

## MAST CELL HEPARIN STIMULATES MIGRATION OF CAPILLARY ENDOTHELIAL CELLS IN VITRO\*

BY RICHARD G. AZIZKHAN, JANE CLIFFORD AZIZKHAN, BRUCE R. ZETTER,‡  
AND JUDAH FOLKMAN

*From the Department of Surgery, Children's Hospital Medical Center and the Department of Microbiology  
and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115*

It is now recognized that angiogenesis, the induction of capillary proliferation, is important for the continued growth of solid tumors (1-3). Kessler et al. (4) reported that mast cells assemble at a tumor site before the ingrowth of new capillaries; it was proposed that mast cells may play a role in tumor angiogenesis. Mast cells are also found in increased numbers in other pathologic states associated with angiogenesis, such as psoriasis (5, 6), chronic inflammation (7, 8), and immune rejection (9, 10). However, the exact function of the mast cells in angiogenesis is not understood.

Previously, Ausprunk and Folkman (11) reported that capillary endothelial cell migration was one of the major components of the growing capillary sprouts that arise in angiogenesis. After bovine capillary endothelial cells were cloned and carried in long-term culture (12) in this laboratory, Zetter (13) developed a quantitative assay in vitro for measuring the migration of these cells. In this assay, bovine capillary endothelial cells were plated sparsely on glass cover slips coated with colloidal gold so that their migration generated a phagokinetic track, the area of which could be measured accurately.

We now report that mast cells release a factor that stimulates migration of capillary endothelial cells. We have investigated the effect of isolated mast cell products on bovine capillary endothelial cell migration, and we demonstrate that heparin is the mast cell factor responsible for capillary endothelial cell migration in vitro.

### Materials and Methods

*Assay for Endothelial Cell Migration.* 3,000 cells (bovine capillary endothelial, MDCK, Swiss 3T3, human fibroblast, or bovine aortic endothelial cells) were seeded onto 22-mm<sup>2</sup> glass cover slips (Rochester Scientific Co., Rochester, N. Y.) that were coated with colloidal gold, which was prepared according to the method of Albrecht-Buehler (14, 15). The cover slips were incubated in tissue culture dishes containing 1 ml of Dulbecco's modified Eagle's medium (DMEM)<sup>1</sup> (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) with 10% calf serum (Gibco Laboratories, Grand Island Biological Co.) at 37°C and 5% CO<sub>2</sub>. After the cells had attached (~2-4 h later), 1 additional ml of conditioned medium (CM) or test medium was added. All media contained a final concentration of 10% calf serum (10% CS). The cultures

\* Supported by grant R01-CA14019 from the U. S. Public Health Service, National Cancer Institute, and by a grant to Harvard University from Monsanto Company, St. Louis, Mo.

‡ Recipient of Glick-Franzheim Fellowship.

<sup>1</sup> Abbreviations used in this paper: BCE, bovine albumin endothelial cell(s); CM, conditioned medium; 10% CS, 10% calf serum; DMEM, Dulbecco's modified Eagle's medium; ECF-A, eosinophil chemotactic factor; GAG, glycosaminoglycan(s); TCA, trichloroacetic acid.

were then incubated for an additional 18 h and the experiment was terminated by replacement of the medium with 2 ml of 10% buffered formalin phosphate (Fisher Scientific Co., Pittsburgh, Pa.). Cells that moved cleared the colloidal gold in their path and left a track. The phagokinetic track images were projected from an inverted microscope (4 × objective) to the screen of a Setchell-Carson 10M915 television by means of an RCA TC1005 video camera (RCA Laboratories, Princeton, N. J.) The images were traced onto sheets of transparent plastic. The areas of these phagokinetic tracks were then measured on a Zeiss MOP-3 digital image analyzer (Carl Zeiss, Inc., New York). To eliminate consideration of the infrequent cell division or cell collisions, only tracks formed by single cells were analyzed. Each point on a graph represents the mean area of 100 migration tracks ± SEM. For a complete description of this assay see Zetter (13).

*Preparation of Mast Cell CM and Lysates.* Mast cells were obtained by lavaging the peritoneal cavities of 200-g Sprague-Dawley rats with Hanks' balanced salt solution without heparin. The cells were purified on a 38% wt/vol bovine albumin gradient using the method of Sullivan et al. (16). An aliquot of a concentrated suspension of the cells was stained with 0.1% toluidine blue and counted with a hemocytometer. Approximately  $2.5 \times 10^5$  mast cells/rat could be harvested, with purity ranging from 85 to 95%. Contaminant cells included erythrocytes, lymphocytes, and occasional macrophages. Mast cell viability after purification as ascertained by trypan blue exclusion was 96–99%. The mast cells were isolated from two to four rats for each experiment. 5 ml of DMEM 10% CS that contain  $1 \times 10^5$  cells/ml were added to a 25-cm<sup>2</sup> Falcon flask (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). After incubation for 36 h at 37°C and 5% CO<sub>2</sub>, ~25–30% of the mast cells had degranulated. The mast cell CM was then removed, filtered through a 0.22- $\mu$ m Millipore filter (Millipore Corp., Bedford, Mass.) to remove any cellular debris, and stored at –20°C for up to 1 mo before use. Mast cell lysates were prepared by sonicating 1-ml suspensions that contain  $1 \times 10^6$  mast cells for three pulses of 15 s each at 50 W (Ultrasonic Instruments International, Westbury, N. Y.). The lysed cells were then diluted with 9 ml of DMEM 10% CS and filtered through a 0.22- $\mu$ m Millipore filter before use.

*Cell Culture and Preparation of Sarcoma 180 and 3T3 CM.* Bovine aortic (17) and bovine capillary endothelial cell (12, 13) cultures were grown and maintained as previously described. Bovine capillary endothelial cell (BCE) passages 6–14 were used for the experiments. 4 h before use, the growth medium was replaced with fresh DMEM 10% CS without tumor CM. Sarcoma 180, canine kidney (MDCK), BALB/c 3T3, and Swiss 3T3 cells, and human fibroblasts from foreskin explants were grown in DMEM 10% CS supplemented with penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml).

The source of tumor CM was a culture of confluent sarcoma 180 cells as described previously (12). To provide 3T3 CM, confluent Swiss 3T3 cells were grown in 75-cm<sup>2</sup> Falcon tissue culture flasks and incubated in fresh DMEM 10% CS for 48 h. Approximately  $1 \times 10^6$  sarcoma 180 cells and  $6 \times 10^5$  Swiss 3T3 cells each conditioned 1 ml of medium. The CM was passed through a 0.22- $\mu$ m Millipore filter before use.

*BCE Cell Proliferation Experiment.* 10,000 capillary endothelial cells (passage 10) were plated on 30-mm Falcon tissue culture dishes in DMEM 10% CS. Plating efficiency of these sparsely plated cells was >90%. After 2 h, the cells had attached, and the medium was replaced with 2 ml of one of the following: mast cell CM, sarcoma 180 CM, or DMEM 10% CS. Cells were refed with test media every 48 h until the sarcoma 180 CM cultures reached confluency at 10 d. Cells were counted on days 7 and 10 using a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). Each point on the graph represents the mean of three samples ± SEM.

*BALB/c 3T3 DNA Synthesis Experiment.* Preparation of quiescent cultures of BALB/c 3T3 cells and the methods for measuring DNA synthesis after treatment with factors have been previously published (18). Mast cell CM was tested for effects on growth of 3T3 cells.

*Isolated Mast Cell Products and Glycosaminoglycans Tested for Endothelial Migration Activity.* Each of the following mast cell products and glycosaminoglycans (GAG) was dissolved in DMEM supplemented with 10% CS and tested in log concentrations from 10 ng to 1 mg/ml: 5-hydroxytryptamine hydrochloride (Sigma Chemical Co., St. Louis, Mo.); bovine trypsin type III (Sigma Chemical Co.); bovine pancreas chymotrypsin (Miles Laboratories, Inc., Elkhart, Ind.); eosinophil chemotactic factor (ECF-A) (1-Val-Gly-Ser-Glu [Miles Laboratories Inc.]);

heparin, grade 1 sodium salt derived from porcine intestinal mucosa (165 U/mg [Sigma Chemical Co.]); heparin derived from beef lung (183 U/mg [gift of M. B. Matthews, University of Chicago, Chicago, Ill.]); chondroitin sulfate A, special grade (Miles Laboratories Inc.); chondroitin sulfate B (dermatan sulfate [Sigma Chemical Co.]); chondroitin sulfate C, special grade (Miles Laboratories Inc.); chondroitin sulfate mixed isomers (Sigma Chemical Co.); hyaluronic acid, grade III (Sigma Chemical Co.); heparan sulfate and keratan sulfate (gifts of M. B. Matthews).

*Other Substances Tested for Endothelial Migration Activity.* Dextran sulfate 40,000 and 500,000 mol wt, dextran 40,000 and 500,000 mol wt, and protamine sulfate (all obtained from Sigma Chemical Co.) were dissolved in DMEM supplemented with 10% CS and tested in the same range of concentrations as for the experiments above.

*Determination of Heat Stability and Dialyzability of Mast Cell Medium.* Aliquots of mast cell CM were heated in H<sub>2</sub>O baths for 5 min at temperatures from 37 to 100°C. The mast cell CM was also dialyzed exhaustively against DMEM in Spectrapor dialysis tubing (Fisher Scientific Co.) with a molecular weight cutoff of 6,000–8,000.

*Incubation of Mast Cell Lysates in Proteolytic Enzymes.* A suspension of  $1 \times 10^6$  mast cells (0.39 mg Lowry protein) (19) in 1 ml of H<sub>2</sub>O was sonicated at 50 W for three pulses of 15 s each (Ultrasonic Instruments International). A 200- $\mu$ l aliquot was diluted to 2 ml with DMEM without serum and incubated with 100 mg of Enzite protease (0.0064 U/mg; Miles Laboratories Inc.). The incubation was carried out at 37°C and pH 7.0 for 4 h on a rotating shaker (Aurthur H. Thomas Co., Philadelphia, Pa.). The immobilized protease was then removed by two sequential centrifugations at 1,000 g for 5 min. The supernate was filtered through a 0.22- $\mu$ m Millipore filter to insure complete removal of the immobilized enzyme. The same procedure was carried out with Enzite trypsin (0.64 U/mg; Miles Laboratories Inc.) at 37°C and pH 8.0 for 4 h. The control samples for this experiment were processed without enzymes in the same manner. Each sample was supplemented with 10% CS and tested on the endothelial cell migration assay. To verify the proteolytic activity of these enzymes under the identical incubation conditions, we labeled confluent BALB/c 3T3 cells in 75-cm<sup>2</sup> flasks with [<sup>3</sup>H]lysine (5 mCi/ml; New England Nuclear, Boston, Mass.) for 48 h. The cells were harvested and dialyzed exhaustively against glass-distilled water to lyse cells and to remove unincorporated [<sup>3</sup>H]lysine. The cell lysates were washed with phosphate-buffered saline and centrifuged. The supernate was decanted and the cellular material suspended in 3 ml DMEM without serum. Aliquots (500  $\mu$ l) of this suspension were incubated alone or with Enzite protease (50 mg/ml) or Enzite trypsin (25 mg/ml). After incubation and removal of the immobilized enzymes, the samples were precipitated with trichloroacetic acid (TCA) to a final concentration of 5% TCA. The samples were placed on individual type HA filters (Millipore Corp.) and washed with 5% TCA and ethanol. The filters were placed in Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted on a Packard scintillation counter for 10 min.

*Heparinase and Chondroitinase ABC Treatment of CM.* Crude heparinase was prepared from *Flavobacterium heparinum* (gift of J. E. Silbert, Boston Veterans Administration Hospital, Boston, Mass.). 20 mg of lyophilized bacteria, suspended in 1 ml of H<sub>2</sub>O, was sonicated at 50 W for six pulses of 15 s each (Ultrasonic Instruments International). The suspension was centrifuged at 1,000 g for 10 min to remove bacterial debris. A 200- $\mu$ l aliquot of crude heparinase solution was incubated at 35°C, pH 7.0, for 12 h with 1,800  $\mu$ l of the following: mast cell CM, sarcoma 180 CM, DMEM 10% CS, and DMEM 10% CS supplemented with heparin (50  $\mu$ g/ml, grade 1 sodium salt, 167 U/mg; Sigma Chemical Co.). 100  $\mu$ l of this enzyme preparation neutralized the anticoagulant activity of 600  $\mu$ g (~100 U) of heparin in 3 ml of fresh whole blood. There was no detectable protease activity (< 1  $\mu$ g/ml) in the heparinase preparation as measured by the method of Schill and Schumacher (20).

Chondroitinase ABC (0.5 U/ml; Miles Laboratories Inc.) was incubated at 37°C, pH 7.4, for 12 h with the following: mast cell CM, sarcoma CM, DMEM 10% CS supplemented with heparin (50  $\mu$ g/ml), or DMEM 10% CS. The heparinase and chondroitinase ABC-treated samples and the non-enzyme-treated controls were assayed for endothelial cell migration activity.

*Statistical Analysis.* One-way analysis of variance was used to detect significant differences

within an experiment. In addition, the Student-Newman-Keuls multiple range test was used to determine between which means there were significant differences ( $C.L. \geq 0.95$ ).

### Results

Mast cell CM caused significant increases in BCE migration in vitro ( $P < 0.01$ ) (Fig. 1). As reported previously, endothelial cell phagokinetic track size was directly proportional to the area of migration (13). In repeated 18-h experiments, mast cell CM resulted in a mean increase in phagokinetic track size of  $>10,000 \mu\text{m}^2/\text{cell}$ . This increase was 100–120% greater than the area of controls that were incubated in either DMEM 10% CS alone or in Swiss 3T3 CM (Fig. 1E). The increase in capillary endothelial cell migration in response to sarcoma 180 CM and mast cell CM were not significantly different ( $P \geq 0.05$ ) (Fig. 1E). The stimulation of capillary endothelial cell migration by mast cell CM was dose-dependent. When the concentration of mast cell CM was increased from 0–100%, a progressive increase in BCE cell migration was observed (Fig. 2). In addition, mast cell lysates and mast cell CM equally stimulated capillary endothelial cell migration.

To control for the specificity of the mast cell effect upon capillary endothelial cells, we examined the migration of four additional cell types incubated in mast cell CM: bovine aortic endothelial, Swiss 3T3, canine kidney cells (MDCK), and human

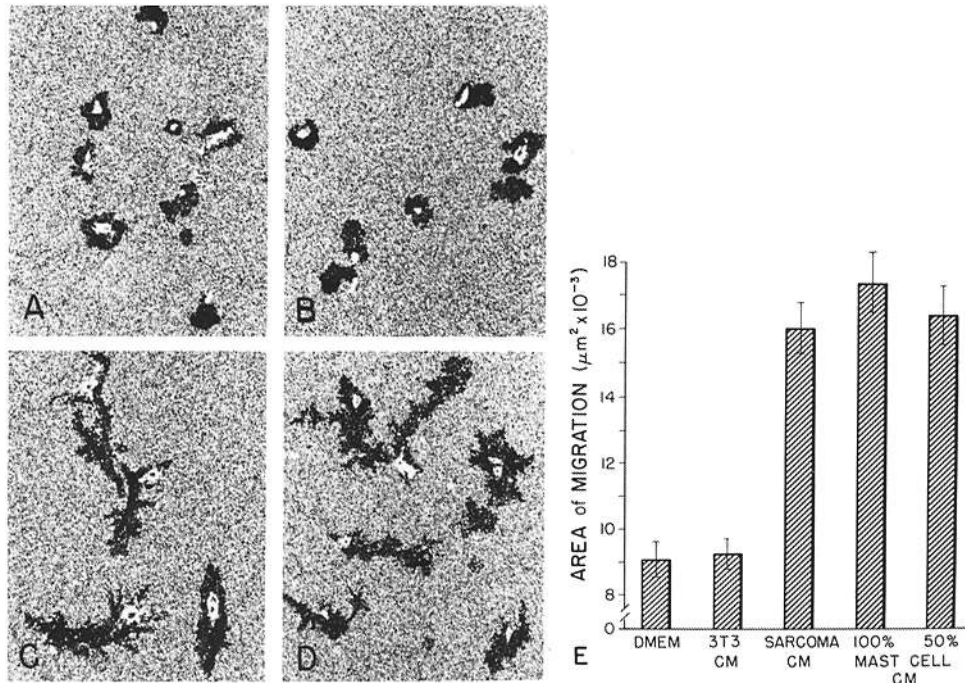


FIG. 1. Migration of BCE in response to media conditioned by sarcoma 180, mast, and 3T3 cells. Test media: (A) DMEM 10% CS not conditioned; (B) DMEM 10% diluted 1:2 with the same medium conditioned by 3T3 cells; (C) sarcoma 180 cells; (D) mast cells. BCE were seeded at a concentration of 3,000 cells in 1 ml DMEM 10% CS onto coverslips coated with colloidal gold. (E) After 18 h, cells were fixed with 10% formalin in phosphate-buffered saline, and the area of the phagokinetic tracks was measured with the aid of a Zeiss MOP-3 digital image analyser as described in Materials and Methods. Each bar represents the area of 100 tracks  $\pm$  SEM.

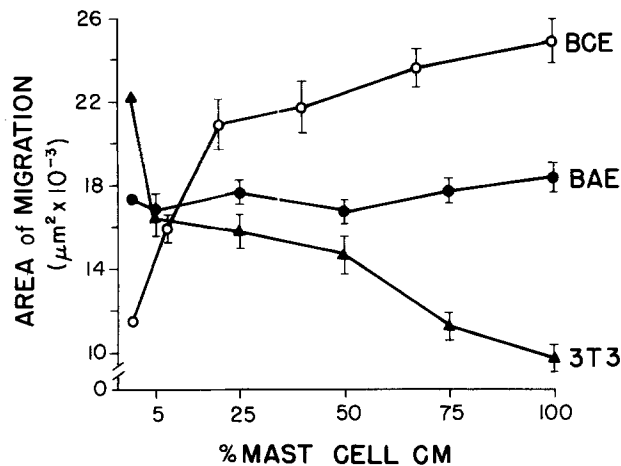


FIG. 2. The effect of mast cell CM on the migration of BCE bovine aortic endothelial cells (BAE), and Swiss 3T3 cells. Using the method described in the legend to Fig. 1, concentrations of mast cell CM from 0 to 100% were incubated with BCE (○), BAE (●), and 3T3 cells (▲). Each point on the graph represents the mean area of 100 phagokinetic tracks  $\pm$  SEM.

fibroblasts grown from foreskin explants. When compared with DMEM 10% CS, mast cell CM did not alter the migration of aortic endothelial cells (Fig. 2), canine kidney (MDCK) cells, or human fibroblasts (data not shown). Swiss 3T3 cell migration, however, was progressively inhibited as the percent of mast cell CM was increased (Fig. 2).

**Effect of Mast Cell CM on Proliferation.** Many factors stimulating migration also stimulate proliferation of endothelial cells (13, 21). We found that mast cell CM did not enhance capillary endothelial cell proliferation over levels achieved with DMEM supplemented with 10% CS ( $P \geq 0.05$ ) (Fig. 3). On the other hand, sarcoma 180 CM, which is known to be mitogenic for capillary endothelial cells (12), stimulated a 500% increase in cell number compared with mast cell CM or DMEM 10% CS ( $P < 0.01$ ). This experiment was repeated three times with nearly identical results. To further evaluate possible mast cell mitogenicity, the effect of mast cell CM on DNA synthesis by quiescent BALB/c 3T3 cells was also investigated. Mast cell CM had no effect on basal incorporation of [ $^3$ H]thymidine but, when added together with serum, it inhibited the stimulation of DNA synthesis by serum (Fig. 4).

**Effect of Mast Cell Products on Capillary Endothelial Cell Migration.** To determine whether endothelial cell migration activity could be assigned to a particular mast cell product, the effect of the following mast cell products on capillary endothelial cell migration was examined: heparin, histamine, ECF-A, chondroitin sulfate (mixed isomers) (Fig. 5), 5-hydroxytryptamine, chondroitin sulfates A, B, and C, trypsin, and chymotrypsin (data not shown). These substances were tested in log increments over a concentration range of 10 ng–1 mg/ml. Only heparin significantly stimulated capillary endothelial cell migration ( $P < 0.01$ ) (Fig. 5). Increased migration could be observed with as little heparin as 1  $\mu$ g/ml, and maximum migration activity occurred at concentrations of 10–50  $\mu$ g/ml. Heparin at 50  $\mu$ g/ml was equivalent to 100% mast cell CM.

**Biochemical Characterization.** The endothelial migration activity in mast cell CM

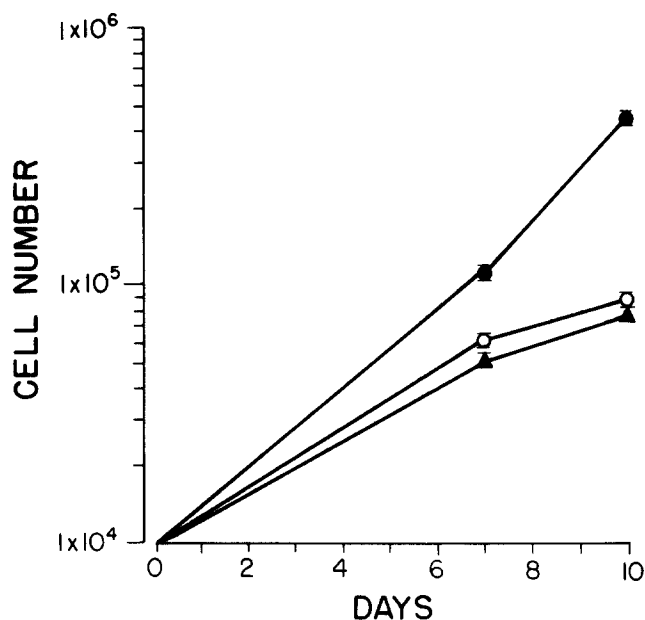


FIG. 3. BCE cell proliferation in response to mast cell CM. 10,000 cells were plated on 30-mm tissue culture dishes and incubated in DMEM 10% CS alone (▲) or diluted 1:2 with mast cell CM (○) or Sarcoma 180 CM (●). The media was changed every 48 h for 10 d. Cells were harvested on days 7 and 10. Each point represents the mean of three samples  $\pm$  SEM.

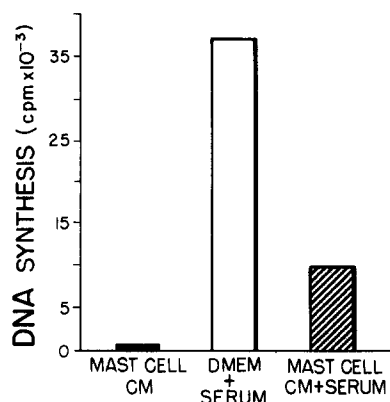


FIG. 4. The inhibition of serum-stimulated DNA synthesis in 3T3 cells by mast cell CM. Growth-arrested 3T3 cells in serum-depleted DMEM were labeled for 72 h with [<sup>3</sup>H]thymidine. Test conditions: calf serum alone (□), mast cell CM with serum (▨) and without serum (■).

was not dialyzable through cellulose with 6,000–8,000 mol wt cutoff. Furthermore, the activity was heat stable to 100°C. Sonicated mast cells (10<sup>5</sup>/ml DMEM 10% CS) resulted in activity equivalent to medium conditioned by the same number of mast cells for 36 h. The capillary endothelial cell migration stimulating activity of sonicated mast cell preparations was not decreased after incubation with Enzite protease or immobilized trypsin before the addition of calf serum (Fig. 6a). Before using these enzymes, their effective proteolytic activity was determined by measurement of the liberation of protein-bound [<sup>3</sup>H]lysine from 3T3 cells prelabeled with [<sup>3</sup>H]lysine.

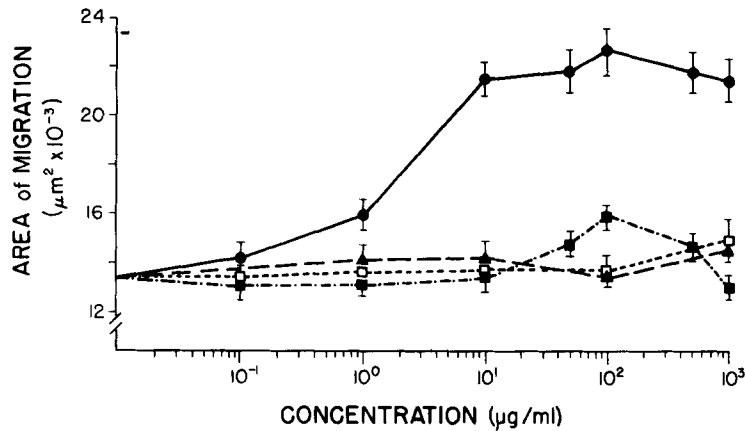


FIG. 5. The effect of isolated mast cell products on BCE migration. The mast cell products were solubilized in DMEM 10% CS and tested at log-dose intervals from 10 ng to 1 mg/ml. The mast cell products tested included: heparin (●), chondroitin sulfate ABC (■), ECF-A (□), and histamine phosphate (▲). Each point represents the mean area of 100 phagokinetic tracks  $\pm$  SEM.

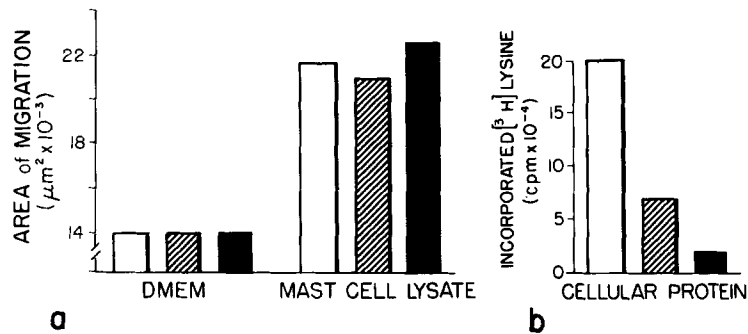


FIG. 6. The effect of proteolytic enzymes on the migration stimulating activity of mast cell lysates. (a) DMEM 10% CS alone, lysates of  $2 \times 10^6$  mast cells suspended in DMEM 10% CS were tested for migration-stimulating activity after a 4-h incubation with 50  $\mu$ g/ml Enzite protease (■) or 25 mg/ml Enzite trypsin (▨) or in the absence of enzymes (□). Each bar represents the mean area of 100 phagokinetic tracks  $\pm$  SEM. (b) Proteolytic activity of the immobilized enzymes was assayed by measuring release of TCA-precipitable [<sup>3</sup>H]lysine from prelabeled 3T3 cell lysates as described in Materials and Methods. The 3T3 cell lysates were incubated at 37°C for 4 h in the absence of enzymes (□), or in the presence of 50 mg/ml Enzite protease (■), or 25 mg/ml Enzite trypsin (▨).

Treatment with immobilized protease released >90% of the incorporated [<sup>3</sup>H]lysine, whereas the immobilized trypsin released 33% (Fig. 6b).

To confirm that heparin is the migration factor in mast cell CM, specific inhibitors of heparin—protamine and heparinase—were studied separately. In a dose-dependent manner, protamine sulfate blocked the effect of mast cell CM on capillary endothelial cell migration, whereas the same concentrations of protamine sulfate in DMEM 10% CS had no effect on migration (Fig. 7a). Furthermore, a ratio of protamine sulfate: heparin of 2:1 was found to completely neutralize the heparin-stimulated BCE migration activity (Fig. 8). Protamine sulfate did not inhibit the capillary endothelial cell migration that was stimulated by sarcoma 180 CM (Fig. 7b).

Heparinase is known to degrade heparin (as well as chondroitin sulfates A, B, and C and heparan sulfate) (22). When mast cell CM or DMEM with heparin (50  $\mu$ g/ml)

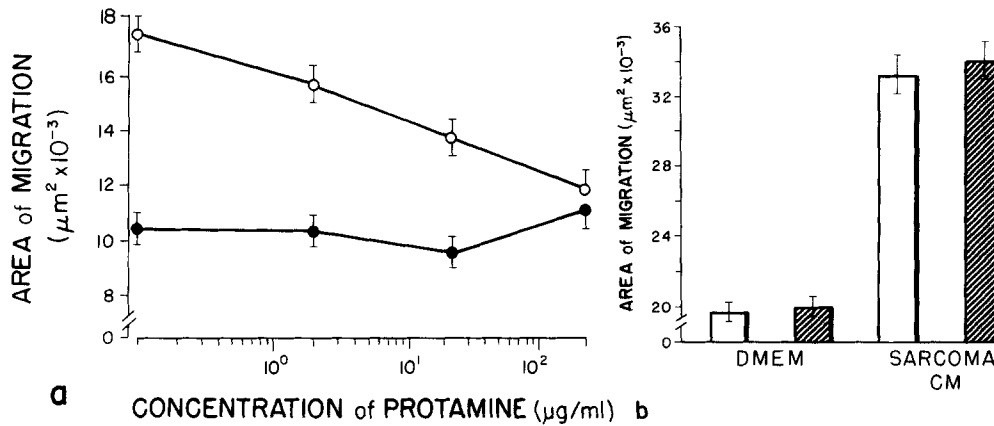


FIG. 7. The effect of protamine sulfate on mast cell-stimulated BCE migration. (a) Protamine sulfate, at concentrations from 100 ng to 200 µg/ml, was added to capillary endothelial cells plated on gold-coated cover slips and incubated in DMEM 10% CS (●) or in the same medium conditioned by mast cells (○). (b) DMEM 10% CS and the same medium conditioned by sarcoma 180 cells was added to BCE cells with (▨) and without (□) protamine sulfate (200 µg/ml). Each point represents the mean area of 100 phagokinetic tracks ± SEM.

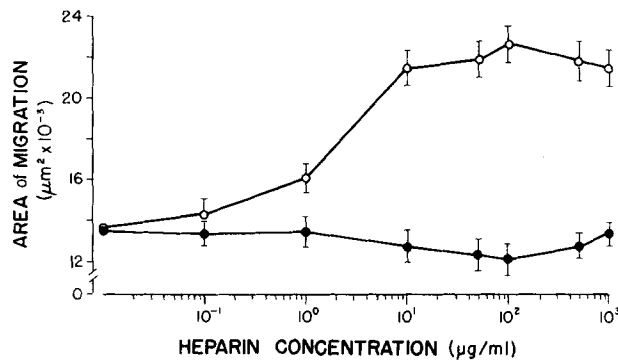


FIG. 8. The effect of protamine sulfate on heparin-induced BCE migration. BCE migration in response to heparin (○) is compared with migration in response to heparin and protamine sulfate together (●). Twice the amount of protamine sulfate was used to neutralize the activity of heparin. Protamine sulfate alone had no significant effect on migration. Each point represents the mean area of 100 phagokinetic tracks ± SEM.

was incubated with heparinase before incubation with capillary endothelial cells, no increased migration occurred. In contrast, sarcoma 180 CM incubated with heparinase stimulated migration of these cells ( $P < 0.05$ ) (Fig. 9). The specificity of heparinase was tested by using chondroitinase ABC, which hydrolyzes chondroitin sulfates but not heparin (23). Chondroitinase ABC was found not to alter mast cell CM- or heparin-stimulated migration ( $P \geq 0.01$ ) (Fig. 10).

**Specificity of Heparin on Cell Migration.** To control for possible nonspecific migration activity of heparin, bovine aortic endothelial cells, canine kidney (MDCK) cells, human fibroblasts, and Swiss 3T3 cells were exposed to heparin in concentrations from 10 ng to 1 mg/ml (Fig. 11 a and b). No increased migration was observed in any cell line, and inhibition of Swiss 3T3 cell migration was observed at concentrations of heparin  $>0.1$  µg/ml (Fig. 11 b).



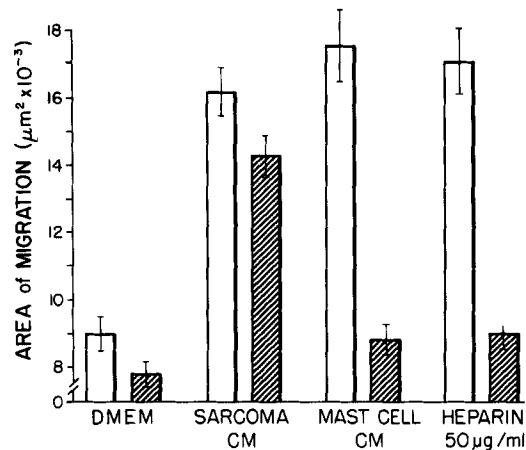


FIG. 9. The effect of heparinase on heparin-stimulated and tumor-stimulated BCE migration. Test media included: DMEM 10% CS alone, DMEM 10% CS in the presence of heparin (50  $\mu\text{g}/\text{ml}$ ), or DMEM 10% CS diluted 1:2 with sarcoma 180 CM or mast cell CM. Each media was tested in the presence (▨) or absence (□) of heparinase. The area of the phagokinetic tracks was measured after 18 h. Each bar represents the mean area of 100 migration tracks  $\pm$  SEM.

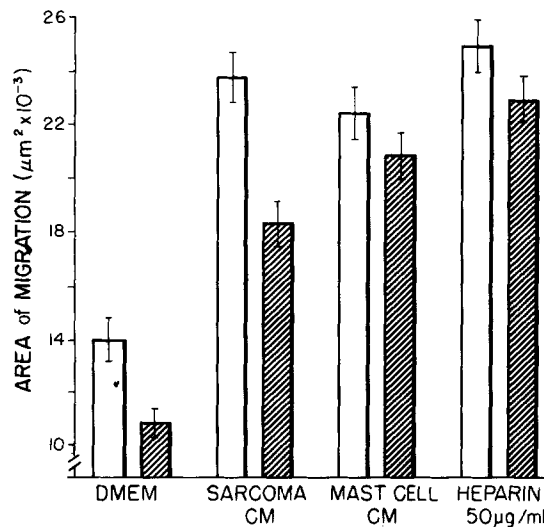


FIG. 10. The effect of chondroitinase ABC on heparin- and tumor-stimulated BCE migration. Test media included DMEM 10% CS alone, DMEM 10% CS supplemented with heparin (50  $\mu\text{g}/\text{ml}$ ), or DMEM 10% CS diluted 1:2 with sarcoma 180 CM or mast cell CM. Each media was tested in the presence (▨) or absence (□) of chondroitinase ABC (0.5  $\mu\text{g}/\text{ml}$ ). Each bar represents the mean area of 100 migration tracks  $\pm$  SEM.

*Effect of Other Polyanionic Substances on Migration.* Heparin was then compared with heparan sulfate in the capillary endothelial cell migration assay. Heparan sulfate stimulated a maximum increase in migration of 15–25% above control (DMEM 10% CS) at a concentration of 100  $\mu\text{g}/\text{ml}$  (Fig. 12). This result was not statistically significant at any concentration. Furthermore, all the major polyanionic GAG were simultaneously compared in the migration assay (Fig. 12). Neither nonsulfated hyaluronic acid, nor sulfated GAG, the chondroitin sulfates A, B, and C, nor heparan

## CAPILLARY ENDOTHELIAL CELL MIGRATION

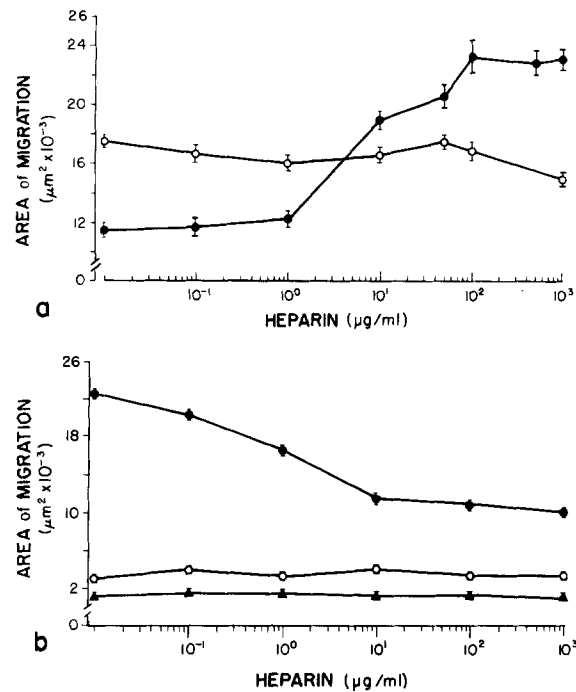


FIG. 11. The effect of heparin on cell migration. Concentrations of heparin from 10 ng to 1 mg/ml were tested for their ability to stimulate the migration of the following five cell types: (a) BCE (●) and bovine aortic (BAE) cells (○) and also (b) of Swiss 3T3 cells (●), MDCK cells (▲), and human fibroblasts (○). All incubations were carried out in DMEM containing 10% CS. Each point represents the mean area of 100 phagokinetic tracks  $\pm$  SEM.

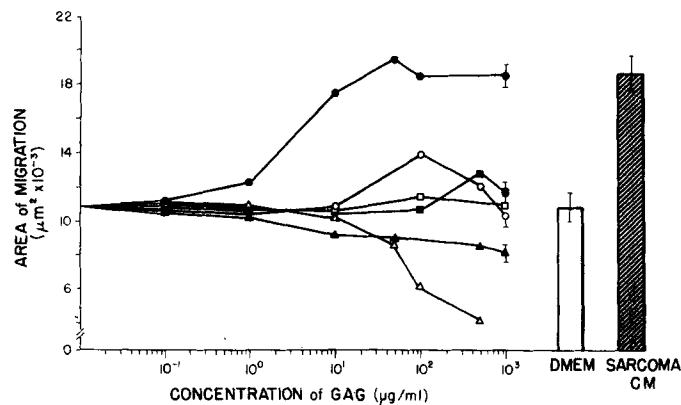


FIG. 12. The effect of GAG on the migration of BCE. Concentrations of GAG ranging from 10 ng to 1 mg/ml were tested in the phagokinetic track assay described in the legend to Fig. 1. Test substances: heparin (●), heparan sulfate (○), chondroitin sulfate B (■), chondroitin sulfate C (□), hyaluronic acid (▲), and keratan sulfate (Δ). Test substances were added in DMEM 10% CS. The controls included: DMEM 10% CS without additives (open bar) and sarcoma 180 CM (hatched bar). Each point represents the mean area of 100 phagokinetic tracks  $\pm$  SEM.

sulfate had any significant effect on capillary endothelial cell migration ( $P \geq 0.05$ ). Keratan sulfate, however, inhibited migration of these cells. Finally, the effect of synthetic polyanions on migration was investigated. Nonsulfated dextran of two

different molecular weights (40,000 and 500,000) did not stimulate capillary endothelial cell migration. However, dextran sulfates of similar molecular weights significantly increased migration ( $P < 0.01$ ) (Fig. 13).

### Discussion

These experiments show that mast cells release a factor that stimulates the migration of BCE in vitro. This factor, which is found in mast cell CM and in lysates of isolated mast cells, is heparin on the basis of the following data: (a) only heparin, of all the mast cell secretory products tested, stimulated capillary endothelial cell migration in vitro; (b) heparin preparations from a variety of sources stimulated capillary endothe-

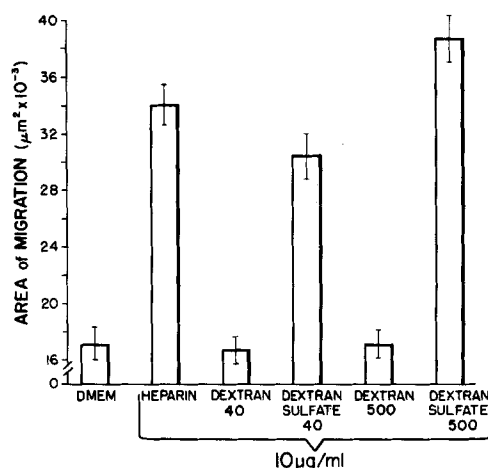


FIG. 13. The effect of dextran and dextran sulfate on BCE migration. Using the phagokinetic track assay, the migration of BCE in response to sulfated and nonsulfated synthetic polysaccharides was examined. Test substances were dissolved in DMEM 10% CS and included: dextran 40,000 mol wt (40); dextran sulfate 40,000 mol wt (40); dextran 500,000 mol wt (500); dextran sulfate 500,000 mol wt (500). The concentration of polysaccharide was 10 μg/ml for each sample. Controls included: DMEM 10% CS and DMEM 10% CS supplemented with heparin (10 μg/ml). Each bar represents the mean area of 100 phagokinetic tracks  $\pm$  SEM.

lial cell migration to the same degree but did not stimulate migration of several other cell types; (c) the migration activity of heparin and mast cell CM was blocked by specific antagonists of heparin (protamine and heparinase) but not by chondroitinase ABC; (d) the migration activity of mast cell CM was resistant to heat (100°C) and incubation with Enzite protease and trypsin. Commercial heparin is also heat stable (24) and rat mast cell heparin has been shown to be resistant to proteolytic enzyme digestion (25).

Neither mast cell CM nor heparin stimulate the migration of aortic endothelial cells and several nonendothelial cell types. Whereas a variety of transformed and neoplastic cells have been shown to produce migration factors for capillary endothelial cells (13), mast cells are the first nontransformed cell type found to produce such a factor.

Although tumor CM and mast cell CM stimulate capillary endothelial cell migration to comparable levels, they differ in one important respect. Tumor CM stimulates capillary endothelial cell proliferation as well as migration. Mast cell CM stimulates

migration only. Thus, endothelial cell migration and proliferation can be uncoupled in vitro. Although our results cannot predict whether separate factors will regulate migration and proliferation in vivo, they raise the possibility that these two events are controlled independently, and that the migration component of neovascularization may be modulated by the action of mast cell products.

The mechanism by which capillary endothelial cell migration is stimulated by heparin is unknown. Although heparin is a densely charged GAG, polyanions with similar charge densities, such as heparan sulfate and the chondroitin sulfates (24), do not significantly alter cell motility. Furthermore, keratan sulfate, a charged sulfated GAG found in large quantities in avascular tissues like the cornea and cartilage, inhibits capillary endothelial cell migration. Heparin is more sulfated than the structurally related GAG (24), heparan sulfate and hyaluronic acid. The synthetic polyanion, dextran sulfate, stimulates capillary endothelial cell migration; however, nonsulfated dextran does not. Although both charge and degree of sulfation may be important in stimulating migration of capillary endothelial cells, these parameters alone do not account for the phenomenon observed.

In other cell systems, cell membrane-heparin interactions have been associated with increased cell membrane motility. Heparin has been implicated in both phagocytosis by Kupffer cells (26, 27) and in pinocytosis by murine macrophages (28) and fibroblasts (29). Heparin also increases pseudopod formation and motility of *Amoeba proteus* (29). Recently, arterial smooth muscle cell proliferation (30, 31) has been shown to be inhibited by heparin.

The ability to stimulate migration of capillary endothelial cells in vitro is a newly discovered property of mast cells and heparin. It is not known if this activity also functions in vivo and, if so, for what reason. However, it is known that mast cells are intimately associated with tumor angiogenesis (4) (and possibly with other forms of angiogenesis). Therefore, it is theoretically possible that one role of mast cells is to enhance the directional elongation of new capillary sprouts by sustained release of heparin. If these observations are upheld by experiments in vivo, one possible type of angiogenesis inhibitor would be a substance that would block the endothelial cell migration effect of mast cells.

### Summary

Migration of capillary endothelial cells is an important component of angiogenesis in vivo. Increased numbers of mast cells have been associated with several types of angiogenesis. We have used a quantitative assay in vitro to demonstrate that mast cells release a factor that significantly increases bovine capillary endothelial cell migration. The factor is present in medium conditioned by mast cells as well as lysates of mast cells. The stimulatory effect of mast cells on migration is specific for capillary endothelial cells. Furthermore, mast cells have no mitogenic activity for capillary endothelial cells.

Of all the secretory products of mast cells tested, only heparin stimulated capillary endothelial cell migration in vitro. Heparin preparations from a variety of sources stimulated capillary endothelial cell migration to the same degree but did not stimulate migration of several other cell types. The migration activity of heparin and mast cell conditioned medium was blocked by specific antagonists of heparin (protamine and heparinase), but not by chondroitinase ABC. The migration activity of

mast cell conditioned medium was resistant to heat (100°C) and incubation with proteolytic enzymes. These results suggest that the role of mast cells in angiogenesis may be to enhance migration of the endothelial cells of growing capillaries.

We thank M. B. Matthews for the gift of mucopolysaccharide reference kit, Dr. Michael Klagsbrun and Dr. Julianne Glowacki for all their help and for reviewing the manuscript, James Stone and R. Clement Darling, III for expert technical assistance, David Fishman for the statistical analysis, Mr. Janis Cirulis for the illustrations, and Pauline Breen for typing assistance.

Received for publication 23 April 1980 and in revised form 13 June 1980.

### References

1. Folkman, J., E. Merler, C. Abernathy, and G. Williams. 1971. Isolation of a tumor factor responsible for angiogenesis. *J. Exp. Med.* **133**:275.
2. Gimbrone, M. A., Jr., S. B. Leapman, R. S. Cotran, and J. Folkman. 1972. Tumor dormancy in vivo by prevention of neovascularization. *J. Exp. Med.* **136**:261.
3. Folkman, J., and R. Cotran. 1976. Relation of endothelial proliferation to tumor growth. *Int. Rev. Exp. Pathol.* **16**:207.
4. Kessler, D. A., R. S. Langer, N. A. Pless, and J. Folkman. 1976. Mast cells and tumor angiogenesis. *Int. J. Cancer.* **18**:703.
5. Szabov, E., and C. Meszaros. 1964. Die rolle der mastocyten in der histogenese der psoriasis. *Z. Haut. Geschlechtskr.* **36**:373.
6. Mottaz, J. H., A. S. Zelickson, E. G. Thorne, and G. Wachs. 1973. Blood vessel changes in psoriatic skin. *Acta Dermato-Venerol.* **53**:195.
7. Rebeck, J. W., J. M. Hodson, R. J. Priest, and C. L. Barth. 1962. Basophilic granulocytes in inflammatory tissues of man. *Trans. N. Y. Acad. Sci.* **103**:409.
8. Dvorak, A. M., M. C. Mihm, Jr., and H. F. Dvorak. 1976. Morphology of delayed-type hypersensitivity reactions in man. II. Ultrastructural alterations affecting the microvasculature and the tissue mast cells. *Lab. Invest.* **34**:179.
9. Dvorak, H. 1971. Role of the basophilic leukocyte in allograft rejection. *J. Immunol.* **106**:279.
10. Smith, S. S., and P. K. Basu. 1970. Mast cells in corneal immune reaction. *Can. J. Ophthalmol.* **5**:175.
11. Ausprunk, D. H., and J. Folkman. 1977. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc. Res.* **14**:53.
12. Folkman, J., C. C. Haudenschild, and B. R. Zetter. 1979. Long-term culture of capillary endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5217.
13. Zetter, B. R. 1980. Migration of capillary endothelial cells is stimulated by tumour-derived factors. *Nature (Lond.)*. **285**:41.
14. Albrecht-Buehler, G. 1977. The phagokinetic tracks of 3T3 cells. *Cell.* **11**:395.
15. Albrecht-Buehler, G. 1977. Daughter 3T3 cells: are they mirror images of each other? *J. Cell Biol.* **72**:595.
16. Sullivan, T. J., K. L. Parker, W. Stenson, and C. W. Parker. 1975. Modulation of cyclic AMP in purified rat mast cells. I. Responses to pharmacologic, metabolic, and physical stimuli. *J. Immunol.* **114**:1473.
17. Kadish, J. L., C. E. Butterfield, and J. Folkman. 1979. The effect of fibrin on cultured vascular endothelial cells. *Tissue Cell.* **11**:99.
18. Klagsbrun, M., R. Langer, R. Levenson, S. Smith, and C. Lillehei. 1977. The stimulation of DNA synthesis and cell division in chondrocytes and 3T3 cells by a growth factor isolated from cartilage. *Exp. Cell Res.* **105**:99.

19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**:265.
20. Schill, W. B., and G. F. B. Schumacher. 1972. The radial diffusion in gel for micro determination of enzymes. *Anal. Biochem.* **46**:502.
21. Wall, R. T., L. A. Harker, and G. E. Striker. 1978. Human endothelial cell migration stimulation by a released platelet factor. *Lab. Invest.* **39**:523.
22. Linker, A., and P. Hovingh. 1965. The enzymatic degradation of heparin and heparitin sulfate. I. The fractionation of a crude heparinase from flavobacteria. *J. Biol. Chem.* **240**:3724.
23. Yamagata, T., H. Saito, O. Habuchi, and S. Suzuki. 1968. Purification and properties of bacterial chondroitinases. *J. Biol. Chem.* **243**:1523.
24. Jaques, L. B. 1976. Heparin and related sulfated mucopolysaccharides. *Methods Biochem. Anal.* **24**:203.
25. Yurt, R. W., R. W. Leid, Jr., K. F. Austen, and J. E. Silbert. 1977. Native heparin from rat peritoneal mast cells. *J. Biol. Chem.* **252**:518.
26. Saba, T. M., J. P. Filkins, and N. R. Di Luzio. 1966. Properties of the "opsonic system" regulating *in vitro* hepatic phagocytosis. *J. Reticuloendothel. Soc.* **3**:398.
27. Kitchen, A. G., and R. Megirian. 1971. Heparin enhancement of Kupffer cell phagocytosis *in vitro*. *J. Reticuloendothel. Soc.* **9**:13.
28. Cohn, Z. A., and E. Parks. 1967. The regulation of pinocytosis in mouse macrophages. II. Factors influencing vesicle formation. *J. Exp. Med.* **125**:213.
29. Dougherty, T. F., G. Scheebeli, and N. Panagiotis. 1964. Stimulation of motility and pinocytosis by heparin in *Amoeba proteus* and murine fibroblasts. *J. Reticuloendothel. Soc.* **1**:363.
30. Guyton, J. R., R. D. Rosenberg, A. W. Clowes, and M. J. Karnovsky. Inhibition of rat arterial smooth muscle cell proliferation by heparin. I. In vivo studies with anticoagulant and non-anticoagulant heparin. *Circ. Res.* In press.
31. Hoover, R. L., R. D. Rosenberg, W. Hoering, and M. J. Karnovsky. Inhibition of rat arterial smooth muscle cell proliferation by heparin. II. In vitro studies. *Circ. Res.* In press.