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CARRAGEENAN STIMULATES REDUCTION OF NITROBLUE TETRAZOLIUM BY HUMAN NEUTROPHILS WITHOUT MEMBRANE DEPOLARIZATION, MYELOPEROXIDASE SECRETION, OR INCREASED OXYGEN CONSUMPTION

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Abstract—Carrageenan, a sulfated polyanionic polysaccharide, is commonly used to induce inflammation in experimental animals, and this model is used to screen for the effectiveness of antiinflammatory drugs. Carrageenan-induced inflammation has been attributed to a variety of autocoids including histamine, bradykinin, complement, superoxide, and prostaglandins. This study examines the effects of carrageenan on human PMN in a serum-free system. Carrageenan was found to stimulate the reduction of NBT by PMNs without stimulating membrane depolarization, oxygen consumption, H_2O_2 production, or myeloperoxidase secretion. Carrageenan stimulates a heat-labile, NBT-reducing system which is unassociated with the usual stimulus–response coupling seen with other PMN activators such as PMA, FMLP, and zymosan.

INTRODUCTION

Carrageenan is an inflammatory, sulfated polysaccharide isolated from a variety of algae. In 1959, Benitz and Hall characterized the morphology and pathology of the inflammatory response to carrageenan (1). Shortly thereafter Winter et al. demonstrated that aspirin, phenylbutazone, and hydrocortisone could partially inhibit carrageenan-induced hind paw swelling in the rat (2). This model

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has since been used to evaluate the effectiveness of antiinflammatory drugs. The mechanism of carrageenan's inflammatory effects have been attributed to histamine and serotonin, at least during the early phase (3); bradykinin (4), especially during the intermediate period (3); complement activation (3); and the production of arachidonic acid metabolites (5, 6). Additionally, esterase and protease activity appear important for the full manifestation of the inflammatory response (3, 4, 7, 8).

More recently Oyanagui demonstrated the importance of the superoxide anion (O_2^-) in carrageenan-induced foot edema (9). Since polymorphonuclear leukocytes (PMNs) predominate in carrageenan-induced lesions (10), we elected to examine the effect of carrageenan on oxidant production and enzyme secretion by PMNs in a serum-free system. This would isolate cellular-derived factors from plasma-derived factors such as bradykinin and complement split products.

MATERIALS AND METHODS

Nitroblue tetrazolium (grade III), zymosan A, kappa carrageenan (type III), dimethyl sulfoxide (DMSO), phenol red, Triton X-100, luminol, sodium pyruvate, *n*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), phorbol-12-myristate-13-acetate (PMA), and horseradish peroxidase (type IV) were purchased from the Sigma Chemical Co., St. Louis, Missouri. Guaiacol was obtained from Mallinckrodt Chemical Works, St. Louis, Missouri, and hydrogen peroxide from Fisher Scientific, Springfield, New Jersey. The fluorescent probe Di-S-C_3(5) (3,3'-dipropylthiodicarbocyanine) was a gift from Dr. Alan Waggoner, Department of Chemistry, Amherst College, Amherst, Massachusetts. Serum-activated zymosan (SAZ) was prepared as previously described (11).

Citrated whole blood was drawn from healthy human volunteers after obtaining informed, written consent. The study was begun after approval was granted by a committee for protection of human subjects. PMNs were isolated by countercurrent elutriation according to a previously described procedure (11). Cells used for all experiments were greater than 90% PMN.

Nitroblue Tetrazolium Reduction by PMNs Stimulated by Carrageenan. PMNs were suspended at 2×10^6/ml in HBSS (HEPES balanced salt solution: 10 mM HEPES, 145 mM NaCl, 5 mM KCl, 5.5 mM glucose, and 1 mM $CaCl_2$, pH 7.3). NBT was present at 1.5×10^{-4} M. The reaction volume was 2.5 ml, and samples were run in triplicate and incubated at 37°C for 20 min in a shaking water bath. Reactions were started by the addition of carrageenan. After the incubation period, samples were removed to an ice-water bath and centrifuged at 1000*g* for 10 min. One milliliter 3 N HCl was added to the cell pellets which were then sonicated and centrifuged again. Two milliliters DMSO were added to the pellet and the absorbancy read at 492 nm.

Oxygen Consumption by PMA- and Carrageen-Stimulated PMNs. Oxygen consumption was determined with a Yellow Springs model 53 electrode and recorder (Yellow Springs, Ohio). PMN were at 2×10^6/ml in HBSS and were stirred at 37°C for 12 min to determine baseline consumption of oxygen. The stimulant (PMA or carrageenan) was added to the reaction mixture, and the oxygen consumption rate followed. The electrode was standardized according to the method of Robinson and Cooper (12).

Hydrogen Peroxide Production by PMA- and Carrageenan-Stimulated PMNs. Reaction mixtures contained PMNs at 2×10^6/ml, 10 units/ml horseradish peroxidase, stimulant or buffer

(HBSS), and phenol red solution (13). Samples were incubated at 37°C for 30 min with shaking, cooled to 4°C, and centrifuged at 1000*g* for 10 min. The supernatants were decanted into 10 μl 5 N NaOH and the absorbancy read at 610 nm. The concentration of H_2O_2 in samples was determined from a standard curve.

Luminol-Dependent Chemiluminescence (CL) of Carrageenan- and Zymosan-Stimulated PMNs. Samples for CL experiments contained PMN at 4×10^5/ml, luminol at 2×10^{-6} M, and SAZ at 0.2 mg/ml or carrageenan (concentration varied). The cells and luminol were preincubated at 37°C for 20 min and the stimulus added at time zero. Samples were counted in a Packard liquid scintillation counter (model 2002, Downers Grove, Illinois) in the out-of-coincidence mode. Samples were counted for 0.2 min every 4 min and incubated at 37°C between readings.

Requirement of Viable PMNs for NBT Reduction. To determine if carrageenan-induced NBT reduction was dependent on living cells, an experiment was performed with viable cells and cells exposed to a 100°C water bath for 5 min. Reaction mixtures contained PMN at 2×10^6/ml, NBT at 1.5×10^{-4} M, and carrageenan (1 mg/ml) or HBSS in a total volume of 2.5 ml. Samples were incubated for 30 min at 37°C and then processed as above.

Effect of Carrageenan on Release of Myeloperoxidase (MPO) and Lactate Dehydrogenase (LDH) from PMNs. Samples contained PMNs at 2×10^6/ml and SAZ at 0.2 mg/ml or carrageenan at 0.5 mg/ml. Samples were incubated in at 37°C water bath with shaking. Aliquots were removed at intervals, centrifuged (250*g*) at 4°C, and the supernatants saved for MPO and LDH analysis. To one set of resting cells Triton X-100 was added (0.1% final) and the supernatants used as the 100% control. All samples were run in duplicate.

MPO was assayed according to the method of Himmelhoch et al. (14). A 2-ml reaction mixture contained 1 ml 0.0268 M guaiacol, 0.5 ml 1.4 mM H_2O_2, 0.25 ml 0.01 M phosphate buffer (pH 7.0), and 0.25 ml supernatant. The reaction was followed at 470 nm for 1 min at room temperature. LDH was assayed according to a previously published method (15).

The effect of carrageenan on the release of other granular enzymes, as β-glucuronidase and lysozyme, was examined. However, it was found that carrageenan affected their assays directly, by either inhibiting enzymatic activity or by binding the substrate. At 1 mg/ml carrageenan did not affect the enzymatic activity of MPO or LDH of 100% lysates.

Effects of Carrageenan on PMN Membrane Depolarization. The activation of PMN has been associated with a change in membrane potential (16). The effect of carrageenan on membrane potential was examined using the fluorescent probe Di-S-C_3(5) according to the method of Jones et al. (17). A fluorescence spectrophotometer (model MPF-3L, Perkin-Elmer Corp., Norwalk, Connecticut), with a magnetic stirrer, was used to measure fluorescence. The excitation and emission wavelengths were set at 622 nm and 665 nm, respectively. PMNs were suspended at 2.4×10^7/ml in a total volume of 3 ml. The fluorescent probe was added at a final concentration of 0.66 μg/ml. The cells and probe were stirred in the photometer and allowed to equilibrate. Cells were then stimulated with 60 μl of carrageenan to yield a final concentration of 0.1 mg/ml. After $2\frac{1}{2}$ min, FMLP was added at a final concentration of 2×10^{-6} M.

RESULTS

Effect of Carrageenan on NBT Reduction, Oxygen Consumption, and Hydrogen Peroxide Production by Human PMNs. The addition of carrageenan to PMNs results in a concentration-dependent stimulation of NBT reduction (Table 1). Attempts to use ferricytochrome *c* as an indicator of superoxide production were unsuccessful as the carrageenan precipitated it from solution. NBT reduction has been considered an indicator of O_2^- production (18). However,

Table 1. Effect of Carrageenan and PMA on NBT Reduction, Oxygen Consumption, and H_2O_2 Production by Human PMNs

Stimulus	NBT reduction[a]	O_2 consumed[b]	H_2O_2 production[c]
Resting	0.086 ± 0.001	Nil	12.5 ± 2.5
Carrageenan			
0.01 mg/ml	0.087 ± 0.003	Nil	15.8 ± 1.7
0.10 mg/ml	0.133 ± 0.005	Nil	15.8 ± 0.5
1.00 mg/ml	0.175 ± 0.005	Nil	5.0 ± 0.25
PMA 10 ng/ml	0.154 ± 0.002	85.5 ± 7.5	82.5 ± 1.7

[a] Absorbancy determined at 492 nm.
[b] Nanomoles per minuts per milliliter of reaction mixture.
[c] Nanomoles per milliliter of reaction mixture.

Picker and Fridovich have suggested any aqueous reduction of NBT is superoxide dismutase (SOD) suppressible (19). We noted SOD at 10 μg/ml suppressed NBT reduction by 42% (data not shown). However, because SOD can inhibit NBT reduction without O_2^- necessarily participating in the reduction, and because ferricytochrome *c* could not be used, oxygen consumption and H_2O_2 production were measured. Table 1 reveals there to be no stimulation of oxygen consumption or H_2O_2 production when PMNs are incubated with carrageenan.

Luminol-Dependent CL of Carrageenan-Stimulated PMNs. Stimulation of PMNs with carrageenan results in a very weak chemiluminescent response in comparison with SAZ-stimulated cells (Table 2). A significant fraction of luminol-dependent CL requires the oxidation of luminol by hypochlorite, the product of the oxidation of chloride by MPO and H_2O_2. It is not surprising that the CL is low, since carrageenan does not stimulate H_2O_2 production. These data confirm the absence of a typical respiratory burst by carrageenan-stimulated PMNs.

Dependence on Viable PMNs for NBT Reduction. The ability of a stim-

Table 2. Chemiluminescence of PMNs Stimulated with SAZ or Carrageenan

Stimulus	Integrated counts over 64 min (×1000)
Resting	2.1
SAZ 0.2 mg/ml	186.6
Carrageenan	
0.01 mg/ml	2.3
0.1 mg/ml	4.4
1.0 mg/ml	5.3

Table 3. Requirement of Viable PMNs for Carrageenan-Induced NBT Reduction

	Blank	Reagent blank	Nonviable stimulated	Nonviable resting	Viable resting	Viable stimulated
NBT	+	+	+	+	+	+
Carrageenan (1 mg/ml)	−	+	+	−	−	+
Cells						
Viable	−	−	−	−	+	+
Nonviable	−	−	+	+	−	−
$A_{492\,nm}$[a]	0.000 [b]	0.025 ± 0.005	0.051 ± 0.001	0.044 ± 0.001	0.086 ± 0.006	0.211 ± 0.005

[a] Results are expressed as means ±*SEM*.
[b] Used to blank spectrophotometer for all other readings.

ulus to initiate NBT reduction unassociated with the reduction of oxygen to O_2^- and H_2O_2 is contrary to current thought (18). For this reason we tested the necessity for viable PMNs. Table 3 demonstrates the NBT-reducing mechanism to be heat sensitive. Carrageenan alone (without PMN) has no ability to reduce NBT. The low absorbancies were primarily due to turbidity produced by carrageenan and/or cells remaining after the processing and extraction of reduced NBT. Thus some heat-labile system in PMNs is required for NBT reduction.

Release of MPO and LDH by Carrageenan- or SAZ-Stimulated PMNs. Figure 1A and 1B illustrate the time course of LDH and MPO release, respectively, from carrageenan- or SAZ-stimulated PMNs. Carrageenan-stimulated cells did not release MPO in excess of resting cells, whereas SAZ-stimulated PMNs released 23% of their MPO in the first 15 min of incubation. The low release of the cytosolic enzyme LDH by carrageenan-stimulated cells suggests that carrageenan is not significantly cytotoxic, i.e., does not decrease membrane integrity.

PMN Membrane Depolarization. Figure 2 is the fluorescence signal of human PMNs incubated with Di-S-C_3(5). When carrageenan was added, no change in fluorescence was detected, indicating the membrane was not depolarized. The addition of FMLP to the same mixture caused a change in fluorescence characteristic of depolarization. Thus, carrageenan does not change the membrane potential of PMNs, and it has no effect on the assay system since FMLP triggered a normal depolarization response in the presence of carrageenan. The FMLP-induced depolarization is followed by a drop in fluorescence below the resting level. This phenomenon, which resembles hyperpolarization, is actually due to the oxidation of the fluorescent probe by hypochlorite produced by secreted MPO oxidizing chloride with H_2O_2 (20, 21). Therefore, it appears that carrageenan does not inhibit the production of H_2O_2 or the secretion of MPO.

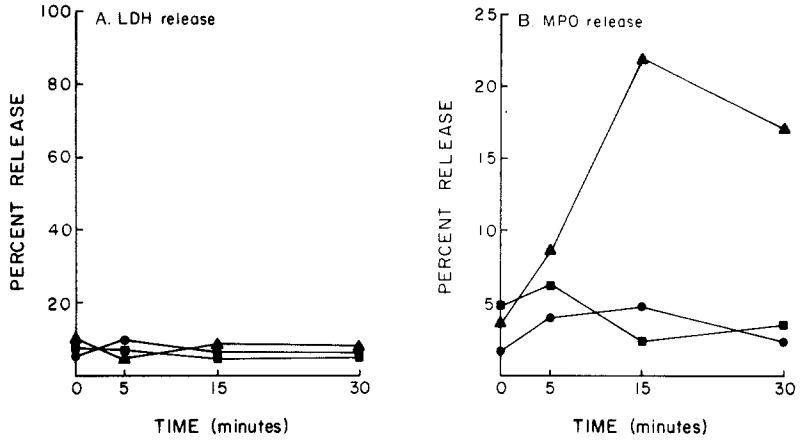

Fig. 1. Time course of LDH (A) and MPO (B) release from resting (●), carrageenan-stimulated (■), or SAZ-stimulated (▲) PMNs.

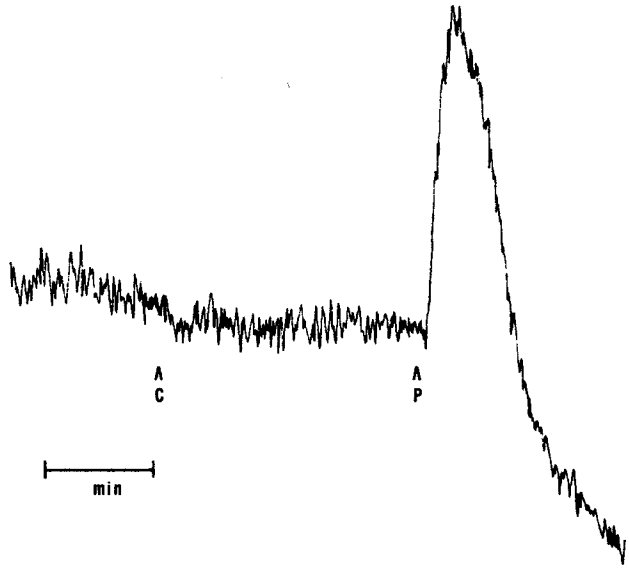

Fig. 2. The effects of carrageenan and FMLP on PMN membrane potential. Carrageenan was added to resting cells in the presence of Di-S-$C_3(5)$ at "C" and the FMLP peptide was added to the same mixture at "P."

DISCUSSION

Since the discovery that steroidal and nonsteroidal antiinflammatory drugs could inhibit carrageenan-induced edema (2), this model has had wide popularity in the study of potential antiinflammatory agents. Despite the model's common usage, a wide variety of mediators has been implicated as causative in carrageenan's inflammatory potential (3–9).

In this paper we report that carrageenan does not appear to stimulate the release of granule enzymes or toxic oxidants one normally associates with the inflammatory response. Rather, there is an isolated activation of an NBT-reducing system. While carrageenan does not stimulate increased oxygen consumption, H_2O_2 production, MPO secretion, or membrane depolarization, it does not suppress these events when another stimulant such as FMLP is used. This statement is supported by Figure 2. When FMLP is added to a mixture of PMNs and carrageenan, there is a depolarization followed by a fall in fluorescence below baseline. This drop in fluorescence has been attributed to an oxidation of the probe, Di-S-C_3(5), by hypochlorite (20, 21), a product of chloride oxidation by H_2O_2 and MPO (22).

In previous studies, NBT reduction by stimulated, intact PMNs was shown to be oxygen dependent and associated with increased metabolism through the hexose monophosphate (HMP) shunt (18). However, these authors also noted a cyanide-insensitive, oxygen-independent NADH-NBT reductase in sonicates of cells. Using endotoxin as a stimulant, Proctor (23) noted stimulation of the HMP shunt to be proportionate to the amount of NBT reduced, and found NBT reduction to be possible, although reduced, under anerobic conditions. Humbert et al. (24) found methylene blue to be capable of stimulating NBT reduction by PMNs from normals and patients with chronic granulomatous disease (CGD). In cell-free systems it was found that methylene blue was acting as an electron carrier between reduced pyridine nucleotides and NBT, suggesting an enzyme or enzyme system was not necessarily required for NBT reduction by viable cells. Others have found the polyanion heparin to stimulate NBT reduction by PMNs (25, 26). Czarnetzki et al. (27) have published a paper that perhaps is most relevant to our work. They incubated the polyanions heparin and chondroitin sulfate A and B with PMNs and noted NBT reduction. Stimulation of the HMP shunt, however, required the presence of both the polyanion and NBT, indicating that the polyanion in some way facilitated the transfer of electrons from NADPH to NBT.

The failure of carrageenan to stimulate oxygen consumption and H_2O_2 production is not surprising. Whitin et al. (28) have found membrane depolarization to be a prerequisite for O_2^- production. Furthermore, others have noted a disparity between SOD-inhibitable cytochrome *c* reduction (O_2^- production) and NBT reduction by zymosan-stimulated PMNs (29), and ample evidence exists

which documents O_2^- production and enzyme secretion can be dissociated (30–33). However, with the absence of membrane depolarization in carrageenan-stimulated PMNs, it is unlikely that NBT reduction is simply a phenomenon of incomplete stimulus-response coupling. Rather, it is more likely that polyanions in some way are capable of stimulating the shuttling of electrons from intracellular reducing agents to NBT. How and whether this process contributes to the in vivo inflammatory response induced by carrageenan will require further study.

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