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Actions of a Leukocytic Inflammatory Substance on Isolated Tissue Responses (38972)

RICHARD P. CARLSON, MARTIN L. OGLETREE, AND ALLAN M. LEFER

Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107
Department of Physiology,

Leukocytic pyrogen (LP) was first prepared from polymorphonuclear (PMN) leukocytes from rabbit peritoneal exudate by Beeson in 1948 (1). For the past 25 yr, LP was investigated very intensively by several groups including those of Wood (2), Moses *et al.* (3), and Atkins and Bodell (4). Petersdorf and Bennett (5) first demonstrated that PMN leukocytes from dog peritoneal exudate released LP. Van Arman and Carlson (6, 7) prepared a substance from exudated leukocytes of the dog synovial space that caused fever when injected intravenously and acute inflammation when injected into synovial space. They named this substance leukocytic inflammatory substance (LIS), since it exhibited several properties which differentiated it from LP.

The purpose of the present study was to determine the effects of LIS on isolated tissue preparations in order to obtain information on the mechanism of the inflammatory action of LIS.

Methods. Preparation of LIS. All equipment used in the experiments was pyrogen-free, (i.e., heated at 200° for 6 hr) except for the glass vials containing the carrageenan solution. No anticoagulant was used in collection and preparation of LIS. Adult dogs of either sex weighing 11–15 kg were injected with 3 mg of carrageenan (Marine Colloids-RENJ 5043) in 0.75 ml of pyrogen free saline (PFS) (Ambot, Cutter Labs.) into the synovial space of both stifles (i.e., knee joints) (8). After 3 hr of acute inflammation, the exudate was aspirated from the stifles and placed into conical centrifuge tubes in an ice bath. If the exudate was heavily contaminated with erythrocytes (i.e., deep red color), it was discarded. The amount of exudate collected varied from 6 to 10 ml, and the WBC count was from 80,000 to 120,000 cells/mm³. Neutrophils made up 95% of the cell population, the remaining 5% being mononuclear cells. All subsequent procedures were carried out at 4°. The exudate was centrifuged at 400*g* for 15 min. After centrifugation, the viscous, clear, cell-free supernatant was discarded and a volume of PFS was added to the pellet which doubled the exudate volume. The pellet and PFS were vigorously mixed for 15 sec and centrifuged at 400*g* for 15 min. After each centrifugation, the supernatant was discarded, and the previous step was repeated. After the second wash and spin, the cell pellet was reconstituted with PFS and mixed. This suspension was incubated, at 37° for 1 min to remove debris from the cell surfaces, and recentrifuged at 400*g* for 15 min. The cell pellet was then resuspended to its original exudate volume with PFS, and the cells were counted and diluted to a concentration of 50 million/ml of PFS. The suspended cells were transferred to 25-ml glass-stoppered Erlenmeyer flasks and incubated with gentle agitation at 37° for 45 min to generate the LIS. After 45 min, the flasks were placed into an ice bath. Finally, the cell suspension was centrifuged at 400*g* for 15 min; the supernatant (LIS) was aspirated and filtered through a 0.22 μm Millipore filter. Bacterial and fungal tests were performed on the filtrates to ensure that the samples were not contaminated.

Isolated lysosomes of cat liver. Livers were rapidly removed from pentobarbital-anesthetized cats and placed into cold 0.25 *M* sucrose and 0.02 *M* Tris buffer at pH 7.3 (9). Homogenates (1:5, w:v) were prepared in a Waring Blendor from 25 g of liver and were centrifuged at 1000*g* for 15 min. The supernatants were recentrifuged at 15,900*g* for 30 min and the pellets were washed with and resuspended in buffer. Each aliquot consisted of 4.6 ml of lysosomal fractions and 0.4 ml LIS or 0.4 ml of 0.9% NaCl as

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a control. The aliquots were incubated at 37° in a shaking incubator for 30 min. Release of lysosomal enzymes was stopped by placing the flasks in an ice bath. The samples were recentrifuged at 15,900*g* for 30 min. The supernatant containing the free enzyme activity was filtered and assayed; the pellets, containing the bound lysosomal enzymes, were lysed with 0.1% Triton X-100 and homogenized. After incubation at room temperature with Triton X-100, the pellets were centrifuged at 15,900*g* for 30 min. The supernatant was filtered and retained for assay. Cathepsin D and β-glucuronidase activities were measured in both lysosomal fractions.

Cat papillary muscle. The muscles were isolated and placed in 10-ml chambers suspended in a modified Krebs-Henseleit solution at 37° (10). Muscles were stimulated at a frequency of 1/sec for a duration of 16.7 msec at 2 V above threshold. All muscles were individually stretched to a length just less than that producing the greatest active tension. Isometric contractions were recorded on an oscillographic recorder using Grass FT-03 force transducers. LIS and myocardial depressant factor (MDF) were studied after an initial equilibration period of about one hour. Each muscle received 0.5 ml of LIS or 0.5 ml of 0.9% NaCl. Muscles were later subjected to MDF activities comparable to that present in shock plasma to test the responsiveness of the muscles.

Cat jejunum. Bradykinin formed from the action of kallikrein upon plasma kininogen was assayed by the isolated cat jejunal strip technique of Ferreira and Vane (11). Strips (2–3 cm long and 5–7 mm wide) were placed in oxygenated (i.e., 95% O_2 + 5% CO_2) Krebs-Henseleit solution at 37°. The strips were stretched to a resting tension of 1.4 g. After an equilibration period of 60 min, 0.5 ml LIS was added to the bath. Bradykinin triacetate (Sigma) at a bath concentration of 5 ng/ml was used as a standard, and 0.9% NaCl as a control. Kallikrein (Bayer) and cat plasma kininogen (12) were employed to form bradykinin.

Cat aortic strip. Helical strips of fresh thoracic aorta were used to obtain consistent results. The dimensions of the strips were 18–20 × 2–3 mm. The strips were placed into 20-ml tissue baths in freshly prepared Krebs-Henseleit solution and were continuously oxygenated with 95% O_2 and 5% CO_2 at 37°. The strips equilibrated for 120 min before the administration of any test agent. A tension of 2.1 g was maintained on the strips. LIS was tested by adding 0.5 ml to the bath; an equal volume of 0.9% NaCl was the control. Norepinephrine at a final concentration of 10 ng/ml was used as a standard.

Platelet aggregation. LIS at 0.02–0.04 ml was added to dog platelet-rich plasma (0.45 ml) and tested directly for platelet aggregation in a Payton Dual Channel Aggregation Module, as well as for its ability to enhance or inhibit ADP-induced platelet aggregation.

$PGF_{2\alpha}$ activity. LIS preparations were assayed for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by a specific radioimmunoassay according to the method of Flynn *et al.* (13).

Biochemical determinations. The supernatants containing LIS and liver lysosomes were assayed for activities of the lysosomal hydrolases, cathepsin D, and β-glucuronidase using the method of Anson (14) and Talalay *et al.* (15), respectively. The protein concentrations of the supernatants of LIS were determined using the method of Lowry *et al.* (16).

Results. LIS at 0.4 ml in the suspension of cat liver lysosomes increased the release of cathepsin D by 9% and β-glucuronidase by 7% compared to control samples incubated at 37° for 30 min (Fig. 1). Thirty-minute samples were chosen, because release of lysosomal hydrolases peaked at 30 min.

Figure 2 shows LIS at 0.5 ml in the bath to have no effect on developed tension of cat jejunum, aortic strip, and papillary muscle, and standards (i.e., bradykinin, norepinephrine, and myocardial depressant factor) were employed in the muscle preparations to show that each isolated muscle system was responsive to appropriate stimuli. Moreover, LIS contained no proteolytic enzymes which could split cat plasma kininogen to form bradykinin when tested on the cat jejunum, nor did LIS inhibit the amount of brady-

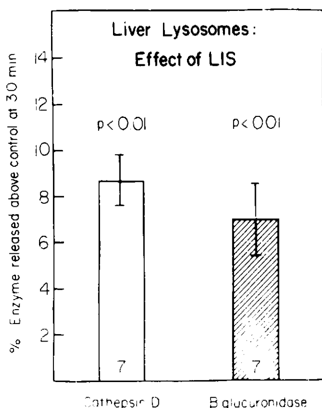

FIG. 1. Effect of 0.4 ml LIS on release of lysosomal hydrolases from cat liver lysosomes after 30 min of incubation at 37°. Bar heights represent means ± SEM. Numbers within bars indicate number of experiments.

kinin formed by the action of pancreatic kallikrein on the plasma kininogen in the system.

Dog platelets were not aggregated by LIS at 0.02–0.04 ml alone, nor was ADP-induced aggregation enhanced or inhibited by LIS. Prostaglandin $F_{2\alpha}$ activity was absent in LIS fractions or present only at concentrations less than 40 pg/ml of LIS when measured by a specific radioimmunoassay.

Table I summarizes the cathepsin D (2.84 ± 0.20 units/mg) and β-glucuronidase (15.5 ± 3.3 units/mg) activities in LIS preparations. The protein concentration by the Lowry method was 0.34 ± 0.04 mg/ml. These hydrolases and protein were released from a suspension of neutrophils at a concentration of 50 million/ml, incubated at 37° for 45 min, and represent only modest amounts of these enzymes.

Since no bacterial or fungal contaminations were detected in any of the LIS preparations, the inflammatory action of LIS is not due to direct microbial action, but rather to a humoral component of the extract.

Discussion. We tested the effects of LIS on the isolated smooth muscle of cat jejunum and thoracic aortic strip, and no change in developed tension was observed at amounts of LIS which produce an acute inflammation in the intact dog (6). This inactivity may be due in part to species specificity of LIS (5, 6, 17) or to the absence of intrinsic activity of the LIS preparation on muscle tissue.

The cat papillary muscle did not respond to LIS despite the fact that MDF, a small peptide generated during shock (18), depressed the developed tension of this preparation. This finding indicates that substances released from inflamed neutrophils do not significantly inhibit myocardial contraction. Experiments by Van Arman and Carlson (6) on isolated guinea pig ileum and rat uterus had previously indicated that LIS did

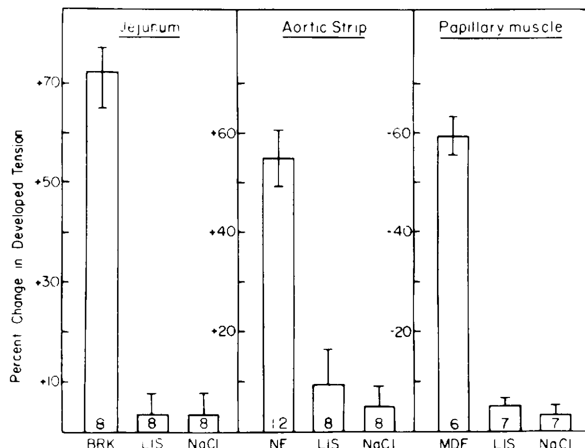

FIG. 2. Responses of isolated cat jejunum, aortic strip, and papillary muscles to 0.5 ml LIS. Bradykinin (BRK) at 5 ng/ml, norepinephrine (NE) at 10 ng/ml, and myocardial depressant factor (MDF) of 60 units/10 ml were the bath concentrations for responses shown. Bar heights represent means ± SEM. Numbers within bars indicate number of experiments.

TABLE I. LYSOSOMAL ENZYME ACTIVITY AND PROTEIN CONCENTRATION OF LIS.

Cathepsin D activity[a]	β-Glucuronidase activity[b]	Protein[c]
2.84 ± 0.20	15.5 ± 3.3	0.34 ± 0.04

All values are means ± SEM for six separate batches of LIS.

[a] 1 Cathepsin D unit equals 1 mEq of tyrosine X 10^{-4} released/hr/mg protein incubation at 37° from bovine hemoglobin.

[b] 1 β-glucuronidase unit equals 1 μg of phenolphthalein released hr/mg protein at 37° from phenolphthalein glucuronide.

[c] Protein concentration in LIS extract, expressed as mg/ml.

not exert activity on these smooth muscle preparations. Since LIS prepared from dogs was able to release lysosomal enzymes from cat liver lysosomes, there is not a strict species specificity for LIS. Thus, the more probable explanation is that LIS has no intrinsic activity on several types of muscle preparations.

The finding that LIS enhances release of lysosomal hydrolases in liver lysosomal fractions indicates that LIS is capable of acting *in vitro*. This means that LIS probably does not require the release of some other active substance, or need to interact with components present in the blood to produce its inflammatory action. Other substances which induce inflammatory effects, such as bacterial endotoxin, appear to require the mediation of other substances. Apparently LIS does not have this requirement.

Experiments designed to test the kinin forming activity of LIS on plasma kininogen were also negative. This finding is consistent with the results of Greenbaum *et al.* (19) who reported the simultaneous release of a kinin-forming enzyme and kininase from stimulated leukocytes; thus, LIS may not have the capacity to release kinins directly during the inflammatory process.

Our studies indicate that cathepsin D and β-glucuronidase, two lysosomal hydrolases, exhibit an enhanced release in the presence of LIS. These hydrolases play a very important part in the propagation of the inflammatory process (20, 21) and may be an important mechanism of the LIS effect. A small amount of lysosomal enzymes were found to exist in the LIS preparation. However, this small amount could not account for the increased release of lysosomal enzymes when LIS was incubated with liver lysosomes. This amount of lysosomal hydrolases originated from PMN leukocytes during the incubation period for generation of LIS. However, these lysosomal hydrolases were probably released without cell lysis, because the WBC counts before and after incubation at 37° for 45 min were the same. Moreover, the neutrophils did not exclude eosin Y, an indicator of nonviable or damaged cells. Therefore, release of the lysosomal hydrolases from the intact cells probably occurred by granule extrusion, exocytosis, or the formation of phagolysosomes.

LIS failed to exert a significant effect on dog platelets, which suggests that LIS does not contain cationic proteins similar to those described by Janoff and Zweifach (22) in leukocytic suspensions. Moreover, the absence of $PGF_{2\alpha}$ activity in LIS may be due to the fact that $PGF_{2\alpha}$ was oxidized to a metabolite (i.e., 15 keto $F_{2\alpha}$) or to the fact that only insignificant amounts of $PGF_{2\alpha}$ are formed by inflamed neutrophils. At present, LIS does not appear to exert its action via release of $PGF_{2\alpha}$, although we have no data to indicate whether other prostaglandins may be involved.

Additional investigation is necessary to further purify LIS and determine its biochemical identity. Development of a sensitive bioassay for its activity would be helpful in this regard. At present, release of lysosomal enzymes appears to be one mechanism of the inflammatory action of LIS, although *in vivo* probably other mechanisms are involved.

Summary. LIS had no action on the mechanical activity of isolated cat vascular, intestinal smooth, or cardiac muscle. No effect on platelet aggregation was observed, and $PGF_{2\alpha}$ activity was absent in LIS preparations. Isolated lysosomal enzyme release was increased significantly when LIS was added to the incubation medium. This action may help to explain the inflammatory action

of this naturally occurring material found in the inflamed synovial fluid of the canine knee joint.

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