 min. The supernatant was discarded and the pellet was suspended in KRP solution. \sim 100,000 cells were incubated at 37°C for 5–60 min in the presence of 125–2,000 μ g/ml STZ in a total volume of 0.4 ml. Cytochalasin B (Sigma) was added in some experiments in order to measure reactive oxygen production by cells whose phagocytic activity was inhibited. Mesangial cells were preincubated with cytochalasin B at a final concentration of 5 μ g/ml for 10 min at 37°C before addition of STZ. Cytochalasin B was dissolved in 0.1% dimethylsulfoxide. The resulting concentration of dimethylsulfoxide in the incubation medium (0.025%) did not influence the cell activity.

Morphological Studies. The incubation was stopped by centrifugation at 3,000 g for 3 min at 4°C. The supernatant was discarded, and the pellet was fixed by immersion in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 1 h at room temperature followed by an overnight washing in 0.2 M cacodylate buffer. For conventional light microscopy, the pellet was stained with periodic acid-Schiff (PAS) reagent and embedded in Epon. Semithin sections (0.5–1 μ m) were stained with toluidin blue at pH 9. In order to estimate the percentage of mesangial cells that were phagocytic and the number of particles engulfed per cell, semithin sections of the cellular pellet were performed at 10 μ M intervals so that the same cell did not appear on different sections. 500 cellular sections were examined at \times 400 magnification and the number of STZ particles included within each of them was counted. This technique underestimates the real number of STZ particles per cell since only one section per cell obtained at random was studied. For transmission electron microscopy, the pellet was postfixated in 2% aqueous osmium tetroxide for 1 h at room temperature, dehydrated in graded ethanol solutions, and embedded in Epon. Thin sections (60–100 nm) were cut and stained with uranyl acetate and lead citrate. Sections were examined with a Philips EM-400 electron microscope (80 kV). In order to verify that the phagocytic cells possessed the same characteristics as the cultured mesangial cells studied under basal conditions, dissociated mesangial cells obtained either before or after a 10 min incubation with 1,000 μ g/ml STZ were examined in parallel. Both preparations were spread out on slides, fixed with absolute ethanol for 10 min, washed, and then treated with a specific antimyosin antiserum (kindly provided by Pr. Mahieu, Liège, Belgium) for indirect fluorescence microscopy.

Phagocytosis Assay. In order to quantitate the amount of zymosan that was bound to or incorporated in the mesangial cells, the particles of zymosan were labeled with ¹²⁵I by the ammonium persulfate method (24) and then opsonized as described above. \sim 150,000 cells were incubated at 37°C for 5–30 min in the presence of 1,000 μ g/ml STZ (0.12 μ Ci/mg) in a total volume of 0.3 ml. At the end of the incubation, 0.5 ml of ice-cold KRP was added and this total volume was layered on ice-cold Ficoll (Pharmacia, Uppsala, Sweden) sodium diatrizoate (Sigma) gradient (specific gravity equal to 1.095 g/cm³) (25).

After centrifugation (400 g, 10 min, 4°C) the free particles of STZ appeared in the pellet and the mesangial cells in the upper phase as demonstrated in parallel studies with [³H]thymidine-labeled cells. ¹²⁵I present in these fractions was counted in a crystal-type scintillation detector (Searle model 1185). Results were expressed either as cpm of ¹²⁵I associated to the cells per tube or as the average number of STZ particles per cell. To calculate the latter value, we utilized the specific activity of STZ (0.12 μCi/mg) and the weight of zymosan particles (1 μg = 37,500 particles).

In order to confirm the value of the percent of phagocytic cells estimated on semithin sections of a 3,000 g pellet, 150,000 mesangial cells were incubated for 20 min with 1 mg/ml unlabeled STZ, and then they were separated from the free particles of zymosan by the same method as that used in the quantitative phagocytosis assay with ¹²⁵I-STZ. The cells were spread out on slides, stained with PAS, and examined under light microscopy.

Determination of Superoxide Anion ($\cdot O_2^-$) Production by Mesangial Cells. Triplicate reaction mixtures containing ~100,000 mesangial cells pretreated or not by cytochalasin B were incubated with or without STZ and in the presence of 75 μM horse heart ferricytochrome C type III (Sigma) in a total volume of 0.4 ml (26). After 5–60 min, the incubation was stopped by centrifugation at 3,000 g for 3 min at 4°C, and 0.3 ml of cell free supernatant was diluted with 2.1 ml of KRP, pH 7.4. The amount of reduced cytochrome generated was calculated from the decrease in absorbance at 550 nm upon addition of a grain of ferricyanide, using an absorbance coefficient of 21.1 mM⁻¹ cm⁻¹. The specificity of the cytochrome *c* reduction was checked in each experiment by using supernatant from reaction mixtures containing 10 μg/ml superoxide dismutase in addition to the other compounds. The amount of reduced cytochrome *c* in these samples was identical to that found in nonincubated blanks. The results of the $\cdot O_2^-$ determinations were expressed as nanomoles of cytochrome *c* reduced per 100,000 cells after subtraction of the appropriate blank value.

Determination of Hydrogen Peroxide (H₂O₂) Production by Mesangial Cells. Duplicate or triplicate reaction mixtures containing ~100,000 mesangial cells were incubated with or without STZ and in the presence of 0.28 mM phenol red sodium salt (Sigma) and 50 μg/ml type II horse radish peroxidase (Sigma) in 0.4 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 5.5 mM dextrose and 140 mM NaCl (27). The incubation was stopped by centrifugation at 3,000 g for 3 min at 4°C. The supernatants were collected and the pH was adjusted to 12.5 by the addition of 10 μl 3 M NaOH. Absorbance was read at 610 nm with a Model 25 Beckman spectrophotometer against a blank containing the same reagents except the cells. Standard curves of absorbance against H₂O₂ concentration (1–20 μM) were obtained and the results expressed as nanomoles of H₂O₂ generated per 100,000 cells.

High Performance Liquid Chromatography (HPLC) of Lipoyxygenase End-products. Flasks of culture containing mesangial cells at confluency were washed three times with RPMI medium devoid of fetal bovine serum. The cells were then incubated at 37°C for 3 h with 5 ml of the same medium containing 15 μCi of [5, 6, 8, 9, 11, 12, 14, 15-³H] arachidonic acid (100 Ci/mmol; The Radiochemical Centre, Amersham, UK). At the end of the incubation, the medium was discarded and the cells were washed twice with 5 ml of RPMI medium containing 5 mg/ml of fatty acid free-bovine serum albumin. The cells were then incubated at 37°C for 16 h with RPMI medium containing 10% fetal bovine serum. Under these conditions the greatest part of tritiated arachidonic acid present in the cells was incorporated into the phospholipids. At the end of this labeling period, the mesangial cells were scraped off their flasks and incubated with or without STZ (1 mg/ml) in 0.5 ml of 20 mM Tris-HCl buffer, pH 7.4, containing 5 mM glucose, 135 mM NaCl, 10 mM KCl, 10 mM NaCH₃COO, and 5 mM CaCl₂. The incubation was carried out for 20 min at 37°C and terminated by precipitation with 1 ml of cold methanol and centrifugation at 22,000 g for 2 min. The supernatant was removed and the pellet was rewashed with 1 ml of methanol and respun. The supernatants were then pooled, acidified to pH 3–3.5 with 1 M HCl, and added to 6 volumes of diethylether and 4 volumes of distilled water. The ether layer was removed and the pooled supernatant extracted again with the same

1840 REACTIVE OXYGEN PRODUCTION DURING PHAGOCYTOSIS

volume of diethylether. Both ether layers were pooled and evaporated to dryness under a nitrogen stream. Unreacted arachidonic acid was separated from its hydroxylated derivatives by silicic acid chromatography using glass columns packed with 0.5 g of silicic acid suspended in hexane/diethylether (90:10, vol/vol) (solvent 1). The dry residue from ether extraction was dissolved in 1 ml of solvent 1, and applied to the column. Two fractions of the sample were collected by using 15 ml of the following two solvents in order: (a) solvent 1 eluting unreacted arachidonic acid and (b) diethylether-methanol (95:5, vol/vol) eluting mono- and dihydroxyacids. The first fraction was discarded and the second fraction was evaporated to dryness under a nitrogen stream. The dry residue was dissolved in 130 μ l of HPLC eluent (hexane/ethanol/acetic acid; 993:6:1, vol/vol) and injected directly into the chromatograph (Varian, model 5000) equipped with a Varian Si 10 column and a UV detector (Pye Unicam). The sample was eluted at a solvent flow of 1.5 ml/min, and fractions were collected at 1-min intervals. The elution of hydroxyacids was monitored by measurement of UV absorbance at 235 nm. Authentic 8-, 9-, 11-, 12-, and 15-(HETE) standards were added to samples before the purification procedure as internal standards. Under the conditions used, 12- and 15-HETE were not clearly separated. [3 H]Radioactivity of each fraction was measured in a liquid scintillation counter (Searle, model Mark II). HETE production was expressed as $\text{cpm} \cdot \text{mg}^{-1} \cdot 20 \text{ min}^{-1}$ (28).

Effects of Lipoxygenase and Cyclooxygenase Inhibitors. Three lipoxygenase inhibitors (29) were tested in phagocytosis studies: eicosatetraenoic acid (ETYA; Roche Pharmaceutical Co. Nutley, NJ), 1-phenyl-3-pyrazolidone (phenidone; Sigma) and nordihydroguaiaretic acid (NDGA; Sigma). Only the first two were used when H_2O_2 production was measured since NDGA behaves also as a potent scavenger of H_2O_2 . An inhibitor of the cyclooxygenase pathway, indomethacin (Merck Chemical Div. Merck & Co., Inc., Rahway, NJ), was also tested in the same experiments. Viability of the cells exposed to these drugs was estimated by trypan blue exclusion.

Results

Morphological Studies of Phagocytosis. Phagocytic activity of the mesangial cells could be observed after 5 min of incubation by light microscopy. STZ particles were in contact with the cell membrane, and in some cases, were partially surrounded by cytoplasmic processes as seen by transmission electron microscopy. Other particles were within intracytoplasmic vacuoles (Fig. 1). The cytoplasm of the phagocytosing cells exhibited multiple microfilaments in the vicinity of the cytoplasmic membrane lining STZ-containing vacuole (Fig. 2). Many cells also exhibited vacuoles containing digested STZ particles. This material was PAS positive under light microscopy. None of these cells were esterase-positive, but they all contained structures that stained positively by indirect immunofluorescence with a specific antimyosin antiserum. Epithelial cells, treated in an identical manner, did not display any phagocytic capability. After 5 min of incubation, the phagocytic activity was observed in 42.2% of cell sections of a 3,000 g pellet and the number of STZ particles per cell section was distributed between 1 and 7 (Fig. 3). As a mean, there were 0.8 particles per cell.

Quantification of Zymosan Uptake. In order to study the time course of phagocytosis, 150,000 mesangial cells were incubated with 1,000 $\mu\text{g}/\text{ml}$ of STZ labeled with ^{125}I (0.12 $\mu\text{Ci}/\text{mg}$). ^{125}I radioactivity associated to the cells increased progressively with time for 20 min until a plateau was reached that corresponded to 4.1% of the total radioactivity present in the incubation medium (Fig. 4). This corresponded to an average of 2.6 particles of ^{125}I -STZ per cell. The value observed after 5 min of incubation was 1.38 particles per cell, thus slightly

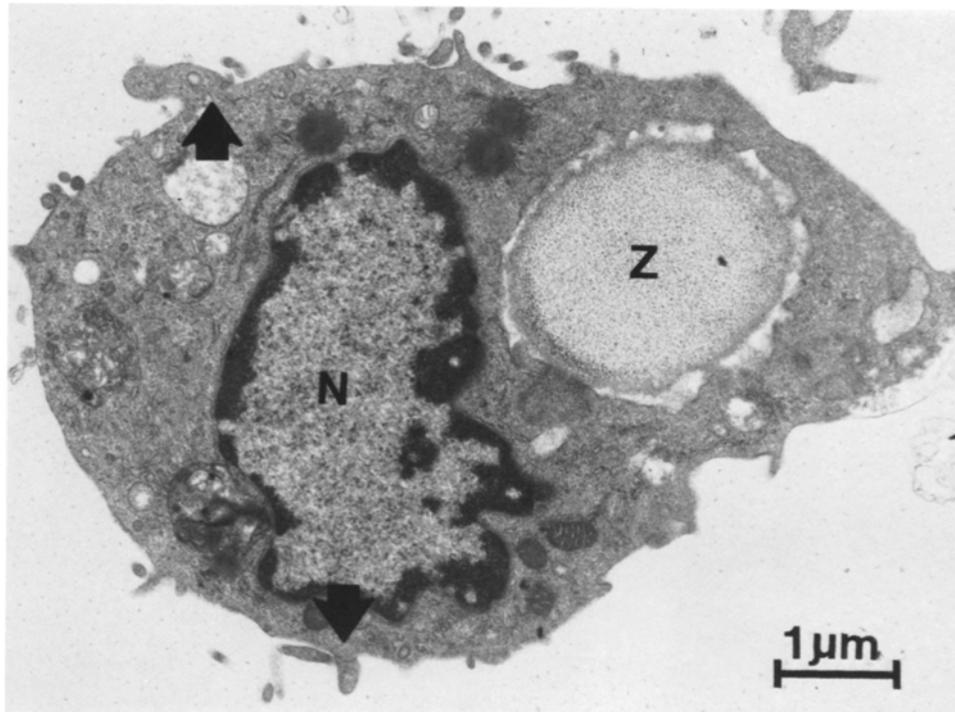


FIGURE 1. Transmission electron micrograph of a mesangial cell after incubation with STZ for 5 min at 37°C. Note the nucleus (N), the STZ-containing vacuole (Z) and many cell processes projecting from the periphery of the cell (arrows). $\times 15,000$.

greater than that derived from the morphological studies. Preheating of the serum used for treatment of the zymosan particles resulted in a marked decrease (50.5%) in ^{125}I binding or uptake.

Parallel experiments were performed with unlabeled STZ. After separation between the cells and the free particles of STZ, 200 cells spread out on slides and stained by PAS were examined. 92 cells (46%) were found to contain one or several particles of zymosan.

$\cdot\text{O}_2^-$ Generation by Mesangial Cells Exposed to STZ. Increasing concentrations of STZ progressively enhanced cytochrome *c* reduction, whereas mesangial cells incubated for 30 min in buffer alone did not reduce cytochrome *c*. At 1,000 $\mu\text{g}/\text{ml}$, the amount of cytochrome *c* reduced was 3.23 ± 0.47 nmol/ 10^5 cells per 30 min. Pretreatment of the cells by cytochalasin B produced a 75% inhibition (0.81 ± 0.06 nmol/ 10^5 cells per 30 min) (Fig. 5). The amount of cytochrome *c* reduced in the presence of a fixed dose (1,000 $\mu\text{g}/\text{ml}$) of STZ accumulated progressively with time till 30–40 min (Fig. 6). There was no detectable reduction of cytochrome *c* when zymosan particles had been preincubated in heated rat serum.

H_2O_2 Generation by Mesangial Cells Exposed to STZ. H_2O_2 was generated by mesangial cells under basal conditions (0.5–0.6 nmol/ 10^5 cells per 30 min), whereas its production by epithelial cells was negligible (Fig. 7). Pretreatment by cytochalasin B had no effect on basal H_2O_2 production. Addition of increasing

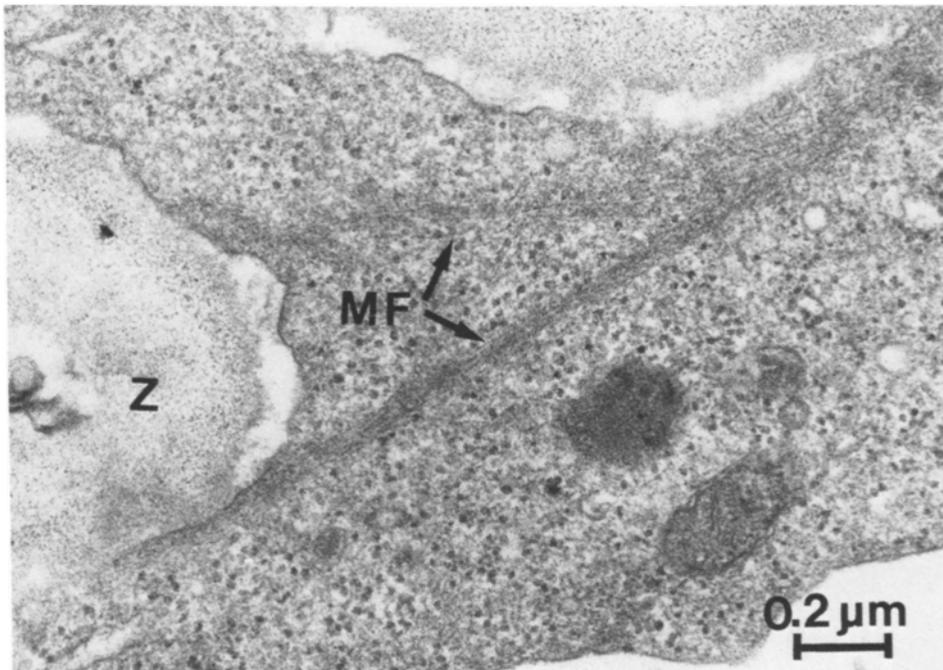


FIGURE 2. Transmission electron micrograph of a mesangial cell after incubation with STZ for 5 min at 37°C. Note the STZ-containing vacuole (Z) and the numerous bundles of microfilaments (MF). $\times 55,000$.

concentrations of STZ (0–2,000 $\mu\text{g}/\text{ml}$) resulted in a progressive increase in H_2O_2 production by mesangial cells but not by epithelial cells. The maximum value ($2.97 \pm 0.16 \text{ nmol}/10^5 \text{ cells per 30 min}$) was reached at 1,000 $\mu\text{g}/\text{ml}$ and corresponded to around 6 times the basal value. There was a plateau of H_2O_2 production between 1,000 and 2,000 $\mu\text{g}/\text{ml}$. Pretreatment of the cells by cytochalasin B resulted in inhibition to a lesser degree than that observed for $\cdot\text{O}_2^-$ production since, at a STZ concentration of 1,000 $\mu\text{g}/\text{ml}$, H_2O_2 generation reached $2.39 \pm 0.09 \text{ nmol}/10^5 \text{ cells per 30 min}$. H_2O_2 accumulated progressively with time and had still not reached a plateau after 60 min of incubation (Fig. 8). Preheating the serum in which zymosan particles were incubated resulted in a marked inhibition of the stimulatory effect of zymosan. In order to compare the effects of opsonized zymosan with those of a soluble stimulatory factor of the oxidative burst, we used phorbolmyristate acetate (32). The maximum value of H_2O_2 production ($0.72 \pm 0.03 \text{ nmol}/10^5 \text{ cells per 30 min}$), obtained at a concentration of 2.5 $\mu\text{g}/\text{ml}$ of this compound, was <25% of that obtained with the phagocytic stimulus at its optimal concentration.

Production of HETE by Mesangial Cells Exposed to STZ. Under basal conditions, mesangial cells released arachidonic acid from the phospholipid stores which, for the most part, remained as such and was only converted to a small extent into 11-, 12-, and 15-HETE (Table I). After exposure to STZ (1,000 $\mu\text{g}/\text{ml}$), there was a marked increase in the release of both free arachidonic acid ($\times 1.92$) and converted products ($\times 2.29$ for the complex 12–15 HETE; $\times 1.86$ for 11-HETE).

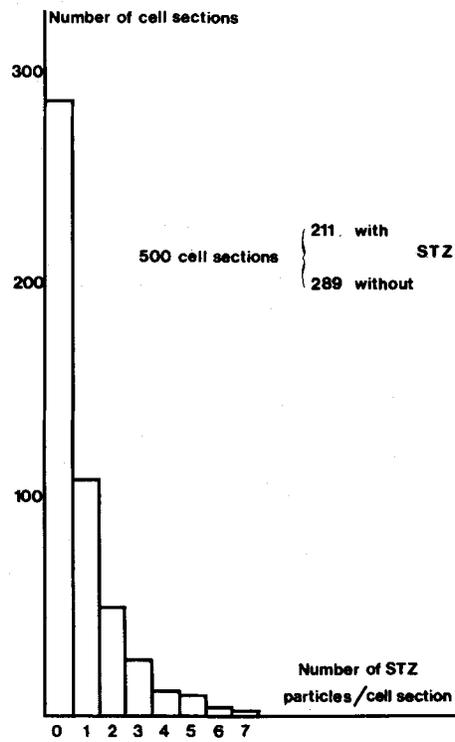


FIGURE 3. Histogram representing the number of mesangial cell sections as a function of the number of STZ particles per cell section.

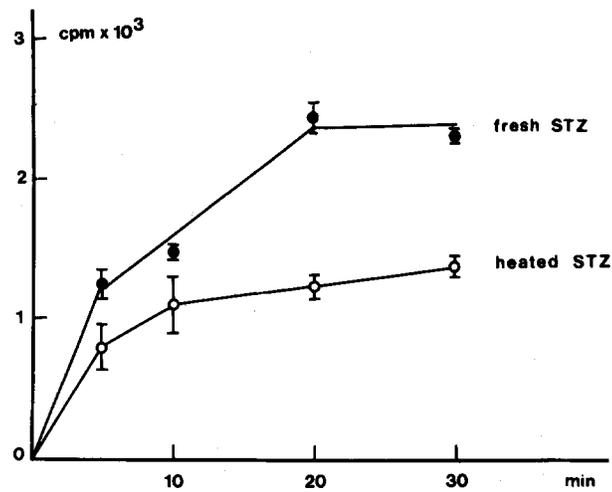


FIGURE 4. Uptake of ¹²⁵I-zymosan by 150,000 mesangial cells as a function of the time of incubation in the presence of 1,000 $\mu\text{g/ml}$ of ¹²⁵I serum-treated zymosan (0.12 μCi) with (○) or without (●) preliminary heating of the serum.

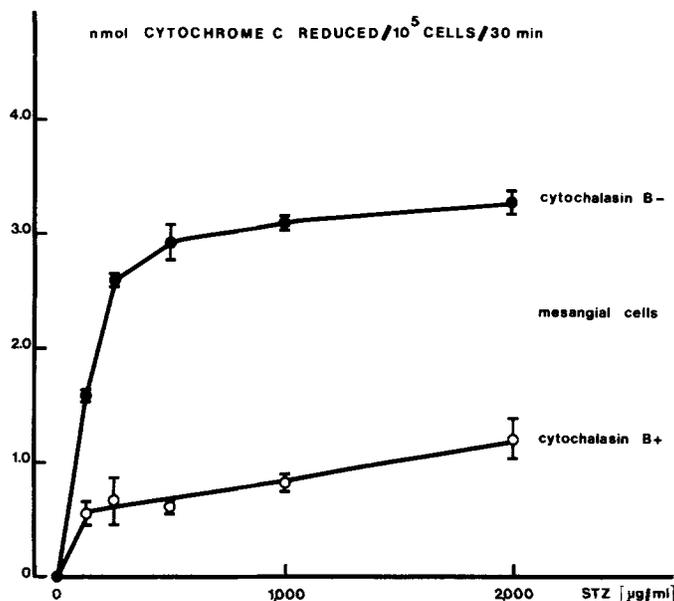


FIGURE 5. Superoxide anion ($\cdot\text{O}_2$) generation by mesangial cells as a function of the concentration of STZ in the incubation medium. Mesangial cells were incubated with (○) or without (●) cytochalasin B. $\cdot\text{O}_2$ generation is expressed as nanomoles of cytochrome *c* reduced per 10^5 cells per 30 min. Data are the means \pm SEM of three experiments.

Effect of Lipoxygenase Inhibitors on H_2O_2 Generation and STZ Uptake by Mesangial Cells. Increasing concentrations of both ETYA and phenidone inhibited progressively H_2O_2 generation (Fig. 9). Inhibition reached 74.8% and 95.8% of the basal value at the highest concentration tested for ETYA and phenidone, respectively. Indomethacin, an inhibitor of the cyclooxygenase pathway did not produce any effect in the range 1–100 μM . This inhibitory effect of the lipoxygenase inhibitors on H_2O_2 production could not be explained by inhibition of the phagocytic process since neither the lipoxygenase inhibitors (ETYA, 40 μM ; phenidone, 100 μM ; NDGA, 20 μM) nor indomethacin (50 μM) diminished the amount of ^{125}I STZ associated to mesangial cells (Table II). This result also suggested that the agents studied were not toxic for mesangial cells at the concentrations used. Absence of toxicity was also demonstrated by trypan blue exclusion after 30 min of incubation of mesangial cells under similar conditions. The percentages of intact cells (mean \pm SEM from four individual results) were: 87.2 ± 1.4 for control cells, and 86.0 ± 1.5 , 86.5 ± 0.9 , and 84.5 ± 1.9 for cells exposed to 40 μM ETYA, 100 μM phenidone, and 20 μM NDGA, respectively.

Discussion

The results of the present study demonstrate that rat cultured mesangial cells are capable of phagocytosing opsonized zymosan particles. Such phagocytosis appears to be specific for this type of cell and not due to the techniques of cell preparations, as rat epithelial cells cultured and prepared in an identical fashion did not exhibit phagocytosis.

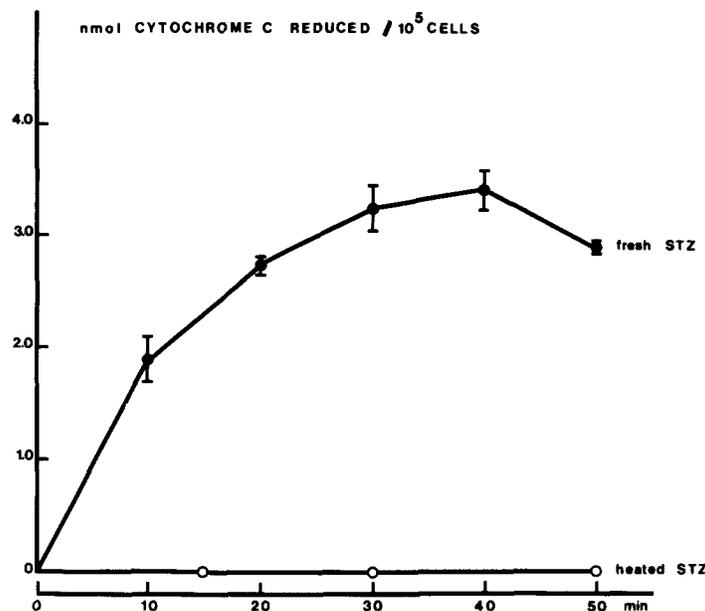


FIGURE 6. Superoxide anion ($\cdot\text{O}_2^-$) generation by mesangial cells as a function of the time of incubation in the presence of serum-treated zymosan with (O) or without (●) preliminary heating of the serum. The concentration of Zymosan was 1,000 $\mu\text{g}/\text{ml}$. $\cdot\text{O}_2^-$ generation is expressed as nanomoles of cytochrome *c* reduced per 10^5 cells. Data are the means \pm SEM of three experiments.

Phagocytosis by rat mesangial cells was associated with a marked stimulation of the production of reactive oxygen species. The amounts of oxygen by-products released by the glomerular mesangial cells in response to phagocytosis were similar to those generated by human polymorphonuclear leukocytes (31), about 30 nmol/ 10^6 cells/30 min, lower than those produced by human monocytes (32) but greater than those generated by human macrophages when obtained after differentiation of monocytes in vitro (32); therefore, contamination by macrophages could not explain the magnitude of $\cdot\text{O}_2^-$, and H_2O_2 production and the great number of phagocytosing cells observed in the present study. In contrast, no increase in H_2O_2 production was observed when rat epithelial cells were studied, providing further evidence for the specificity of this property of the mesangial cells. The release of the oxygen by-products was diminished by pretreatment of the cells with cytochalasin. This result agrees with the findings of Hed and Stendahl (33) who reported that exposure to cytochalasin of human neutrophils stimulated by C_3b -opsonized zymosan but not by IgG-opsonized particles completely inhibited the oxidative burst. Our observation that pretreatment of the particles with heated serum nearly abolished the oxidative burst also suggests a role for a complement-mediated process.

Simultaneously with the production of $\cdot\text{O}_2^-$ and H_2O_2 , the phagocytosing mesangial cells released high amounts of free arachidonic acid from their phospholipid stores. An identical response to the phagocytic stimulus has also been reported with alveolar macrophages in culture and attributed to stimulation of

1846 REACTIVE OXYGEN PRODUCTION DURING PHAGOCYTOSIS

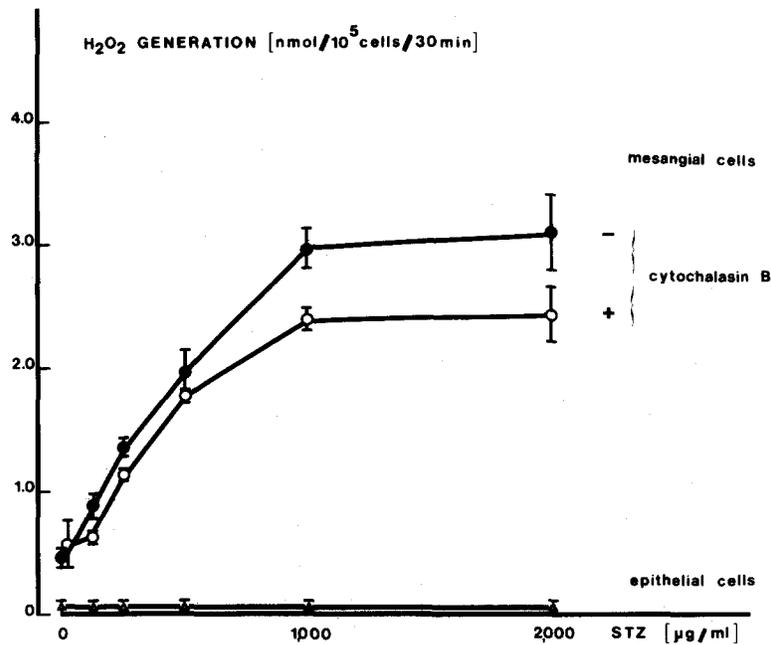


FIGURE 7. Hydrogen peroxide (H₂O₂) generation by epithelial (Δ) or mesangial cells as a function of the concentration of STZ in the incubation medium. Mesangial cells were incubated with (○) or without (●) cytochalasin B. Results are expressed as nanomoles of H₂O₂ generated per 10⁵ cells and per 30 min. Data are the means ± SEM of six experiments.

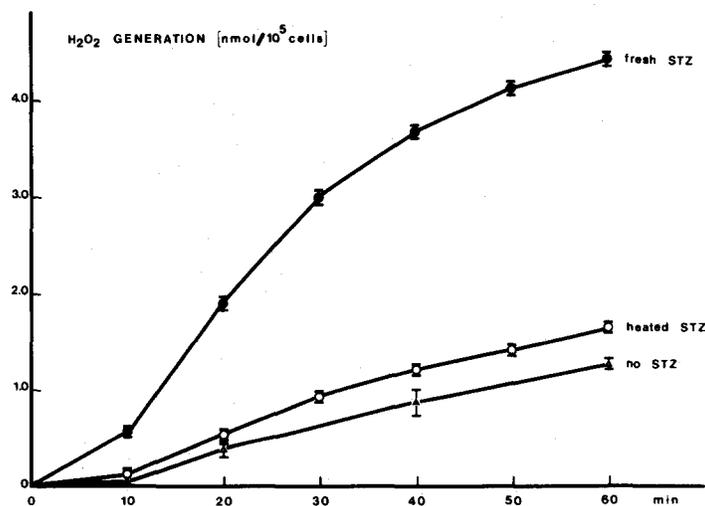


FIGURE 8. Hydrogen peroxide (H₂O₂) generation by mesangial cells as a function of time of incubation under basal conditions (Δ) or in the presence of 1,000 μg/ml of serum-treated zymosan with (○) or without (●) preliminary heating of the serum. Results are expressed as nanomoles of H₂O₂ generated per 10⁵ cells. Data are the means ± SEM of three experiments.

TABLE I
Release of Arachidonic Acid and HETE from Mesangial Cells in Response to Phagocytosis

	Arachidonic acid	12-HETE + 15-HETE	11-HETE
Unstimulated control (3)	71,090 ± 4,514	422 ± 57	789 ± 116
STZ (3)	136,394 ± 14,724	967 ± 7	1,467 ± 58

Cells were exposed to 1 mg·ml⁻¹ STZ during 20 min. Results are expressed as cpm·mg⁻¹·20 min⁻¹. The number of experiments is indicated between parentheses.

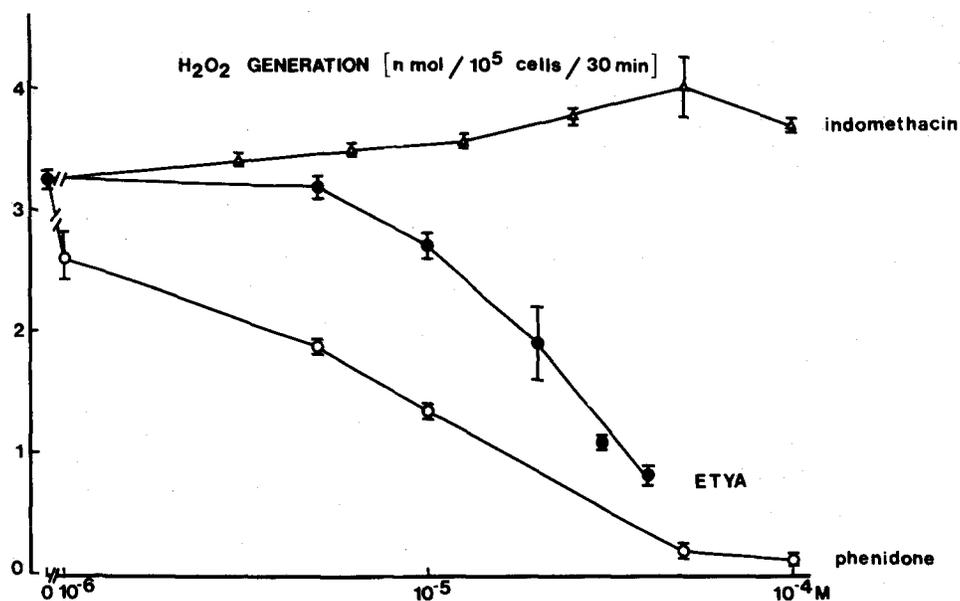


FIGURE 9. Hydrogen peroxide (H₂O₂) generation by mesangial cells in the presence of 1,000 μg/ml of STZ as a function of the concentration of ETYA (●), phenidone (○) or indomethacin (Δ) in the incubation medium. Results are expressed as nanomoles of H₂O₂ generated per 10⁵ cells and per 30 min. Data are the means ± SEM of three experiments.

TABLE II
Effect of Cyclooxygenase and Lipoxygenase Inhibitors on Phagocytosis of ¹²⁵I-STZ by Mesangial Cells

Inhibitor tested	Percentage of control phagocytosis
ETYA (40 μM)	125.5 ± 4.9 (10)
Phenidone (100 μM)	92.6 ± 5.9 (10)
NDGA (20 μM)	97.0 ± 11.1 (6)
Indomethacin (50 μM)	108.4 ± 5.6 (4)

Means ± SEM are given. The numbers of individual values are indicated between parentheses.

1848 REACTIVE OXYGEN PRODUCTION DURING PHAGOCYTOSIS

lysosomal phospholipase (34). A part of released arachidonic acid had been transformed into 11-, 12-, and 15-HETE. This modification of the lipoxygenase activity has also been observed with phagocytosing macrophages (25, 34–37). Our results further suggest that the lipoxygenase pathway may be a significant source of the reactive oxygen species since treatment of the cells by two different inhibitors of the lipoxygenase activity produced a marked decrease in H_2O_2 production, whereas indomethacin, an inhibitor of the cyclooxygenase pathway, did not produce any effect. However, the lipoxygenase inhibitors did not affect the phagocytosis itself. It is thus likely that hydroperoxy intermediates of arachidonic acid (HPETE), which have the capacity to generate highly reactive free radicals when they are transformed into hydroxy derivatives (HETE), are formed early in the phagocytic sequence and then trigger the oxidative burst. A similar hypothesis has been advanced by Smith and Weidemann (38) with peritoneal macrophages. Their conclusion was based on the inhibitory effect of ETYA alone and has been discussed by Ingraham et al. (25). These authors pointed out that ETYA may be reacylated into the phospholipids of the cell membrane and thus may modify the activity of other membrane-associated enzymes responsible for formation of reactive oxygen species. Moreover, Ingraham et al. (25) showed that small doses of ETYA, which had no effect on the respiratory burst of phagocytosing alveolar macrophages, inhibited arachidonic acid metabolism. For that reason we have also used another inhibitor of the lipoxygenase pathway, phenidone (39). This drug, whose structure is completely different from that of a fatty acid, cannot be reacylated. The similarity of results obtained with both inhibitory drugs in our study supports the hypothesis that the generation of reactive oxygen species is the consequence of the activation of the lipoxygenase pathway, itself secondary to the phagocytic process.

The reactive oxygen species and the lipoxygenase products formed during the phagocytic process by rat mesangial cells may play a role in inflammatory process that affect the glomerulus. H_2O_2 is especially effective as a microbicidal agent (15). When the toxic oxygen derivatives are released outside the cells, they can injure other cells and membranes, particularly endothelial cells (40, 41) and can modify cellular proliferation (42). They also stimulate PG synthesis by the neighboring glomerular cells (43) and generate the production from arachidonic acid of a chemotactic lipid (44). Furthermore, 12-HETE, which is the main hydroxyderivative of arachidonic acid produced by rat isolated glomeruli (28, 45) and rat glomerular epithelial cells (45), is also considered to have several important physiological activities. It stimulates chemotactic and chemokinetic responses as well as the hexose monophosphate shunt (46) and the release of lysozyme from specific granules in polymorphonuclear leukocytes (47). 12-HETE is also a strong immunosuppressor able to inhibit erythrocyte rosette formation and concanavalin-induced blast transformation of lymphocytes (48). These effects may be secondary to its incorporation into the membrane components (phospholipids and triglycerides) of leukocytes resulting in a change in the membrane properties of these cells (49). The synthesis of 12-HETE by the glomerular mesangial cells during phagocytosis thus may, in conjunction with the release of reactive oxygen products, play a role in the mediation of the inflammatory response of the glomerulus in experimental or spontaneous glomerulonephritis.

Recently the capability of mesangial cells to produce other mediators of the immune responses (50) and of the local inflammatory process (51) has also been reported.

Summary

To investigate the phagocytic capability of glomerular mesangial cells and the biochemical events associated with phagocytosis, rat cultured mesangial cells were incubated in the presence of opsonized zymosan (STZ) and production of reactive-oxygen species and lipoxygenase products were determined. Mesangial cells were identified on the basis of morphologic (presence of microfilaments and pattern of staining by an anti-myosin antiserum) and physiologic (contractile activity in response to angiotensin II) characteristics. No contamination by esterase-positive cells was observed. Electron microscopy revealed that the phagocytic process started after 5 min of incubation, and affected ~50% of the cells. Superoxide anion ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2) generation by mesangial cells exposed to STZ increased with time and STZ concentration. Cells incubated with zymosan particles treated with heated serum produced undetectable amounts of $\cdot\text{O}_2^-$ and 6 times less H_2O_2 than cells exposed to STZ. Pretreatment by cytochalasin B produced a marked decrease in STZ-stimulated production of reactive oxygen species. [^3H]Arachidonic acid was incorporated into mesangial cell phospholipids and its release and conversion into monohydroxyeicosatetraenoic acids (HETE) was measured by radiometric high performance liquid chromatography (HPLC). Incubation with STZ markedly stimulated the release of arachidonic acid from its phospholipid stores and its transformation into 11-, 12-, and 15-HETE. Lipoxygenase inhibitors inhibited STZ-stimulated H_2O_2 production, whereas they did not modify the phagocytic process as shown by the absence of any effect on the uptake of ^{125}I -STZ by the mesangial cells. This study demonstrates that a high percentage of rat cultured mesangial cells phagocytose opsonized particles. The phagocytic process results in an oxidative burst that appears to be dependent on stimulation of the lipoxygenase pathway.

We thank Mrs. N. Knobloch for secretarial assistance and Professor M. Schambelan for critical review of the manuscript.

Received for publication 31 March 1983 and in revised form 30 August 1983.

References

1. Michael, A. F., W. F. Keane, L. Rajj, R. L. Vernier, and S. M. Mauer. 1980. The glomerular mesangium. *Kidney Int.* 17:141.
2. Burkholder, P. M. 1982. Functions and pathophysiology of the glomerular mesangium. *Lab. Invest.* 46:239.
3. Foidart, J., J. Sraer, F. Delarue, P. Mahieu, and R. Ardaillou. 1980. Evidence for mesangial glomerular receptors for angiotensin II linked to mesangial cell contractility. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 121:333.
4. Ausiello, D. A., J. I. Kreisberg, C. Roy, and M. J. Karnovsky. 1980. Contraction of cultured rat glomerular cells of apparent mesangial origin after stimulation with angiotensin II and arginine vasopressin. *J. Clin. Invest.* 65:754.

1850 REACTIVE OXYGEN PRODUCTION DURING PHAGOCYTOSIS

5. Sraer, J., J. Foidart, D. Chansel, P. Mahieu, B. Kouznetzova, and R. Ardaillou. 1979. Prostaglandin synthesis by mesangial and epithelial glomerular cultured cells. *FEBS (Fed. Eur. Biochem. Soc.) Let.* 104:420.
6. Foidart, J. M., J. B. Foidart, and P. Mahieu. 1981. Synthesis of collagen and fibronectin by glomerular cells in culture. *Renal Physiol.* 3:183.
7. Farquhar, M. G., and G. E. Palade. 1962. Functional evidence for the existence of a third cell type in the renal glomerulus. *J. Cell. Biol.* 13:55.
8. Haakenstad, A. O., G. E. Striker, and M. Mannik. 1976. The glomerular deposition of soluble immune complexes prepared with reduced and alkylated antibodies and with intact antibodies in mice. *Lab. Invest.* 35:293.
9. Striker, G. E., M. Mannik, and M. Y. Tung. 1979. Role of marrow-derived monocytes and mesangial cells in removal of immune complexes from renal glomeruli. *J. Exp. Med.* 149:127.
10. Mancilla-Jimenez, R., B. Bellon, J. Kuhn, M. F. Belair, M. Rouchon, P. Druet, and J. Bariety. 1982. Phagocytosis of heat-aggregated immunoglobulins by mesangial cells. An immunoperoxidase and acid phosphatase study. *Lab. Invest.* 46:243.
11. Dubois, C. H., J. B. Foidart, M. B. Hautier, C. A. Dechenne, M. J. Lemaire, and P. R. Mahieu. 1981. Proliferative glomerulonephritis in rats: evidence that mononuclear phagocytes infiltrating the glomeruli stimulate the proliferation of endothelial and mesangial cells. *Eur. J. Clin. Invest.* 11:91.
12. Foidart, J. B., C. A. Dechenne, P. Mahieu, C. E. Creutz, and J. De Mey. 1979. Tissue culture of normal rat glomeruli. Isolation and morphological characterization of two homogeneous cell lines. *Invest. Cell. Pathol.* 2:15.
13. Camazine, S. M., G. B. Ryan, E. R. Unanue, and M. J. Karnovsky. 1976. Isolation of phagocytic cells from the rat renal glomerulus. *Lab. Invest.* 35:315.
14. Schreiner, G. F., J. M. Kiely, R. S. Cotran, and E. R. Unanue. 1981. Characterization of resident glomerular cells in the rat expressing Ia determinants and manifesting genetically restricted interactions with lymphocytes. *J. Clin. Invest.* 68:920.
15. Weissmann, G., J. E. Smolen, and H. M. Korchak. 1980. Release of inflammatory mediators from stimulated neutrophils. *N. Engl. J. Med.* 303:27.
16. Sraer, J., J. D. Sraer, D. Chansel, H. Jueppner, R. D. Hesch, and R. Ardaillou. 1978. Evidence for glomerular receptors for parathyroid hormone. *Am. J. Physiol.* 235:F96.
17. Tucker, S. B., R. V. Pierre, and R. E. Jordon. 1977. Rapid identification of monocytes in a mixed mononuclear cell preparation. *J. Immunol. Methods.* 14:267.
18. Kreisberg, J. I., and M. J. Karnovsky. 1983. Glomerular cells in culture. *Kidney Int.* 23:439.
19. Sterzel, R. B., J. H. Ehrich, H. Lucia, D. Thomson, and M. Kashgarian. 1982. Mesangial disposal of glomerular immune deposits in acute malarial glomerulonephritis of rats. *Lab. Invest.* 46:209.
20. Latta, H. 1973. Ultrastructure of the glomerulus and juxta-glomerular apparatus. In *Handbook of Physiology. Section 8: Renal Physiology.* American Physiological Society, Washington, DC. p. 1.
21. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* 121:153.
22. Scott, W. A., N. A. Pawlowski, M. Andreach, and Z. A. Cohn. 1982. Resting macrophages produce distinct metabolites from exogenous arachidonic acid. *J. Exp. Med.* 155:535.
23. Sraer, J., L. Baud, M. Bens, E. Podjarny, D. Schlondorff, R. Ardaillou, and J. D. Sraer. 1983. Glomeruli cooperate with macrophages in converting arachidonic acid to prostaglandins and hydroxyeicosatetraenoic acids. *Prostaglandins, Leukotrienes and Medicine.* In press.

24. Webb, L. S., B. B. Keele Jr., and R. B. Johnston Jr. 1974. Inhibition of phagocytosis-associated chemiluminescence by superoxide dismutase. *Infect. Immun.* 9:1051.
25. Ingraham, L. M., R. S. Weening, M. F. Clarke, L. A. Boxer, and R. L. Baehner. 1982. Relation of respiratory burst and arachidonate metabolism during phagocytosis by guinea pig alveolar macrophages. *J. Lab. Clin. Med.* 99:908.
26. Goldstein, I. M., D. Roos, H. B. Kaplan, and G. Weissmann. 1975. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J. Clin. Invest.* 56:1155.
27. Pick, E., and Y. Keisari. 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *J. Immunol. Methods.* 38:161.
28. Sraer, J., M. Rigaud, M. Bens, H. Rabinovitch, and R. Ardaillou. 1983. Metabolism of arachidonic acid via the lipoxygenase pathway in human and murine glomeruli. *J. Biol. Chem.* 258:4325.
29. Jim, K., A. Hassid, F. Sun, and M. J. Dunn. 1982. Lipoxygenase activity in rat kidney glomeruli, glomerular epithelial cells, and cortical tubules. *J. Biol. Chem.* 257:10294.
30. Shah, S. V. 1981. Light emission by isolated rat glomeruli in response to phorbol myristate acetate. *J. Lab. Clin. Med.* 98:46.
31. Reiss, M., and D. Roos. 1978. Differences in oxygen metabolism of phagocytosing monocytes and neutrophils. *J. Clin. Invest.* 61:480.
32. Nakagawara, A., C. F. Nathan, and Z. A. Cohn. 1981. Hydrogen peroxide metabolism in human monocytes during differentiation in vitro. *J. Clin. Invest.* 68:1243.
33. Hed, J., and O. Stendahl. 1982. Differences in the ingestion mechanisms of IgG and C3b particles in phagocytosis by neutrophils. *Immunology.* 45:727.
34. Hsueh, W., U. Desai, F. Gonzalez-Crussi, R. Lamb, and A. Chu. 1981. Two phospholipase pools for prostaglandin synthesis in macrophages. *Nature (Lond.)* 290:710.
35. Scott, W. A., J. M. Zrike, A. L. Hamill, J. Kempe, and Z. A. Cohn. 1980. Regulation of arachidonic acid metabolites in macrophages. *J. Exp. Med.* 152:324.
36. Scott, W. A., N. A. Pawlowski, M. Andreach, and Z. A. Cohn. 1982. Resting macrophages produce distinct metabolites from exogenous arachidonic acid. *J. Exp. Med.* 155:535.
37. Scott, W. A., N. A. Pawlowski, H. W. Murray, M. Andreach, J. Zrike, and Z. A. Cohn. 1982. Regulation of arachidonic acid metabolism by macrophage activation. *J. Exp. Med.* 155:1148.
38. Smith, R. L., and M. J. Weidemann. 1980. Reactive oxygen production associated with arachidonic acid metabolism by peritoneal macrophages. *Biochem. Biophys. Res. Commun.* 97:973.
39. Blackwell, G. J., and R. J. Flower. 1978. 1-Phenyl-3-pyrazolidone: an inhibitor of cyclooxygenase and lipoxygenase pathways in lung and platelets. *Prostaglandins.* 16:417.
40. Sacks, T., C. F. Moldow, P. R. Craddock, T. K. Bowers, and H. S. Jacob. 1978. Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes. An in vitro model of immune vascular damage. *J. Clin. Invest.* 61:1161.
41. Weiss, S. J., J. Young, A. F. Lo Buglio, A. Slivka, and N. F. Nimeh. 1981. Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. *J. Clin. Invest.* 68:714.
42. Vladimirov, Y. A., V. I. Olenev, T. B. Suslova, and Z. P. Cheremisina. 1980. Lipid peroxidation in mitochondrial membrane. *Adv. Lipid. Res.* 17:173.
43. Baud, L., M. P. Nivez, D. Chansel, and R. Ardaillou. 1981. Stimulation by oxygen free radicals of prostaglandin production by rat renal glomeruli. *Kidney Int.* 20:332.
44. Perez, H. D., B. B. Weksler, and I. M. Goldstein. 1980. Generation of a chemotactic lipid from arachidonic acid by exposure to a superoxide-generating system. *Inflam-*

1852 REACTIVE OXYGEN PRODUCTION DURING PHAGOCYTOSIS

- matory*. 4:313.
45. Dunn, M. J., A. S. Petrusis, L. S. Scharschmidt, K. Jim, and A. Hassid. 1982. The use of glomerular cell culture to evaluate cyclooxygenase and lipoxygenase products of arachidonic acid metabolism in the kidney. *In Biochemistry of Kidney Functions*. F. Morel, editor. Elsevier/North-Holland Biomedical Press, Amsterdam. p. 3.
 46. Goetzl, E. J., J. M. Woods, and R. R. Gorman. 1977. Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration by 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid. *J. Clin. Invest.* 59:179.
 47. Stenson, W. F., and C. W. Parker. 1980. Monohydroxyeicosatetraenoic acids (HETEs) induce degranulation of human neutrophils. *J. Immunol.* 124:2100.
 48. Gualdè, N., M. Rigaud, H. Rabinovitch, J. Durand, J. L. Beneytout, and J. C. Breton. 1981. Inhibition de la réponse lymphocytaire par les métabolites libérés par la lipoxygenase des macrophages chez la souris. *C. R. Acad. Sci. Paris.* 293:359.
 49. Stenson, W. F., and C. W. Parker. 1979. 12-L-Hydroxy-5,8,10,14-eicosatetraenoic acid, a chemotactic fatty acid, is incorporated into neutrophil phospholipids and triglycerides. *Prostaglandins.* 18:285.
 50. Lovett, D. H., J. L. Ryan, and R. B. Sterzel. 1983. A thymocyte-activating factor derived from glomerular mesangial cells. *J. Immunol.* 130:1796.
 51. Lovett, D. H., R. B. Sterzel, M. Kashgarian, and J. L. Ryan. 1983. Neutral proteinase activity produced in vitro by cells of the glomerular mesangium. *Kidney Int.* 23:342.