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CARRAGEENAN-STIMULATED RELEASE OF ARACHIDONIC ACID AND OF LACTATE DEHYDROGENASE FROM RAT PLEURAL CELLS

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Abstract—Cells isolated from the rat pleural cavity consist mainly of macrophages, mast cells, eosinophils, and lymphocytes. Isolated pleural cells labeled with [^{14}C]arachidonic acid released appreciable amounts (~12%) of radiolabel upon exposure to pharmacological concentrations of carrageenan (1–100 μg/ml). The release of radiolabel was decreased by an inhibitor of phospholipase A_2 (*p*-bromophenacyl bromide) but not by an inhibitor of arachidonate cyclooxygenase (indomethacin). The released products were arachidonic acid and, to a much lesser extent, prostaglandin E_2 and leukotriene C_4. The release of radiolabel was associated with release of cytosolic lactate dehydrogenase over the same range of carrageenan concentrations. Time–course studies indicated that release of radiolabel preceded that of lactate dehydrogenase. Since *p*-bromophenacyl bromide blocked stimulated release of radiolabel but did not prevent release of lactate dehydrogenase, it is unlikely that increase in arachidonate causes carrageenan-induced cell damage. Nevertheless, the question of whether the activation of phospholipase A_2 in the pleural cells, most probably the macrophages, was sufficient to initiate the carrageenan-induced inflammatory response requires further study. Cytotoxicity which was apparent with as little as 5 μg/ml of carrageenan, may have been a significant consequence of carrageenan action.

The sulfated polysaccharide, carrageenan, is a potent inflammatory agent (for review, see Ref. 1). It is widely used in various experimental models of inflammation, such as paw edema [2] and pleural effusion [3, 4] in rats. The intrapleural injection of 500 μg carrageenan in rats, for example, induces progressive accumulation of a protein-rich exudate that contains large numbers of neutrophils. The infiltration of plasma protein, the appearance of neutrophil chemotactic activity, and the accumulation of neutrophils are closely related [5]. Edema produced by carrageenan is dependent on mobilization of neutrophils [6, 7]. The response to carrageenan is neither associated with histamine release from mast cells nor inhibited by antihistamines [7–9]. Instead, high concentrations of leukotriene B_4 and prostaglandin E_2 (PGE_2)§ are detectable in carrageenan-induced exudates [10–12]. Moreover, indomethacin and aspirin, which inhibit the metabolism of arachidonic acid via lipoxygenase and cyclooxygenase pathways in neutrophils in such exudates [13], markedly suppress the inflammatory response to carrageenan [13–16]. Other inhibitors of the arachidonate lipoxygenase and cyclooxygenase pathways, nordihydroguaiaretic acid and BW755C at higher dosages, have also been shown to inhibit exudate formation and neutrophil infiltration induced by carrageenan [14, 17]. The results imply that the carrageenan-induced inflammatory responses are mediated, at least in part, by metabolites derived from arachidonic acid metabolism.

Initially, our interest was to study the possibility that carrageenan exerts its effect by interacting with a specific cell population(s) within the pleural cavity, although there is evidence against this idea in that removal of 87% of pleural resident cells does not affect carrageenan pleurisy [7]. As we report here, cells isolated from the pleural cavity and labeled with [^{14}C]arachidonic acid released appreciable amounts of radiolabeled material upon exposure to carrageenan. The radiolabeled products were mainly arachidonic acid and, to a much lesser extent, PGE_2 and leukotriene C_4 (LTC_4). In addition, however, we observed that the release of the radiolabel was accompanied by the release of lactate dehydrogenase (LDH; L-lactate:NAD oxidoreductase; EC 1.1.1.27) into the incubation medium, and thus our interest shifted to the possibility that the two events may be causally related. For comparison, the effects of carrageenan on two nonphagocytic cell systems (i.e. cultured rat submandibular gland cells and a human mesothelioma cell line) were examined.

MATERIALS AND METHODS

Materials. Carrageenan (Viscarin carrageenan, RENJ-6755) and Hanks' balanced salt solution were

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§ Abbreviations: PGE_2, prostaglandin E_2; LTC_4, leukotriene C_4; LTD_4, Leukotriene D_4; Hanks–HEPES–albumin, Hanks' medium containing *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and bovine serum albumin; LDH, lactate dehydrogenase; RIA, radioimmunoassay; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; and BPB, *p*-bromophenacyl bromide.

purchased from Marine Colloids, Inc., Springfield, NJ, and GIBCO, Long Island, NY, respectively. The lactate dehydrogenase assay kit (No. 226-UV), indomethacin, *p*-bromophenacyl bromide (BPB), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), bovine serum albumin (fatty acid-free), Triton X-100, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), ethidium bromide and fluorescein diacetate were purchased from the Sigma Chemical Co., St. Louis, MO. Dimethyl sulfoxide was obtained from Pierce, Rockford, IL, and arachidonic acid was purchased from the Aldrich Chemical Co., Milwaukee, WI.

Male Sprague–Dawley rats (220–270 g body wt) were obtained from Taconic Farms, Inc., Germantown, NY. The rats were housed at ~24° and were maintained on Purina laboratory chow and distilled water *ad lib.*

Primary cultured rat submandibular gland cells were supplied by Dr. Chu S. Lo, F. E. Herbert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD. The human mesothelioma cell line was provided by Dr. James B. McMahon, DCT, DTP, National Cancer Institute, National Institutes of Health.

Preparation of agents. Carrageenan was dissolved in sterile saline solution. The ionophore A23187 or BPB was dissolved in dimethyl sulfoxide, followed by dilution with Hanks–HEPES–albumin as required. Indomethacin (14.32 mg) was dissolved in dimethyl sulfoxide (200 μl) and neutralized with 1 N NaOH (40 μl); the solution was immediately diluted to 10 ml with Hanks–HEPES–albumin to give a 4 mM solution. Further dilution was made where necessary. In all cases, the final concentration of dimethyl sulfoxide in the medium was below 0.1%; appropriate solutions were prepared for the control samples.

Isolation of mixed pleural cells: Labeling with [^{14}C]arachidonic acid. Male Sprague–Dawley rats (220–270 g) were killed in an ether-saturated chamber. The chest was opened by making an incision below the diaphragm and cutting along both sides of the rib cage. Cells were collected by washing the cavity with 2 × 6 ml Hanks' medium, and the washes were centrifuged at 850 *g* for 10 min at 4°. The cells were washed once in 30 ml of Hanks' medium buffered with 25 mM HEPES, pH 7.4, and centrifuged before resuspension in Hanks–25 mM HEPES that contained 0.1% fatty acid-free bovine serum albumin (Hanks–HEPES–albumin) to give a density of 1.0×10^6 cels/ml.

[^{14}C]Arachidonic acid (56.9 mCi/mmol in ethanol, New England Nuclear, Boston, MA) was evaporated to dryness, reconstituted in Hanks–HEPES–albumin medium, and then added to the cell suspension to give final concentrations of 0.75, 1.5, or 3 μM arachidonate. Incorporation of [^{14}C]arachidonic acid was allowed to proceed for 90 min at 37° in an atmosphere of 95% air–5% CO_2. After incubation, free [^{14}C]arachidonic acid was removed from radiolabeled cells by washing twice with 30 ml of Hanks–HEPES–albumin. Both cell fraction and medium were assayed for radioactivity to verify the extent of incorporation of radiolabel into cells. The final cell pellets were resuspended in Hanks–HEPES–albumin to give 1×10^6 cells/ml. Aliquots (0.36 ml) were transferred into 1.5-ml Eppendorf tubes for studying the release of radiolabel and LDH.

Labeling of adherent pleural cells and other monolayer cultures with [^{14}C]arachidonic acid. Cell preparations enriched in the macrophages (85–95%) were prepared by incubation (37°) of the mixed pleural cells obtained as described above in culture plates (Falcon 3847 Primaria™, 1.6 cm diameter, flat bottom, Becton Dickinson Labware, Oxnard, CA) for 2 hr in 95% air–5% CO_2. The nonadherent cells were removed by washing the cultures twice with Hanks–HEPES–albumin medium. The adherence of cells varied with the number of mixed pleural cells added to the well: with increasing numbers of cells, i.e. 1.0, 1.5, 2.0, and 2.5×10^6 cells per well, the percentage of cells that adhered decreased, i.e. 86 ± 5, 83 ± 1, 71 ± 1, and 62 ± 6 (N = 3) respectively. Upon labeling with 1.5 μM [^{14}C]arachidonic acid, total cellular radiolabel was linearly proportional to the number of adherent cells. In subsequent experiments, 1.5 ml of pleural cell suspensions containing 1×10^6 cells/ml were routinely plated and incubated with 1.5 μM [^{14}C]arachidonic acid for 2 hr at 37°.

Primary cultured rat submandibular gland cells and the human mesothelioma cell line were supplied as monolayer cells. The cells were labelled with [$^{+114}$C]arachidonic acid as described above, and the radiolabeled cells were washed and then covered with 0.36 ml of Hanks–HEPES–albumin for release studies.

Release studies on monolayer cells or pleural cell suspension. Unless specified otherwise, 40 μl of NaCl or carrageenan solution previously warmed to 37° was added to the cells (0.36 ml) at 37°. At the indicated times, the cell medium was centrifuged (Beckman Microfuge B, 4°, 90 sec). A portion of the supernatant fluid was analyzed for arachidonic acid and its metabolites; another portion was assayed for radioactivity in a liquid scintillation spectrometer. To study the effect of drugs on the carrageenan-stimulated release, indomethacin or BPB was incubated with the cells for 10 min at 37° prior to the addition of carrageenan.

Although a high concentration (2.5%) of bovine serum albumin has been used to trap the released arachidonic acid and its metabolites [18], we found that 0.1% fatty acid-free bovine serum albumin was sufficient. Accordingly, a 0.1% concentration was used to minimize binding of drugs.

Measurement of arachidonic acid by high performance liquid chromatography (HPLC). Arachidonic acid was identified and quantified by HPLC on a Partisil 5 ODS-3 reverse phase column (4.6 mm i.e. × 25 cm). Medium from six cultures was pooled, from which 2-ml samples were used in the assay. To each 2-ml sample was added 6 ml acetonitrile (HPLC grade, UV cutoff 190 nm, Fisher Scientific, Fair Lawn, NJ). The mixture was shaken and kept at −20° overnight to allow separation of the phases. Upon centrifugation, the organic phase was collected and evaporated to dryness under nitrogen; 90–94% of the radioactivity was recovered in the organic phase. The dried residue was reconstituted in 200 μl

acetonitrile, and a 50-μl aliquot was analyzed for arachidonic acid by HPLC (Altex Chromatograph model 110A, Altex Scientific Inc., Berkeley, CA) equipped with a Schoeffel variable wavelength ultraviolet absorbance detector (Kratos Analytical, Ramsey, NJ). The mobile phase was a linear gradient of acetonitrile/water/acetic acid ranging from 70:30:0.01 to 100:0:0 (by vol.) over a 30-min elution period at a flow rate of 1 ml/min. The eluate was monitored at 193 nm and was collected at 1-min intervals. Radioactivity was determined by liquid scintillation spectroscopy. Standard arachidonic acid (10–100 ng) was chromatographed under the same conditions, and the height of each peak was measured. A standard curve of peak height versus arachidonic acid concentration was constructed.

Radioimmunoassay (RIA) for PGE_2 and LTC_4. The levels of PGE_2 and LTC_4 in the incubation medium were assayed with a PGE_2 [^{125}I] and LTC_4 [^{3}H] kit (New England Nuclear). Aliquots of 25, 50, and 100 μl of the incubation medium (either undiluted or diluted 1:10) were assayed. The sensitivities of the assays for PGE_2 were in the range of 0.25 to 25 pg and for LTC_4 20–1600 pg. The extent of cross-reactivity between PGE_2 and other known prostaglandins and eicosanoids was less than 4% and that between LTC_4 and most known prostaglandins and other eicosanoids was less than 10% except for 11-*trans* LTD_4 and LTD_4 (cross-reactivity was 60.5 and 55.3% respectively). Thus, inclusion of some LTD_4 in the measurement could not be ruled out.

Lipid analysis. Lipids from the cell suspensions or monolayers prelabeled with [^{14}C]arachidonic acid were extracted by a modified procedure of Bligh and Dyer [19]. Briefly, 1.5 ml of chloroform and methanol mixture (1:2) was added to 400 μl of the cell suspension to form a miscible system. A further 500 μl of chloroform and 500 μl of 2 M KCl in H_2O were then added to achieve separation of organic and aqueous phases. The organic phase was washed once with 2 M KCl. The final chloroform layer was mixed with the Lipid standards, PC, PS, PI, and PE, either individually or in combination. The mixture was dried under nitrogen, reconstituted in chloroform, and applied to the plate. The solvent system of Allan and Cockcroft [20], $CHCL_3/CH_3OH/CH_3COOH/H_2O$ = 75/45/3/1, by vol., was used to separate the phospholipids on silica gel plates (precoated, silica gel 60F-254, E. Merck). Spots were visualized by iodine vapor. The gel was scraped from the plates in bands of 1.0 cm, suspended in Aquasol gel (Aquasol/H_2O= 7.5/2.5, v/v) and asayed for radioactivity by liquid scintillation counting. The identity of radioactive substances was established by cochromatography with the authentic standards.

Assay of lactate dehydrogenase. The activity of LDH in medium and cell lysates was analyzed by the Sigma No. 226-UV assay kit. Carrageenan, Triton X-100, and all drugs at concentrations used did not interfere with the assay.

For total intracellular LDH activity, cells were lysed with 0.1% Triton X-100. Completeness of extraction was tested by sonification, which did not promote additional release of LDH.

Assay of plasma membrane integrity. Cell counts were determined in a hemocytometer. Cell viability was determined by fluorescein diacetate and ethidium bromide dyes [21]. Viable cells were identified by the green cytoplasmic fluorescence exhibited by cells upon incubation with fluorescein diacetate, whereas membrane damage was indicated by orange fluorescence of the nucleus by ethidium bromide. At least 500 cells per sample were examined.

Presentation of data. All values reported are mean ± SE of the number of samples indicated. The released LDH activity is expressed as a percentage of the total cellular LDH activity.

RESULTS

Incorporation of [^{14}C]arachidonic acid into cellular lipid and distribution of radioactivity in pleural cells. In pleural cell suspensions (1×10^6 cells/ml), incorporation of radiolabel was related to the concentration of [^{14}C]arachidonic acid, and near maximal incorporation (40,001 ± 4,846 dpm/10^6 cells) was achieved with 1.5 μM. Most (72–82%) of the total radioactivity incorporated into cells was localized in cellular lipid. The principal lipids labeled were PC and PE. The remaining radiolabel was distributed among PI, PS, and the neutral lipids. A similar pattern of incorporation was observed with the adherent pleural cells (Table 1).

Relationship between carrageenan-stimulated release of radiolabel and lactate dehydrogenase. In the absence of carrageenan, there was some release of ^{14}C-label (Fig. 1A), but no significant leakage of LDH was observed at any of the time points studied (Fig. 1B). This spontaneous release generally accounted for less than 5% of the total cellular radiolabeled compounds. Carrageenan (10 μg/ml) stimulated significant release of ^{14}C-label into the medium by 15 min (Fig. 1A). There was no release of LDH at 15 min, but by 30–45 min there was significant release of the enzyme (Fig. 1B). Stimulated release of ^{14}C-label and LDH occurred with as little as 5 μg/ml of carrageenan and was maximum at 25–100 μg/ml (Fig. 2). Although the release of LDH was associated with the release of ^{14}C-label in response to different concentrations of carrageenan, there was some dissociation between the two

Table 1. Incorporation of [^{14}C]arachidonic acid into cellular lipids of pleural cells

Pleural cells	Percent incorporation				
	PC	PE	PI	PS	NL
Suspension	65	14	5	3	13
Adherent	61	19	9	4	7

Values are the average of two chromatographs. The differences between the values in the two experiments are less than 10%. Total cellular lipids were extracted from [^{14}C]arachidonic acid-labeled cells and chromatographed (solvent system: $ChCl_3/CH_3OH/CH_3COOH/H_2O$ = 75/45/3/1, by vol.) on silica gel plates. Spots were visualized by iodine vapor. Gel was scraped from the plates in bands of 1.0 cm and assayed for radioactivity by liquid scintillation spectroscopy. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; and NL, neutral lipids.

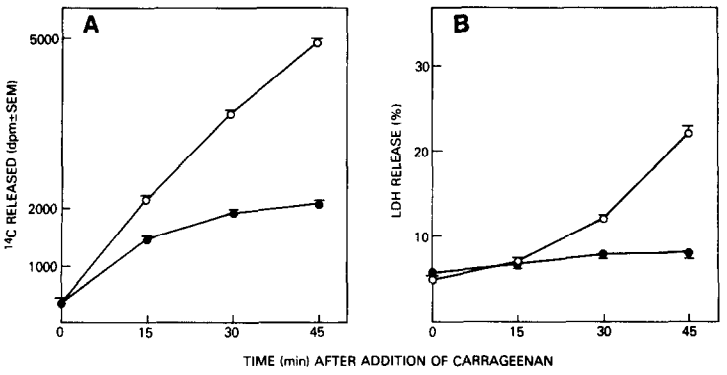

Fig. 1. Time course of release of radiolabel and LDH from [^{14}C]arachidonic acid-labeled pleural cells in response to carrageenan. The isolated pleural cells were incubated (37°) with [^{14}C]arachidonic acid in a buffered salt solution (Hanks–25 mM HEPES–0.1% bovine serum albumin, pH 7.4) for 90 min in 95% air–5% CO_2. The ^{14}C-labelled cells were washed twice and resuspended in the buffered salt solution to give 1×10^6 cells/ml. Saline or carrageenan (40 μl) was added to the cell suspension (360 μl) and, at the indicated times, the medium was withdrawn and centrifuged, and the supernatant fluid was analyzed for LDH and radioactivity. Shown in the figure are the released radioactivity (panel A) and LDH (expressed as a percentage of total cellular LDH, 164 ± 5 units/liter, in panel B) in a representative of three experiments. The values are mean ± SE for three cultures. Maximum release represented 12 ± 1% of the total cellular radiolabel. Key: cells incubated with saline (●—●); and cells incubated with carrageenan (10 μg/ml final concentration; O—O).

responses in that release of ^{14}C-label but not that of LDH was appreciable at 1 μg/ml carrageenan (Fig. 2).

Effects of BPB and indomethacin on stimulated release of radiolabel and LDH from pleural cells. Cell damage did not appear to be a consequence or cause of the release of radiolabel. Addition of BPB, a phospholipase A_2 inhibitor, partially blocked the carrageenan-stimulated release of radiolabel but did not prevent release of LDH (Fig. 3). In contrast, the arachidonate cyclooxygenase inhibitor, indomethacin, had no significant effect on the release of either material (Fig. 4).

Cell viability studies. To rule out the possibility that the release of LDH from cells incubated with carrageenan was due to the toxic effect of [^{14}C]arachidonic acid, the cells were incubated (37°) with carrageenan for 45 min in the absence of [^{14}C]arachidonic acid. Under these conditions, carrageenan at concentrations of 10 and 100 μg per ml

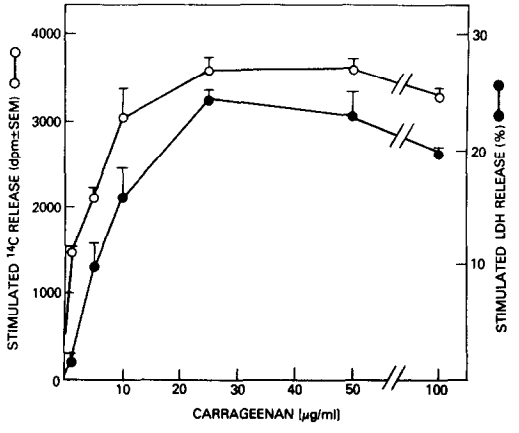

Fig. 2. Increases in the release of radiolabel and LDH from [^{14}C]arachidonic acid-labelled pleural cells in response to increasing concentrations of carrageenan. Labeling of cells and the measurement of the release radiolabel and LDH were performed as described under Materials and Methods. The incubation interval was 45 min. The values shown have been corrected for spontaneous release (release of ^{14}C-label and LDH in the absence of carrageenan was 850 ± 80 dpm and 3 ± 1% respectively) and are mean ± SE for three cultures. Total cellular LDH per 0.36×10^6 cells was 102 ± 7 units/liter.

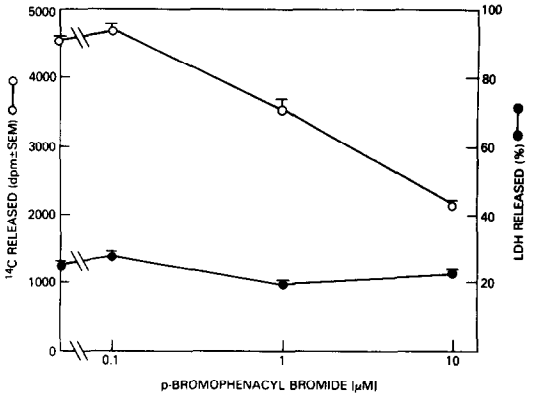

Fig. 3. Effect of *p*-bromophenacyl bromide on carrageenan-stimulated release of radiolabel and LDH from [^{14}C]arachidonic acid-labeled pleural cells. Vehicle (0.1% dimethyl sulfoxide) or BPB at the concentration indicated was incubated (37°) with the cells for 10 min, at which time carrageenan (100 μg/ml) was added. Forty-five minutes later, the released radioactivity and LDH in the incubation medium were assayed. The drug at the concentrations used did not interfere with the assay. Total cellular LDH per 0.36×10^6 cells was 133 ± 3 units/liter; total cellular radiolabel was 16,444 ± 703 dpm. Values are mean ± SE for three cultures.

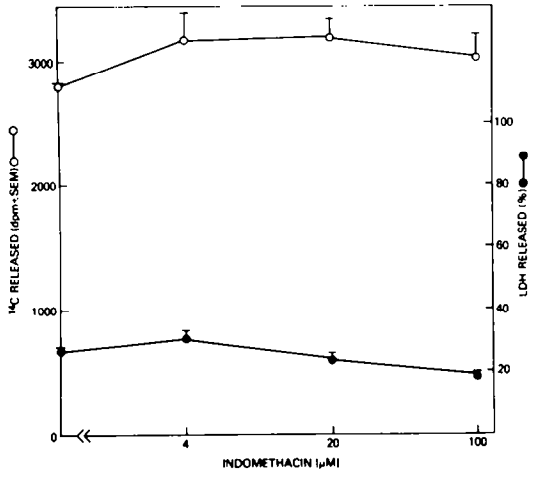

Fig. 4. Effect of indomethacin on carrageenan-stimulated release of radiolabel and LDH from [^{14}C]arachidonic acid-labeled pleural cells. Vehicle (0.1% dimethyl sulfoxide) or indomethacin (4, 20, or 100 μM final concentration) was added to the cell suspension, and 10 min later carrageenan (100 μg/ml) was added. After a further incubation of 45 min, the released radioactivity and LDH in the incubation medium were assayed. Indomethacin at the concentrations used did not interfere with the assay. Although the slight increase in radioactivity was not statistically significant, subsequent studies showed enhancement of [^{14}C]arachidonic acid release by indomethacin. Values are mean ± SE for three cultures. Total cellular LDH: 104 ± 7 units/liter per 0.36 × 10^6 cells.

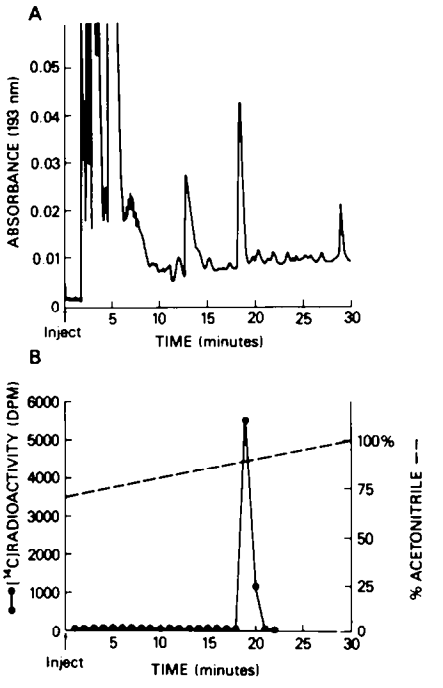

Fig. 5. HPLC elution profile of an acetonitrile extract of the incubation medium from carrageenan-treated pleural cells. To have sufficient samples for the measurement, medium from six tubes (0.36 × 10^6 cells/tube) was pooled for the extraction and chromatography. The mobile phase consisted of a linear gradient of acetonitrile–water–acetic acid (70:30:0.01) to acetonitrile–water–acetic acid (100:0:0) over 30 min at a flow rate of 1 ml/min. Panel A shows the absorbance at 193 nm, and panel B shows the radiochromatographic distribution.

caused release of 15 and 21% of the cellular LDH respectively.

Membrane damage resulting from exposure of pleural cells to carrageenan was further demonstrated by staining with ethidium bromide. Upon incubation with 100 μg/ml of carrageenan for 45 min, 25 ± 4% of the cells were nonviable as compared to the 8 ± 2% nonviable cells in the control cells.

Identification of ^{14}C-label in cell medium. Under the conditions described in Materials and Methods, negligible amounts (<5%) of PGE_2 and LTC_4 were extracted, whereas over 90% of the arachidonic acid was recovered in the acetonitrile extract. With our HPLC systems and elution conditions, the standard arachidonic acid was eluted at 19 min. Shown in Fig. 5 are the elution profiles of an acetonitrile extract of medium separated from pleural cells after incubation with carrageenan. Panel A shows the absorbance measured at 193 nm, and panel B shows the distribution of radioactivity of the same sample. A single radiolabeled peak was eluted from the column at a retention time identical to that of authentic arachidonic acid, i.e. 19 min. Based on the radioactivity of the peak in panel B and the height of the corresponding peak in panel A, the released [^{14}C]arachidonic acid was calculated to have a radiospecific activity of 8.99 ± 0.18 μCi/μmol which is equivalent to 29.6 ± 0.6 nCi/μg (N = 3). This conversion index was used to quantify arachidonic acid in all the release studies.

Carrageenan-stimulated release of arachidonic acid and PGE_2: Effect of indomethacin. Both carrageenan (100 μg/ml) indomethacin (20 μM) stimulated release of arachidonic acid from the pleural cells and, in combination, the agents further increased this release (Table 2). Carrageenan also stimulated formation of PGE_2, whereas indomethacin did not. Indeed, indomethacin blocked the formation of PGE_2, presumably by inhibiting the cyclooxygenase activity.

Responsiveness of adherent pleural cells to carrageenan. The cells that adhered to culture plates were predominantly macrophages (90 ± 5%) and mast cells (5 ± 2%). These cells responded to the same concentration range of carrageenan as mixed pleural cells (Fig. 6). Significant stimulation of ^{14}C release was observed with as low as 1 μg/ml of carrageenan, and further increases in the release were observed with increasing concentrations. In a separate experiment, the released radiolabel was analyzed for arachidonic acid (by HPLC) and for PGE_2 and LTC_4 (by RIA). The results in Table 3 show that carrageenan, at a concentration of 100 μg/ml, caused a 2.5-fold increase in the release of arachidonic acid and a substantial production of PGE_2 and LTC_4. Still greater production of the compounds was observed with 500 μg/ml. The data suggest that adherent cells were more responsive to carrageenan than cell suspension (cf. Tables 2 and 3).

Table 2. Effect of carrageenan and/or indomethacin on arachidonic acid release and PGE_2 production by pleural cells

	Arachidonic acid (ng)	PGE_2 (ng)
Vehicle	50.1 ± 2.1	0.029 ± 0.001
Carrageenan (100 μg/ml)	99.3 ± 5.3	0.074 ± 0.003
Indomethacin (20 μM)	84.0 ± 4.0	0.013 ± 0.001
Carrageenan (100 μg/ml) + Indomethacin (20 μM)	140 ± 5.0	0.013 ± 0.0004

Vehicle (0.05% dimethyl sulfoxide), indomethacin, or carrageenan at the concentration indicated was incubated (37°) with the cells (1×10^6/ml) for 55 min. In the experiment in which the effect of indomethacin on the release stimulated by carrageenan was examined, the cells were first incubated with indomethacin for 10 min, at which time carrageenan was added and incubation was continued for another 45 min. Arachiidonic acid and PGE_2 were analyzed as described under Materials and Methods. Each value is the mean ±SE of triplicate samples.

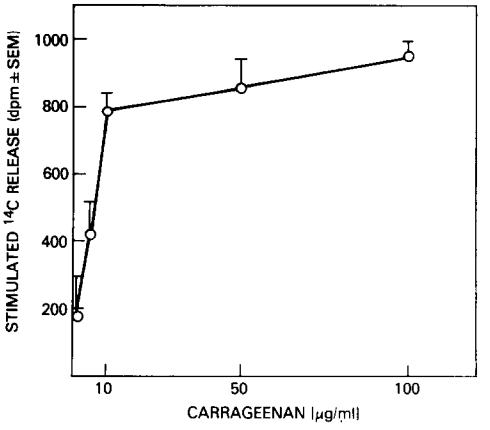

Fig. 6. Release of radiolabel from [^{14}C]arachidonic acid-labeled adherent pleural cells in response to increasing concentrations of carrageenan. Pleural cells were incubated (37°) with [^{14}C]arachidonic acid in culture plates for 2 hr in 9% air–5% CO_2. The non-adherent cells were removed by washing twice with Hanks–25 mM HEPES–0.1% bovine serum albumin. The indicated concentration of carrageenan was added to the adherent cells, and 45 min later the radioactivity released into the incubation medium was assayed. The values shown have been corrected for spontaneous release and are mean ± SE for three cultures.

At 10 and 100 μg/ml, carrageenan caused 11 ± 1 and 18 ± 2% LDH release respectively.

Studies with rat submandibular gland cells and human mesothelioma cells. These cell cultures did not respond to a wide range of concentrations of carrageenan (1–500 μg/ml; data not shown). Shown on panel A of Fig. 7 are the released radiolabel from control and carrageenan-treated submandibular gland cells prelabeled with [^{14}C]arachidonic acid. Although unresponsive to carrageenan, these cells released significant amounts of radiolabel upon exposure to the ionophore A23187 (1 μM) in a time-dependent manner (Fig. 7, panel B).

DISCUSSION

Inflammatory reactions induced by the intrapleural injection of carrageenan have been widely used as a model in the study of acute inflammation. That arachidonate metabolites mediate plasma exudation as an early event in the carrageenan-induced response was demonstrated by the efficacy of the aspirin-like drugs in suppressing this response [7, 16, 22]. Since inhibitors of arachidonate cyclooxygenase are much more effective than those of arachidonate lipoxygenase in suppressing the carrageenan-induced inflammation [14], product(s) derived from the cyclooxygenase pathway must play an important role in mediating the response. Among the reported vasoactive metabolites of arachidonic acid, PGE_2 is known to have vasodilatory activity [23, 24]. Synergistic effects of LTD_4 and PGE_2 on increased vascular permeability have also been noted [24].

The present data indicate that carrageenan in pharmacological concentrations induces release from

Table 3. Effect of carrageenan on arachidonic acid release, PGE_2 and LTC_4 production by adherent pleural cells

	Arachidonic acid (ng)	PGE_2 (ng)	LTC_4 (ng)
Saline	72.0 ± 1.8	0.032 ± 0.001	ND*
Carrageenan			
100 μg/ml	177.3 ± 6.3	0.135 ± 0.001	0.720 ± 0.050
500 μg/ml	205.3 ± 5.1	0.234 ± 0.010	0.830 ± 0.040

Pleural cells (1.5×10^6/1.5 ml) were incubated (37°) with [^{14}C]arachidonic acid in culture plates for 2 hr at 37° (95% air–5% CO_2). The nonadherent cells were then removed. The adherent cells (~1.2×10^6 cells/well) were incubated (37°) with either saline or the indicated concentrations of carrageenan for 45 min, at which time the medium was withdrawn and centrifuged, and the supernatant fraction was extracted and analyzed for arachidonic acid, PGE_2, and LTC_4 as described in Materials and Methods. Values were determined from pooled samples of three separate cultures.

* Not detectable.

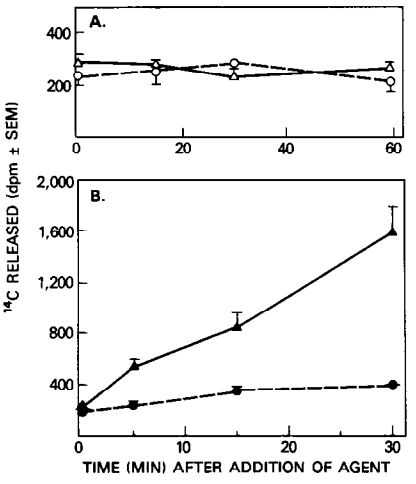

Fig. 7. Time course of release of radiolabel from [^{14}C]arachidonic acid-labeled rat submandibular gland cells in response to carrageenan (panel A) or the ionophore A23187 (panel B). Primary cultured submandibular gland monolayers were incubated (37°) with [^{14}C]arachidonic acid in Hanks–25 mM HEPES–0.1% bovine serum albumin (pH 7.4), which contained 1.3 mM Ca^{2+}, for 2 hr in 95% air–5% CO_2. The ^{14}C-labelled cells were washed twice and were incubated with either carrageenan (100 μg/ml) or A23187 (1 μM) for the time indicated. The radioactivity released into the incubation medium at each time point was analyzed as described under Materials and Methods. Values are mean ± SE for three cultures. Key: cells incubated with saline (○), carrageenan (△), 0.05% dimethyl sulfoxide (●), and A23187 (▲).

resident pleural cells. This action could contribute, therefore, to the initial stages of the inflammatory response *in vivo*, but it should be noted that the mechanism underlying the inflammatory response to carrageenan is not clearly understood. A scheme, proposed by Vinegar *et al.* [7], ascribes the edema formation as being dependent on the mobilization of neutrophils and largely independent of resident pleural cells. As exudate neutrophils convert arachidonate to thromboxane and prostaglandins [14], it is possible that arachidonate released from pleural macrophages may serve as substrate for the incoming neutrophils, but many other sites of arachidonate metabolism can be envisaged.

Our previous studies [5, 15] indicated that an inflammatory exudate of 1.1 to 1.5 ml was induced following the injection of 500 μg carrageenan (in 0.1 ml saline) into the pleural cavity of rats. As the total measurable fluid in the cavity was estimated to be less than 0.01 ml [15] in untreated rats and up to 1.5 ml in carrageenan-injected rats, concentrations of 1–100 μg/ml of carrageenan used in the present study are pharmacologically relevant. We detected both arachidonic acid and PGE_2 in the incubation medium of unstimulated pleural cells; the production of PGE_2 but not the release of arachidonic acid was suppressed when the cells were incubated with indomethacin. The results are thus consistent with the reports that, in resting cells, phospholipase A_2 and cyclooxygenase are active, the latter being capable of converting the released arachidonic acid to the prostaglandins [24, 25]. Carrageenan about doubled the release of arachidonic acid and PGE_2 from the pleural cells. The increase in the release of PGE_2 caused by carrageenan was blocked, as expected, by indomethacin. However, the drug enhanced the release of arachidonic acid induced by carrageenan; indeed, the effects were nearly additive. Indomethacin is known to cause release of arachidonic acid from bone marrow-derived macrophages, presumably by inhibition of the lysophosphatide acyltransferase [18], the principal enzyme thought to control the level of cellular free arachidonic acid [18, 26].

Pathways of phospholipid metabolism implicated in the release of arachidonic acid from leukocytes include (see review in Ref. 27): (1) deacylation of phospholipids (mainly PC) by a phospholipase A_2 and (2) cleavage of arachidonic acid from 1,2-diacylglycerol (derived mainly from PI by the action of phospholipase C) by a diacylglycerol lipase A_2. It seems likely that arachidonic acid is released from the pleural cells largely through the first pathway for the following reasons. First, BPB is a potent inhibitor of phospholipase A_2 in macrophage homogenate [18] as well as in other systems [28, 29], and it did inhibit the carrageenan-stimulated release of [^{14}C]arachidonic acid (Fig. 3), although there is evidence that this agent can inhibit phospholipase C–diacylglycerol lipase A_2 in platelet [30]. Second, carrageenan failed to stimulate hydrolysis of inositol phospholipids in pleural cells prelabeled with [^{3}H]inositol (Lo and Beaven, unpublished observation). This result implies that the observed release of arachidonic acid was not dependent on activation of phospholipase C. Also of note is that, under our experimental conditions for radiolabeling, over 60% of the [^{14}C]arachidonic acid was incorporated into PC, a pattern of incorporation similar to that reported for mouse peritoneal macrophages [25]. Nevertheless, our data do not exclude the possibility that arachidonic acid was released from several classes of phospholipids.

An interesting finding was that the release of LDH from the isolated pleural cells occurred with as little as 5 μg/ml of carrageenan. Increased LDH levels were detected in the exudate after the intrapleural injection of 500 μg carrageenan [31]; however, others have reported no change in LDH levels in the pleural exudate [32]. Although arachidonic acid at concentrations above 1 μM has been reported to be toxic to rat mast cells [33] and mouse macrophages [34], we have demonstrated conditions at which carrageenan caused release of LDH from pleural cells in the absence of exogenous arachidonic acid. Moreover, BPB suppressed the release of radiolabels but did not prevent the release of LDH. Our interpretation of the data is that release of arachidonic acid and cytotoxicity are separate events and that leakage of LDH from the pleural cells may be due to a toxic action of carrageenan.

There are indications that phagocytosis of macromolecules can lead to macrophage and neutrophil injury [35–37]. Cytotoxic effects of carrageenan on guinea pig and mouse macrophages (though at much higher concentrations, i.e. 300–200 μg/ml, than those used here) and on exudate neutrophils have been reported [36, 37]. Ultrastructural studies by

these investigators showed that carrageenan was taken up by the cells, stored in lysosomes (which subsequently swelled and ruptured), and caused cell death. Carrageenan at 300–2000 μg/ml, however, was not toxic to the lymphocytes. Recently, it has been demonstrated that highly purified carrageenan, at a concentration of 100 μg/ml, is toxic to human macrophages but not to lymphocytes [38]. Because lymphocytes are generally nonphagocytic, it was inferred that the polysaccharide had to be phagocytosed in order for its toxic effects to be realized. These findings may be consistent with the results of our time–course studies which showed a delay in the release of LDH from carrageenan-treated cells as compared to the immediate release of the radiolabel.

Although our data do not reveal the source of the released arachidonic acid and its metabolites, the following evidence points to the macrophages as the most likely candidate: (1) Macrophages constitute 70–85% of pleural cell populations of rats. Also, arachidonic acid is one of the major unsaturated fatty acids at the 2-position of phosphoglycerides in macrophage [39, 40]. (2) Among the identifiable cell populations in the pleural wash, few eosinophils and lymphocytes adhered to the culture plate under our experimental conditions. Adherent cells were at least equally and perhaps more responsive to carrageenan than mixed pleural cells. Most of the adherent cells were macrophages. (3) On addition of carrogeenan, the adherent cells released significant amounts of LTC_4; macrophages are known to synthesize and release leukotriene C into the extracellular milieu in response to the phagocytic zymosan particles [41]. (4) The inflammatory response to carrageenan *in vivo* was neither associated with histamine release from mast cells nor inhibited by the antihistamines [8, 9]. Pleural mast cells obtained from rats previously injected with carrageenan appeared intact [15], although this appeared not to be the case for injection of carrageenan into the rat paw [42].

Finally, the time course and concentration dependency of the release observed in the present study are compatible with the *in vivo* action of carrageenan. Taken together, the results suggest that the primary effect of carrageenan as an inflammatory agent is probably on the activation of phospholipase A_2, though its cytotoxic effect may initiate further inflammatory reactions. Furthermore, although the cells of the pleura [7], the infiltrating neutrophils [11, 13], and other tissues [11] have been proposed as target cells for carrageenan action, our data suggested that the macrophage, in addition, may serve as an important source of mediators in this and other acute inflammatory reactions, as has been suggested by Rouzer *et al.* [41].

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