Demonstration of the Chemotactic Properties of Collagen[1] (34719)

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One of the important cellular responses to injury is the infiltration of polymorphonuclear (PMN) cells into the wounded area (1–3). Presumably, these cells first marginate or adhere to the walls of the microcirculation in the injured area, then emigrate between the endothelial cells which make up the wall of the vessel and finally migrate by "crawling" from the vessel through the ground substance to the site of the injury. This margination, emigration and migration of PMN's is collected under the term "chemotaxis" (2–4).

A major methodological problem with the determination of chemotaxis *in vivo* is that for hydrodynamic reasons these PMN cells can be passively trapped or sequestered within the mechanically injured site of injection (4). For this reason Boyden (5) and Ward (6) have developed the quantitative *in vitro* technique of separating white blood cells in a chamber from the substance under study with a Millipore filter with pores of 650 mμ in diameter. They then fix and stain these filters and count the number of cells which have penetrated into these pores and hence are "stuck" on the filter after staining.

Because of our interest in the chemistry of connective tissue necrosis, and the recent criticisms of *in vitro* determinations of chemotaxis, we have developed a semiquantitative *in vivo* technique which is described below for various connective tissue components; particularly soluble collagen.

Materials and Methods. Rat skin, after excision, shaving, and mincing, was extracted with 10 ml/g of 0.15 *M* NaCl in the cold in a Lourdes tissue blender for 45 min. This tissue suspension was then stored in the cold overnight and centrifuged at 20,000*g* for over 1 hr at 2°. The resulting supernatant (90–95% recovery) was then dialyzed against 200 vol of distilled water to precipitate euglobulins (including soluble collagen) and complement, recentrifuged, and the final supernatant was lyophilized. This saline extract of the skin was called S_1. The morphology and histochemistry of the residue from this extraction procedure suggests that relatively intact cells and fibers were left behind (7–9). This S_1 then probably represents a euglobulin-free sample of the extracellular, extrafibrillar components of rat skin. Electrophoretically, S_1 is similar to serum and thus contains a wide variety of the various noncollagenous proteins of the connective tissue.

The insoluble residue from this procedure was then re-extracted in 0.5 *M* NaCl; salt soluble collagen was prepared from this extract and purified according to Piez *et al.* (10). The final product had an intrinsic viscosity of about 13.8 dl/g, a specific optical rotation of $[-358]^{D}_{20°}$ and absorbed very little energy at 280 mμ.

Solutions of this purified soluble collagen were prepared in 0.05 *M* tris buffer (pH 7.5) containing 0.15 *M* NaCl to a final concentration of 1 mg/ml.

Gelatin was prepared from this soluble collagen by acidification to pH 4.0 and heating for 30 min at 50°. The optical rotation of this solution dropped to $[-120]^{D}_{20°}$.

Crystalline soybean trypsin inhibitor (SB TI), trypsin, chymotrypsin (chymo) and crude bacterial collagenase (b-coll'ase) were obtained from Worthington Biochemicals.

Solutions of these enzymes were made up in Tris buffered saline (the b-coll'ase con-

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tained 0.01 *M* $CaCl_2$) to contain 20 μg/ml. Other solutions of these enzymes were made up to 1 mg/ml and added to the solutions of S_1 or collagen (1 mg/ml) to make a final concentration of 20 μg/ml. These latter enzyme–substrate mixtures were incubated at 20° for 1 hr; the reaction was stopped by adding SBTI to make a final concentration of 20 μg/ml from a SBTI solution containing 1 mg/ml. All reaction mixtures were used immediately after incubation. In this fashion, various proteolytic degradation products of connective proteins from S_1 and from collagen were obtained.

The cutaneous collagenolytic activity (cut. coll'ase) of the S_1 prepared previously was activated by treatment with equal amounts of trypsin at pH 5.5 for 30 min at 22° (11, 12). After adding sufficient SBTI to completely inhibit all the trypsin, the active collagenolytic activity so released remained in solution in 65% saturated ammonium sulfate. After dialysis, lyophilization, and molecular sieving chromatography on Sephadex-100, the excluded collagenolytic activity was lyophilized and made up in acetate buffer (pH 5.5) to a final concentration of 200 μg/ml. This collogenolytic activity was purified some 100-fold from its concentration in S_1 and 10,000-fold from its concentration in rat skin. At this concentration and pH, significant amounts of a large number of degradation products could be visualized using polyacrylamide gel electrophoresis from both soluble collagen and the supernatant from purified native and highly insoluble collagen (Carrillo and Houck, to be published).

Millipore filters (13-mm diam) with a pore size of 450 mμ were used as follows: the abdominal area of 200 g male Sprague-Dawley rats were washed with pHisoHex, shaven; and the epidermis was very carefully removed with a sterilized, dull scalpel; every attempt was made to avoid bleeding; animals which bled were not used; these dissected areas were covered with filters which had been washed in 0.15 *M* NaCl with the marked side up; these filters were then covered with filtering discs (S&S Co. No. 470) of the same outside diameter; to this gauze disc was added 0.1 ml of the sample and this sample disc was then covered by wax paper (in which the Millipore filters were packed) to inhibit water loss *via* evaporation; this whole sandwich or filter disc and paper, was then covered with an adhesive bandage. This is illustrated in Fig. 1.

About four of the Millipore filter "sandwiches" were placed on each animal and the rat was placed in a restraining chamber for the appropriate period of time. After this time, the filters were removed, rinsed in distilled water, fixed in methanol, re-rinsed, and stained with standard Ehrlich's hemotoxylin. These filters were then rinsed with acid alcohol, water, and then dipped into bluing agent (2 g of $NaHCO_3$, 20 g of $MgSO_4$/liter H_2O), fixed with increasing alcohol concentration, and cleared in xylene (5). This stained filter was then inspected microscopically, under oil immersion, for cells which had crawled *through* the 450 mμ wide pores and had emerged on the marked (up) disc of the Millipore filter. These filters were then evaluated at 0; ±, less than 5 cells/field; +, ca. 100 cells/field; and ++, more than 200 cells/field. An example of these judgments is shown in the photomicrographs in Fig. 2.

Experimental Results. One-tenth-ml samples of S_1, enzymes, collagen, and gelatin were applied in triplicate to five groups, of four rats each, with one buffer control being

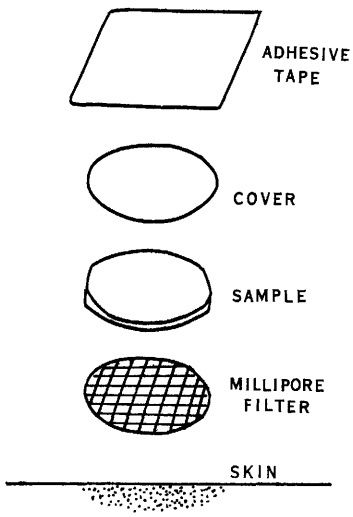

FIG. 1. Schematic diagram of the Millipore filter package applied to an abraded specimen of rat skin.

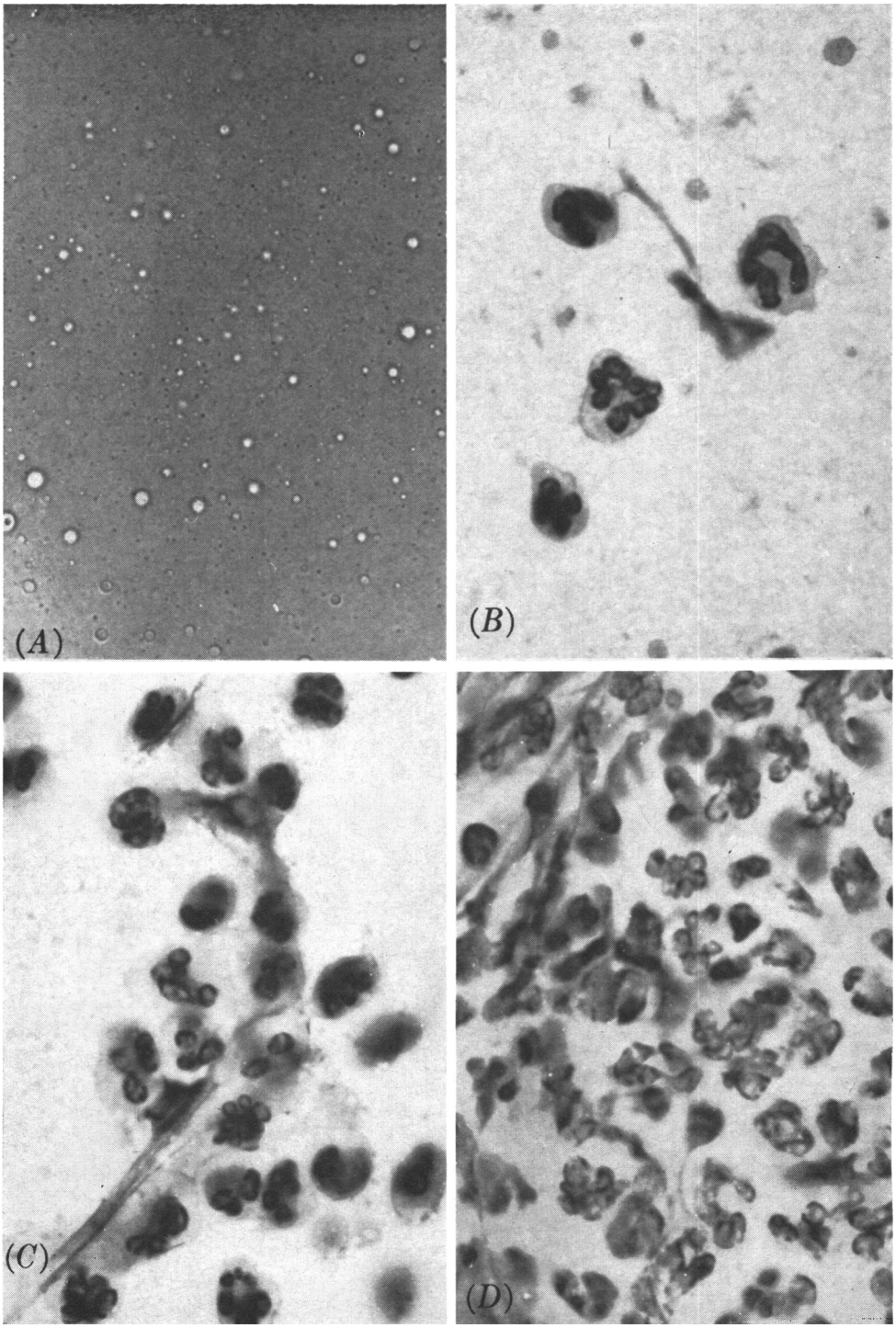

FIG. 2. Typical photomicrograph of Millipore filters demonstrating 0 (A); ± (B); + (C); and ++ (D) degrees of chemotactic infiltration of PMNs. Final magnification is ×3600.

the fourth sample for every animal. The filters were removed, stained, and studied from each group of four animals at 3, 3.5, 4, and 5 hr after application. The mean results of these samples are presented in Table I. All results were quite similar in each of the triplicate samples on all four rats. In this fashion, the kinetics of PMN infiltration into the Millipore filters in response to a variety of samples was determined.

These results suggest that, after about 4 hr, all samples except buffer controls, melted collagen (gelatin) and cut. coll'ase were mildly chemotactic. Bacterial collagenase, which undoubtedly contains bacterial toxins that are intrinsically chemotactic (2), was most rapidly and notably chemotactic in this system, with collagen being the next most chemotactic material.

It must be emphasized that there was a remarkable similarity in the replicate results for each sample. The only variables that did influence the results were bleeding during the removal of the epithelium and using rats heavier than 220 g. The older (heavier) the animal, the thicker the skin, and the less chemotaxis that could be demonstrated.

The various samples described above, as well as those representing the proteolytic digestion products of S_1 or collagen, were tested in triplicate at various dilutions for 4 hr on four rats each for chemotaxis as described. These mean results are presented in Table II. Again, these results were remarkably similar within the same rat or between four different animals.

TABLE I. The Kinetics of PMN Migration Through a Millipore Filter (450 mμ) in Response to Various Proteins.

Materials	Time after application (hr)			
	3	3.5	4	5
Buffer (Tris, 15 *M* NaCl)	0	0	0	0
S_1 (1 mg/ml)	0	±	±	±
Trypsin + SBTI (20 μg/ml)	0	0	±	±
Chymo + SBTI (20 μg/ml)	0	0	±	±
Collagen (1 mg/ml)	0	±	+	+
Gelatin (1 mg/ml)	0	0	0	0
B-coll'ase (20 μg/ml)	±	±	+	+
Cut. coll'ase (20 μg/ml)	0	0	0	0

The data in Table II shows firstly, that proteolytic digestion of S_1 increased its chemotactic properties markedly, *i.e.*, 100 μg/ml of S_1 was inactive whereas 10 μg/ml of S_1-enzyme digest was still slightly active chemotactically. Secondly, solutions containing as little as 100 μg/ml of soluble collagen were still chemotactic, while the bacterial collagenase could be diluted to a concentration of 0.02 μg/ml with a trace of chemotaxis still remaining. The chemotactic activity of the collagen–bacterial collagenase incubation mixtures were equivalent to that of the bacterial collagenase alone and hence collagenolysis products from this enzyme did not increase the chemotactic activity of the collagen itself. Further, trypsin digestion of soluble native collagen did not alter its chemotactic properties. Remarkably enough, however, the chemotactic properties of soluble collagen were markedly improved by partial digestion with cutaneous collagenase. Even 0.1 μg of these collagenolysis products were still chemotactic while less than 10 μg of the intact collagen alone was not chemotactic. Thus, collagenolysis products from cutaneous collagenase activity were 100 times more active chemotactically than was the intact collagen molecule!

Discussion and Conclusions. The imposed requirement that PMNs migrate through Millipore filter holes which were considerably smaller than the normal diameter of these cells seem sufficiently rigorous to exclude any but the most directed movement by these cells. The advantage of this system over that of Ward (6) for the study of chemotaxis is that this is an *in vivo* system; the disadvantage is that it is not as quantitative as the *in vitro* technique of Ward. Comparison of our results with those obtained by Ward suggest that the *in vivo* technique might be 10 times more sensitive than using washed cells *in vitro* (Ward, personal communication). Finally, the buffer controls were always zero in the *in vivo* method; whereas, in the *in vitro* chamber technique, a few cells moved into the filter in the controls.

The major findings using this *in vivo* method to study chemotaxis were: (i) that soluble

TABLE II. The Effects of Dilution upon the Number of PMNs Which had Migrated Through a Millipore Filter (450 mμ) After 4 hr.

Materials	Dilution				
	0	1:10	1:100	1:1000	1:10,000
S_1 (1 mg/ml)	±	0	0	0	0
Trypsin + SBTI (20 μg/ml)	±	0	0	0	0
Chymo + SBTI (20 mμ/ml)	±	0	0	0	0
S_1 + trypsin	+	+	±	0	0
S_1 + chymo	+	+	±	0	0
Collagen (1 mg/ml)	+	±	0	0	0
B-coll'ase (20 μg/ml)	+	+	+	±	0
Collagen + B-coll'ase	+	+	+	±	0
+ trypsin	+	±	0	0	0
Cut. coll'ase	0	0	0	0	0
Collagen + cut. coll'ase	++	++	++	++	+

collagen, but not the gelatin made from this collagen, was markedly chemotactic; (ii), that bacterial collagenase digested collagen was probably not more chemotactic than undigested collagen, and (iii) that cutaneous collagenase digested collagen was considerably more chemotactic than intact soluble collagen!

The native soluble collagen is markedly viscous in solution and the digestion of this material into smaller products would release collagen-derived polypeptides (11, 12), which would be capable of a more rapid diffusion away from the sample disc through the filter into the dermis. Thus, the more rapid establishment of a concentration gradient of these collagen-derived products could be established with a concomitant increase in the number of PMNs chemotactically responding to this gradient. Bacterial collagenase, however, reduces collagen to small tetrapeptides which may be too small to interact chemotactically with the PMN, since peptides less than 8 residues long are not chemotactic (2).

Finally, these findings suggest the possibility that collagenolysis may serve chemotactically the function of producing polymorphonuclear infiltration during necrotic inflammation, when collagenolysis is occurring rapidly in wounded tissue (9).

Summary. An *in vivo* method for measuring chemotaxis has been described using Millipore filters. This method demonstrated that soluble collagen, but not gelatin, was quite chemotactic. Further, the products of collagenolysis produced by cutaneous collagenase, but *not* by bacterial collagenase, were extraordinarily chemotactic for polymorphonuclear leukocytes within 4 hr after testing at a concentration of about 0.1 μg.

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