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PROTEASES RELEASED IN ORGAN CULTURE BY ACUTE DERMAL INFLAMMATORY LESIONS PRODUCED IN VIVO IN RABBIT SKIN BY SULFUR MUSTARD: Hydrolysis of Synthetic Peptide Substrates for Trypsin-like and Chymotrypsin-like Enzymes[1]

KAZUYUKI HIGUCHI,[2,6] AKIRA KAJIKI,[2,7] MASAHIRO NAKAMURA,[2,8] SUSUMU HARADA,[2,7] PEGGY J. PULA,[2] ALAN L. SCOTT,[3] and ARTHUR M. DANNENBERG, Jr.[2-5,9]

Departments of [2]Environmental Health Sciences, [3]Immunology and Infectious Diseases, and [4]Epidemiology
School of Hygiene and Public Health
[5]Department of Pathology, School of Medicine
The Johns Hopkins University, Baltimore, Maryland 21205

Abstract—The purpose of these studies was to identify some of the extracellular proteolytic enzymes associated with the development and healing of acute inflammatory lesions. Lesions were produced in the skin of rabbits by the topical application of the military vesicant, sulfur mustard (SM). Full-thickness, 1-cm^2 central biopsies of the lesions were organ-cultured for one to three days, and the culture fluids were assayed for proteases with a variety of substrates. When compared to culture fluids from normal skin, the culture fluids from both developing and healing SM lesions had three to six times the levels of proteases hydrolyzing two synthetic peptide substrates: (1) *t*-butyloxycarbonyl-Leu-Gly-Arg-4-trifluoromethylcoumarin-7-amide(Boc-Leu-Gly-Arg-AFC, herein abbreviated LGA-AFC), and (2) *N*-benzoyl-phenylalanine-β-naphthyl ester (BPN). LGA-AFC is a substrate for trypsin, plasmin, plasminogen activator, thrombin, kallikrein, and the C3 and C5 conver-

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[6] On leave of absence from the Research Institute for Diseases of the Chest, Faculty of Medicine, Kyushu University, Fukuoka, Japan
[7] On leave of absence from the Second Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan
[8] On leave of absence from First Department of Internal Medicine, Kurume University, Kurume, Japan
[9] To whom reprint requests should be addressed.

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tases; BPN is a chymotrypsin and cathepsin G substrate. The culture fluids did not consistently hydrolyze four other synthetic peptide substrates or the proteins [^{14}C]-casein and [^{14}C]elastin. In order to determine the likely sources of LGA-AFCase and BPNase activity, we counted the number of granulocytes (PMNs), macrophages (MNs) and activated fibroblasts in histologic sections of developing and healing SM lesions, and we measured the levels of these enzymes in serum, in culture fluids of PMN and MN peritoneal exudate cells, and in culture fluids of two fibroblast cell lines. In SM lesions, serum and fibroblasts seemed to be the major source of LGA-AFCase, and serum alone the major source of BPNase. Tissue PMNs and MNs seemed to be only minor sources. The crusts of healing lesions, which were full of dead PMNs, seemed to be a rich source of both enzymes. In the SM lesion culture fluids, whether LGA-AFC and BPN were hydrolyzed by endopeptidases or only by exopeptidases could be determined by evaluating complex formation with α-macroglobulin proteinase inhibitors (αM). Endopeptidases, but not exopeptidases, are entrapped and inhibited by αM, because an internal peptide band in αM must first be hydrolyzed before molecular rearrangement (required for proteinase inhibition) occurs. The catalytic site of endopeptidases that are entrapped and inhibited by αM is known to remain active on (and reachable by) small synthetic peptide substrates such as LGA-AFC and BPN. In sodium dodecyl sulfate–polyacrylamide gel preparations of SM lesion culture fluids, we found electrophoretic bands that both stained for αM with specific antibody with the immunoperoxidase technique and hydrolyzed LGA-AFC and/or BPN. Thus, at least some of the SM lesion enzymes that hydrolyzed LGA-AFC and BPN were endopeptidases. These proteinases probably played a local extracellular role in the inflammatory process before they were inhibited by extravasated serum inhibitors, such as αM.

INTRODUCTION

There are many reports in the literature on the identification of the inflammatory mediators released from isolated leukocytes (1–4) but few that measure which mediators are released into the extracellular fluids of intact inflammatory lesions at different stages of development and healing (discussed in reference 5). We, therefore, organ-cultured such lesions and measured the proteolytic enzymes released into the culture fluids. The lesions were produced by the military vesicant sulfur mustard (SM). Similar to ultraviolet light, SM damages the DNA of epidermal cells, initiating a slowly developing acute inflammatory process (5–8).

The culture fluids did not hydrolyze [^{14}C]casein or [^{14}C]elastin. Evidently, the serum proteinase inhibitors present in the extracellular fluids of the lesions were sufficient to inactivate the proteinases soon after their release by cells in the local inflammatory site (see reference 9).

Two small, synthetic peptide substrates, however, were hydrolyzed: (1) Boc-Leu-Gly-Arg-AFC (LGA-AFC), a substrate for plasmin, thrombin, and other trypsin-like enzymes, and (2) *N*-benzoyl-phenylalanine-β-naphthyl ester (BPN), a substrate for chymotrypsin-like enzymes. The levels of LGA-AFCase and BPNase were elevated during the entire inflammatory process, and the major

sources of these proteases were extravasated serum, activated fibroblasts and, in healing lesions, the crusts.

MATERIALS AND METHODS

The SM was applied topically at various times so that 2-h, 1-day, and 2-, 3-, 6-, and 10-day lesions were present when the animal was sacrificed. The lesions were cut into 1.0-cm^2 full-thickness explants and organ-cultured for one, two and three days (5). The culture fluids, which extracted the extracellular fluids from the lesions, were collected, cleared by centrifugation, and assayed for proteases. The explants were also evaluated histologically for epidermal cell death, leukocyte infiltration, fibroblast activation, crust formation, and healing (re-epithelialization), so that the levels of proteases could be correlated with histopathology of the lesions. Details on the organ culture and histologic techniques are given in references 5 and 10.

Trypsin-like Protease (adapted from references 11–13). Boc-Leu-Gly-Arg-AFC (LGA-AFC) is a trypsin, plasmin, and plasminogen activator and kallikrein substrate (see Table 1). Boc designates *t*-butyl-oxycarbonyl, and AFC designates 7-amino-4-trifluoromethylcoumarin, which is coupled to the carboxyl group of arginine as the 4-trifluoromethylcoumarin-7-amide. The amide linkage is split by the enzyme to release the fluorescent AFC.

This substrate (containing L-amino acids) was obtained from Enzyme Systems Products, (P.O. Box 2033, Livermore, California 94550, catalog No. 58-AFC). It was dissolved in dimethylformamide (DMF) to a concentration of 20 mM. Fifty microliters of the AFC substrate, 900 μl of 0.05 M TES buffer (pH 8.2), and 50 μl of the lesion culture fluid were incubated for 24 h at 37°C. TES designates *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (Sigma Chemical Corp., St. Louis, Missouri, catalog No. T-1375). The fluorescence of the incubated solutions was read at 0 and 24 h in an AMINCO spectrofluorometer with an excitation wavelength of 400 nm, and an emission wavelength of 505 nm. As a control, the substrate solution without enzyme (50 μl) was incubated for 24 h in 950 μl of the TES buffer, and the increase in its fluorescence was subtracted from the increase in the fluorescence produced by the lesion culture fluids. The pH of the incubated solutions was usually 8.1.

The AFC standard (Sigma, catalog No. A-8401), used to set the "sensitivity vernier" at 10, consisted of 50 μl of an AFC solution (20 μM in DMF) in 950 μl of TES buffer. Thus, 1.0 μM AFC was equivalent to 10 fluorescence units.

The pH optimum of LGA-AFCase in SM lesion culture fluids and serum was between 7.6 and 8.6. Little or no spontaneous hydrolysis of LGA-AFC occurred in the pH range 5.0–9.0. Also, the hydrolysis of LGA-AFC by lesion culture fluids and by serum was the same in TES, Tris, and phosphate buffers at the same pH.

Chymotrypsin-like Esterase (14, 15). *N*-benzoyl-D,L-phenylalanine-β-naphthyl ester (BPN), a substrate for chymotrypsin-like enzymes, was obtained from Sigma Chemical Co. (catalog No. B-2379). Culture fluids (1.0 ml) were mixed with 2.5 ml of the dilute BPN substrate and incubated 1 h at 37°C in a shaking water bath. (Dilute BPN was prepared by dissolving 8 mg BPN in 4.0 ml acetone and adding this solution to 76 ml of 0.1 M sodium phosphate buffer, pH 6.5.) The hydrolysis was stopped by placing the tubes in cracked ice.

The pH was measured on 0.5 ml of the reaction mixture (it was 6.6), and the remaining 3.0 ml was mixed with 1.5 ml of sodium veronal buffer (0.12 M, pH 8.6) to bring the pH to 7.6 for diazo-coupling. Then, naphthanil diazo blue B (NDBB) (0.5 ml) (Dupont, Organic Chemicals Dept., Wilmington, Delaware 09898) was added. (This diazotizing solution contained 80 mg of NDBB in 20 ml of cold water.) After exactly 3 min in the ice bath, trichloroacetic acid (0.5 ml of a 40% solution) was added to stop the coupling reaction.

Table 1. Hydrolysis of Protease Substrate, Boc-Leu-Gly-Arg-AFC, by Culture Fluids from SM Lesions and Several Purified Proteases. Inhibition by Plasmin Inhibitor, Aprotinin

Culture fluids from[a]	Fluorescence units/ml (A)[b]	Fluorescence units/ml in presence of aprotinin (B)[c]	Percent of protease activity remaining (B/A)
normal skin	8.3	3.4	41
2-h lesions	7.4	2.3	31
1-day lesions	20.3	9.7	48
2-day lesions	23.1	9.8	42
3-day lesions	16.4	5.8	35
6-day lesions	21.1	6.9	33
10-day lesions	21.9	7.3	33
Serum (1:40)[d]	90.3	66.6	74
Trypsin (2.5 ng/ml)[e]	64	0	0
Kallikrein (0.005 units/ml)[e]	34	16	47
Plasmin (0.5 μg/ml)[e]	26	0	0
Urokinase (5 units/ml)[e]	16	30	190

[a] After 24 hr, the culture fluids (2.5 ml) were collected and centrifuged to remove the suspended cells and debris. Fifty μl of culture fluid was added to the substrate solution and incubated for 24 hr. The increase in fluorescence over that of the supplemented RPMI medium alone is listed. The data from a representative rabbit from the series are presented.

[b] See Materials and Methods.

[c] Aprotinin (2.5 μg/ml) (from Sigma Chemical Co.).

[d] A 1:40 dilution of serum is its approximate concentration in culture fluids from SM lesions. Sera from four rabbits were assayed.

[e] Several concentrations of the purified proteases (from Sigma Chemical Co.) were assayed. In each case, a representative concentration from the straight-line portion of the hydrolysis–concentration curve is listed.

Each sample was then shaken vigorously with 5.0 ml of ethyl acetate (to extract the red-purple color) and centrifuged. The supernates (3.0 ml) were removed, and their optical densities (OD) were read at 540 nm in a Bausch & Lomb Spectronic 20 spectrophotometer. Because of their high BPNase content, the first-day culture fluids (from all SM lesions and from normal skin) were diluted with equal parts of culture medium RPMI 1640 before assay.

A blank containing the RPMI 1640 culture medium was run simultaneously, and its OD was subtracted from that produced by the lesion culture fluids. Positive controls of 1.0 μg/ml of crystallized α-chymotrypsin in water (EC 3.4.31.1) (Sigma Chemical Co., catalog No. C-4129) were assayed with each group of culture fluids. In this assay system, 1.0 μg of the α-chymotrypsin produced an increase in OD of 0.300 OD units/ml, and 1.0 μg of β-naphthol produced an increase in OD of 0.02 units/ml.

The BPNase of SM lesion culture fluids (and serum) showed a broad pH optimum between pH 5.4 and 6.8 with acetate and phosphate buffers. Spontaneous hydrolysis of the BPN occurred above pH 7.3. The enzymatic hydrolysis of BPN was usually about 20% higher in acetate buffer at pH 5.6 and 6.6 than it was in phosphate buffer at pH 6.6.

Other Protease Substrates. [^{14}C]Casein, [*methyl*-^{14}C] methylated α-casein (with a specific activity of 2.3 μCi/mg), was obtained from New England Nuclear Corp. (Boston, Massachusetts 02118, catalog No. NEC-735) and used as previously described (9).

[^{14}C]Elastin, [*methyl*-^{14}C] methylated soluble elastin (with a specific activity of 0.015 mCi/mg), obtained from New England Nuclear Corp., was used similarly.

[^{14}C]Suc(OMe)-Ala-Ala-Pro-Val-anilide, i.e., methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valyl-anilide, (aniline[^{14}C (U)]), an elastase substrate, was obtained from New England Nuclear Corp. (catalog No. NEC-780, 9.5 mCi/mmol, 0.001 mCi in 0.2 ml). For the elastase assay, 0.05 ml of SM lesion culture fluid, 0.03 ml of working substrate [0.04 ml of the above anilide in 1.9 ml 0.1 M Tris HCl buffer (pH 9.0)], and 5.0 ml Econofluor (New England Nuclear, catalog No. NEF-941) were incubated 5 h at 37°C. The [^{14}C]anilide released by elastase activity entered the scintillation fluid layer, where its radioactivity could be counted in a scintillation counter (16).

Granulocyte (PMN) and Macrophage (MN) Rabbit Peritoneal Exudate Cells and Rabbit Fibroblast Cell Lines. PMN exudate cells were obtained 4 h after the intraperitoneal injection of 0.1% glycogen in physiologic saline. MN exudate cells were obtained four days after a similar injection of 0.5% glycogen. Details of these procedures are described in reference 10. The PMN exudate cells contained 95–100% neutrophils. The MN exudate cells contained 98–100% mononuclear cells (macrophages with variable numbers of lymphocytes).

With the trypan blue dye-exclusion method (10), we found that the PMNs were 94 ± 2% viable before culture and 71 ± 5% viable after culture for 24 h. Similarly, the MNs were 87 ± 3% viable before culture and 79 ± 1% viable after culture.

The cells were cultured, or frozen and thawed and extracted, in serum-free supplemented RPMI 1640, as described in reference 10. Followng culture or extraction, the cells and debris were removed from the RPMI 1640 by a brief centrifugation (2000 rpm × 10 min). Then, the supernates were frozen and stored at −70°C until assayed.

The fibroblast cell lines CCL-193 and CRL-1414 were obtained from the American Type Culture Collection. They were cultured in medium RPMI 1640, first with 10% calf serum, and then in serum-free medium, as described in reference 10. They also were frozen and thawed and extracted as above.

Protein Determinations. The protein-dye binding method of Bradford, modified by Bio-Rad Laboratories was used (5, 17).

RESULTS

Proteases (in SM Lesion Culture Fluids) Hydrolyzing Boc-Leu-Gly-Arg-AFC (LGA-AFC), a Substrate for Plasminogen Activator, Plasmin, and Other Trypsin-like Proteases

When compared to culture fluids from normal skin, culture fluids from both peak (one-day) and healing (six- and ten-day) SM lesions had four to six times the LGA-AFCase activity per milliliter (Figure 1). Culture fluids from healing

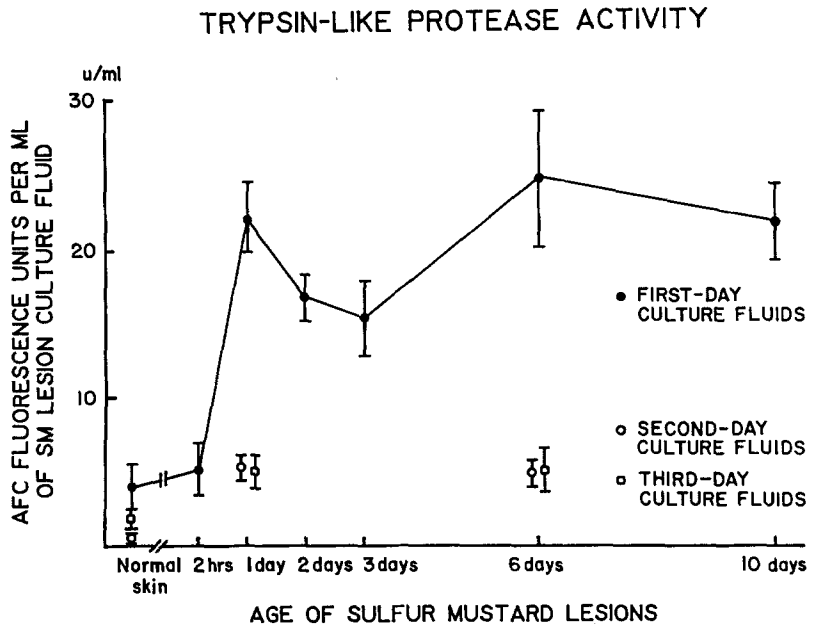

Fig. 1. Trypsin-like protease (plasminogen activator, plasmin, and other) activities in first-, second-, and third-day culture fluids from 1.0-cm^2 dermal sulfur mustard lesions. *t*-Butyloxycarbonyl-Leu-Gly-Arg-4-trifluoromethylcoumarin-7-amide (LGA-AFC) was the substrate. The fluorescence unit is defined in Materials and Methods. The LGA-AFCase activity of each 1.0-cm^2 explant is 2.5 times the fluorescence units shown on the ordinate, since each explant was cultured in 2.5 ml of fluid (not 1.0 ml).

The means and their standard errors are depicted. First-day culture fluids from normal skin and 2-hour SM lesions showed LGA-AFCase activities significantly different from those of 1-, 2-, 3-, 6-, and 10-day SM lesions ($P < 0.01$). The one-tailed Student's *t* test was used. The lesion culture fluids used for these LGA-AFCase assays (and those in Figure 2) came from Experiment II of reference 10.

lesions, but not peak lesions, had 2½ times the LGA-AFCase activity per milligram of protein than such fluids from normal skin had (Figure 2).

Proteases (in SM Lesion Culture Fluids) Hydrolyzing N-*Benzoyl-Phenylalanine β-Naphthyl Ester (BPN), a Substrate for Chymotrypsin-like Enzymes*

When compared to culture fluids from normal skin, culture fluids from both peak (one-day) and healing (six- and ten-day) SM lesions had four and five

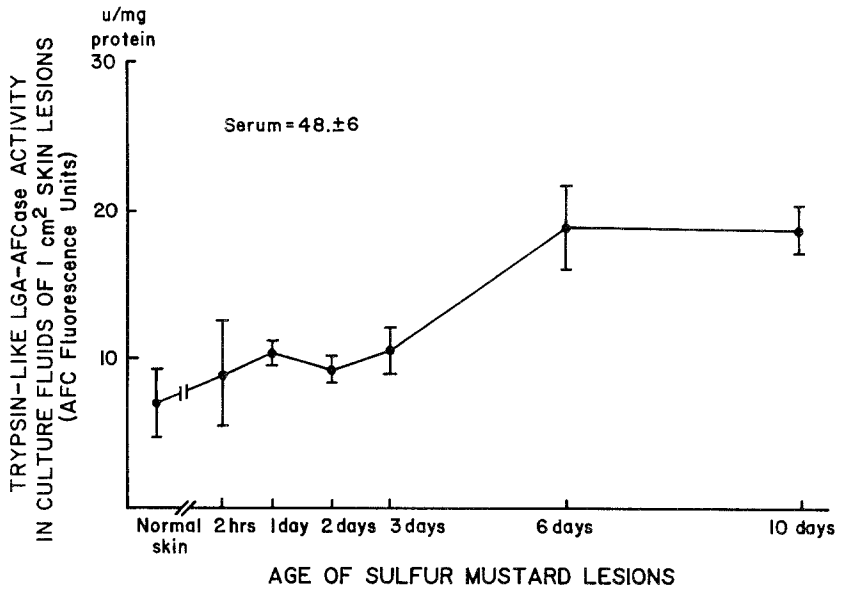

Fig. 2. Trypsin-like (LGA-AFCase) activity per milligram of protein in first-day culture fluids from 1.0-cm^2 dermal SM lesions of various ages. The culture fluids from 6- and 10-day SM lesions showed significantly higher LGA-AFCase activity per milligram of protein than did culture fluids from normal skin and from 1-, 2- and 3-day SM lesions ($P \leq 0.025$). The one-tailed Student's t test was used. The LGA-AFCase activities per milligram of protein in the second- and third-day culture fluids (not shown) were the same or lower than those in first-day culture fluids, which is consistent with a loss of extravasated serum enzymes and a low (or absent) local enzyme release by the cells in the explant.

times the BPNase activity per milliliter (Figure 3). Culture fluids from healing lesions, but not peak lesions, had two times the BPNase activity per milligram of protein than such fluids from normal skin had (Figure 4).

Second- and Third-Day Culture Fluids from SM Lesions

The culture fluids were collected each day and replaced with fresh, supplemented RPMI 1640 medium for the next day of culture. Since most of the unbound extravasated serum in the lesions was extracted during the first day of culture (5, 18), the concentration of protein in second- and third-day culture fluids was decreased (5). If the cells in the SM lesions were releasing apprecia-

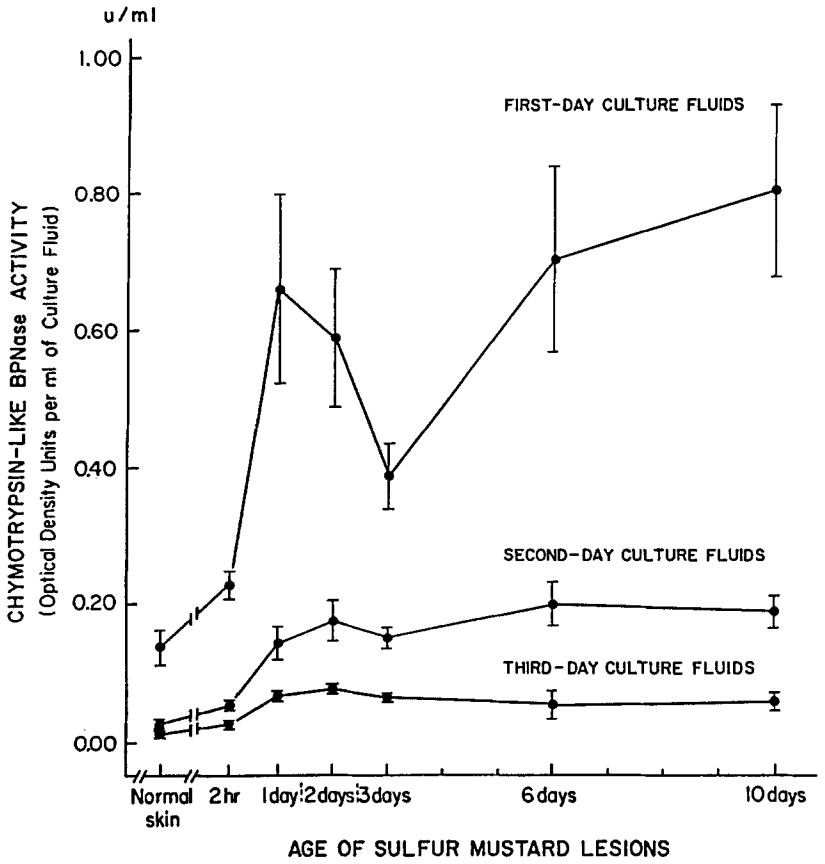

Fig. 3. Chymotrypsin-like esterase activity in first-, second-, and third-day culture fluids from 1.0-cm^2 dermal sulfur mustard lesions. *N*-Benzoyl-D,L-phenylalanine-β-naphthyl ester (BPN) was used as the substrate. The optical density unit is defined in Materials and Methods. The BPNase activity for each 1.0-cm^2 explant is 2.5 times the OD units shown on the ordinate, since each biopsy was cultured in 2.5 ml of fluid (not 1.0 ml).

The means and their standard errors are shown. First-day and second-day culture fluids from 1-, 2-, 3-, 6-, and 10-day lesions showed BPNase activities significantly different from those of corresponding culture fluids from normal skin ($P < 0.002$). Third-day culture fluids showed similar results ($P < 0.014$). The one-tailed Student's *t* test was used. The lesion culture fluids used for these BPNase assays (and those in Figure 4) came from Experiment I of reference 10.

ble amounts of LGA-AFCase and BPNase, the concentration of these enzymes per milligram of protein in the culture fluids should increase during the second and third day of culture. No such increase occurred (see Figure 2 and 4). Therefore, during organ culture, these proteases did not seem to be preferentially released over other protein constituents by the cells in the lesions.

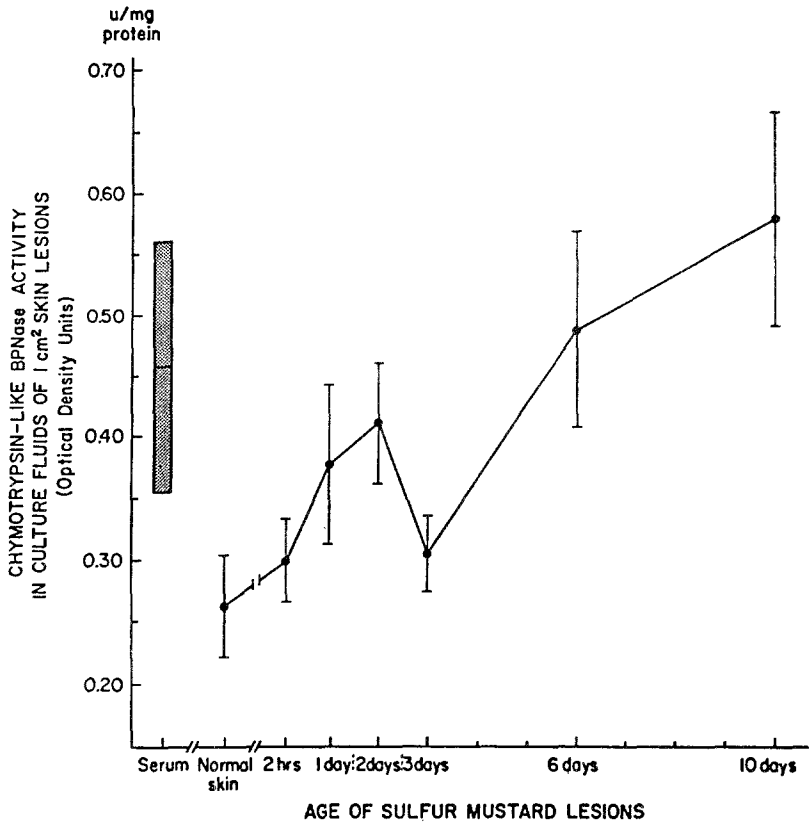

Fig. 4. Chymotrypsin-like esterase (BPNase) activity per milligram of protein in first-day culture fluids from 1.0-cm^2 dermal sulfur mustard (SM) lesions of various ages. The culture fluids from 2-, 6-, and 10-day SM lesions showed significantly higher BPNase activity per milligram of protein than did culture fluids from normal skin ($P < 0.022$). The one-tailed Student's t test was used. The BPNase activities per milligram of protein in the second- and third-day culture fluids (not shown) were the same or lower than those in first-day culture fluids, which is consistent with a loss of extravasated serum enzymes and a low (or absent) local enzyme release by the cells in the explant.

Plasmin and Plasminogen Activator: Probable Sources of LGA-AFCase Activity in Lesion Culture Fluids

Aprotinin is known to inhibit plasmin but not plasminogen activator (19). We, therefore, added aprotinin to SM lesion culture fluids in order to determine the percentage of LGA-AFC hydrolysis that could be due to plasmin. As controls, we also tested the effect of aprotinin on several commercially available proteinases.

Aprotinin (in the appropriate concentrations) partly inhibited the LGA-AFCase found in culture fluids (Table 1). It fully inhibited trypsin and plasmin, partly inhibited kallikrein, and apparently enhanced the activity of urokinase (Table 1). Thus, the SM lesion culture fluids probably contained both aprotinin-inhibitable and noninhibitable types of proteases, probably plasmin and plasminogen activator, respectively, because the culture fluids contained extravasated serum (5, 9, 18), and serum is rich in these two enzymes (or their proenzymes).

In pilot experiments, no plasmin or plasminogen activator could be detected in the culture fluids, either by the fibrin plate method (20, 21) or by the sensitive [^{125}I]fibrin method (22). The latter method was performed by Dr. Pamela J. Jensen, who is with Dr. Gerald S. Lazarus's group at the University of Pennsylvania. We confirmed these results in our own laboratory, using the same method under her guidance. The ability to split LGA-AFC, a small peptide substrate, and the lack of ability to activate plasminogen or hydrolyze fibrin suggest that, in the culture fluids, plasminogen activator and plasmin were bound to the macroglobulin inhibitors (see below).

LGA-AFCase and BPNase Activities in Serum

LGA-AFCase activity per milligram of protein was 2½–5 times higher in serum than in SM lesion culture fluids (Figure 2), whereas BPNase activity per milligram of protein in serum was similar to that in the culture fluids (Figure 4).

The studies presented in Table 1 [and those of others (23, 24)] showed that LGA-AFC or the related substrate, LGA-MCA (4-methylcoumarin amide) was hydrolyzed by plasminogen activator (urokinase), plasmin, kallikrein, α-thrombin, factor Xa, and C3 convertase, C5 convertase and C1s of the complement system. Differences between serum and lesion culture fluids in LGA-AFCase and BPNase activities per milligram of protein could also be due to differences in the inactivation and/or clearance of such proteases within the lesions.

Cellular and Tissue Sources of Proteases Hydrolyzing LGA-AFC and BPN

Cells. Explants, 1 cm^2, of normal skin, one-day SM lesions, and six-day SM lesions contained an average of 16,000, 880,000, and 290,000 PMNs; 100,000, 3,700,000, and 500,000 macrophages (MNs); and 1,000,000, 1,900,000, and 7,400,00 activated fibroblasts, respectively (see reference 10).

Rabbit peritoneal exudate PMNs and MNs and rabbit fibroblast cell lines (obtained and cultured as described in reference 10) released into culture the amounts of LGA-AFCase and BPNase listed in Table 2. From the number of cells of each type in the lesions and from the amount of LGA-AFCase and BPNase released from one million free cells in culture, we could make a rough estimate of the contribution of each cell type to the enzyme content of the SM lesion culture fluids (Table 3).

The SM lesions (and serum) used to obtain the data presented in Tables 2 and 3 were the same as those used to obtain the data presented in reference 10. The many assumptions made to produce these rough estimates are discussed therein. For comparative purposes, the amount of these enzymes extracted from frozen and thawed PMNs, MNs, and fibroblasts are also listed in Table 2.

Serum. The contribution of serum enzymes to the SM lesion culture fluids was also estimated (Table 3). For this estimation, we assumed that 85% of the protein in the culture fluids was serum-derived (see reference 10) and that intravascular and extravascular serum had the same composition.

Crusts. Healing SM lesions have crusts containing fibrin, inspissated serum, large numbers of PMNs (mostly dead) (10), and variable, but smaller, numbers of MNs (again, mostly dead) (10). We removed most of the crust of six-day lesions with a scalpel, taking care not to compress the underlying lesion appreciably. Then, the remainder of the crust was removed (and discarded), but, in the process, the base was unavoidably compressed. [Squeezing the lesions (and normal skin) increases the amount of each enzyme released into the culture fluids (10), probably due to the cell injury and the alteration of the physical properties of the ground substance.] The crusts and the one- and six-day lesion bases (lesions without epidermis or crust) were cultured for 24 h, and the culture fluids were then assayed for LGA-AFCase and BPNase.

The crusts of the six-day lesions released two to three times the LGA-AFCase and BPNase into the culture fluids that the bases of six-day lesions released ($P < 0.01$) (Table 2). Together, the crust plus the base released three to four times the amount of these enzymes that the intact lesion released ($P < 0.01$) (Table 2). Thus, the crusts made an appreciable contribution to the LGA-AFCase and BPNase content of intact six-day SM lesion culture fluids, but we could only guess at the amount of this contribution (see Table 3). Intact lesions had less surface area exposed than split lesions and had not been compressed or squeezed.

The bases of one-day lesions released into the culture fluids over three times the amount of LGA-AFCase that the bases of six-day lesions released ($P < 0.001$) (Table 2). However, the bases of one-day lesions released about the same amount of BPNase into the culture fluids as the bases of six-day lesions released (Table 2).

Serum had 2½–5 times the level of LGA-AFCase per milligram of protein

Table 2. Hydrolysis of Protease Substrates, Boc-Leu-Gly-Arg-AFC and *N*-Benzoyl-phenylalanine-β-naphthyl Ester (BPN) by Culture Fluids from SM Lesions and their Crusts, by PMNs, Macrophages (MNs), and Fibroblasts, and by Serum[a]

Culture fluids (CFs) or extracts	Number of samples	LGA-AFCase (fluorescence units/ml)	Protein concentration (mg/ml)	Number of samples	BPNase (optical density units/ml)	Protein concentration (mg/ml)
CFs from intact 6-day SM lesions	6	15.5 ± 1.3	0.9 ± 0.03	6	0.56 ± 0.04	0.9 ± 0.03
CFs from crusts of 6-day lesions	8	40.7 ± 8.9	0.5 ± 0.1	8	1.24 ± 0.28	0.5 ± 0.1
CFs from 6-day lesions without crusts	8	14.6 ± 1.9	0.8 ± 0.04	8	0.41 ± 0.05	0.8 ± 0.04
CFs from 1-day lesions with epidermis removed	8	54.5 ± 4.6	1.3 ± 0.1	8	0.33 ± 0.05	1.3 ± 0.1
CFs from 5×10^6 PMNs/ml	4	0.5 ± 0.3	0.04 ± 0.01	7	0.50 ± 0.19	0.03 ± 0.01
Extracts of 5×10^6 PMNs/ml	4	0.6 ± 0.2	0.12 ± 0.02	7	1.39 ± 0.39	0.20 ± 0.05
CFs from 5×10^6 MNs/ml	5	1.7 ± 0.7	0.08 ± 0.01	7	0.16 ± 0.09	0.11 ± 0.03
Extracts of 5×10^6 MNs/ml	5	2.4 ± 0.7	0.35 ± 0.06	5	1.31 ± 0.90	0.53 ± 0.16
CFs from 2.4×10^6 fibroblasts/ml	5	61 ± 13	0.14 ± 0.01	4	0.15 ± 0.06	0.07 ± 0.03
Extracts of 2.4×10^6 fibroblasts/ml	5	189 ± 18	0.64 ± 0.03	5	1.09 ± 0.17	0.46 ± 0.12

Serum						
1:29	6	83 ± 10	1.74 ± 0.28	6	0.96 ± 0.21	2.09 ± 0.04
1:45		53 ± 6	1.13 ± 0.18		0.62 ± 0.13	1.35 ± 0.02

[a] *General*. The results are presented as enzyme activity per milliliter of culture fluid. The enzyme activity per milligram of protein can be derived from this table by dividing the activity per milliliter of culture fluid by the protein concentration (mg/ml).

Culture fluids (CFs) from crusts and from "surfaceless" SM lesions: Crusts from two 6-day SM lesions on each of three or four rabbits were removed, and cultured separately for 24 h in 2.5 ml of RPMI 1640. After centrifugation, the supernates were assayed for these enzymes. For comparison, intact 6-day lesions, the "crustless" bases of these lesions, and "surfaceless" 1-day lesions were also cultured.

PMN and MN extracts: 10 million PMN or MN peritoneal exudate cells (in 2.0 ml) were frozen and thawed five times and centrifuged. The supernates were assayed for these enzymes. See Materials and Methods.

PMN and MN culture fluids (CFs): 10 million PMN or MN exudate cells were cultured in 2.0 ml of RPMI 1640 for 24 h and centrifuged. The supernates were assayed for these enzymes. See Materials and Methods.

No corrections were made in the PMN and MN extracts (or CFs) for the 3 ± 1% MN in the PMN exudates and the 1 ± 1% PMNs in the MN exudates. The MN group contained variable numbers of small mononuclears that resembled small lymphocytes (10).

Fibroblasts: The two fibroblast cell lines were freed from the culture flasks with trypsin (see Materials and Methods), washed three times by centrifugation, and cultured 4 h in the supplemented (serum-free) RPMI 1640 medium. The average cell concentration was 3.6×10^6 cells in 1.5 ml. Aliquots of the same cell suspension were used to obtain both the extracts and the culture fluids.

The means and standard errors are listed. Four to seven rabbits were used for the PMN or MN preparations. Three separate cell cultures were maintained for each of the two fibroblast cell lines.

Sera: Dilutions of serum of 1:29 and 1:45 approximate the amounts of serum protein in first-day culture fluids from 1-day and 6-day SM lesions, respectively (9, 10).

Comment. In contrast to our findings in this table, BPNase was previously found to be absent in rabbit PMN (see reference 14). This inconsistency is probably explained by differences in the methodology employed in each case.

Table 3. Sources of Extracellular LGA-AFCase and BPNase in 1.0-cm^2 SM Lesions of Various Ages: Rough Estimates of Percentages of These Enzymes Derived from Serum, PMN, Macrophages (MNs), Fibroblasts, and Crusts[a]

Source	LGA-AFCase	BPNase
Normal skin: enzyme units/2.5 ml culture fluid	10.0	0.35
Serum	540.0 %	145.0 %
PMN	0.0	0.1
MN	0.9	0.7
Fibroblasts	300.0	2.9
1-day SM lesions: enzyme units/2.5 ml culture fluid	51.3	1.65
Serum	420.0 %	102.0 %
PMN	0.4	1.3
MN	6.2	5.6
Fibroblasts	113.0	1.2
6-day SM lesions: enzyme units/2.5 ml culture fluid	60.5	1.77
Serum	220.0 %	79.5 %
PMN	0.1	0.4
MN	0.7	0.7
Fibroblasts	370.0	4.1
Crusts	33.0	33.0

[a] This table presents a very rough estimate of the sources of the LGA-AFCase and BPNase in first-day culture fluids of 1.0-cm^2 explants from normal skin, 1-day SM lesions and 6-day SM lesions. To prepare this table, we assumed that the amount of these enzymes released in cell culture by PMNs, MNs, and fibroblasts was the same as the amount released by these cells when they were present within the organ-cultured SM lesion. The serum protein in the culture fluids was considered to be 85% of the protein concentration present (see reference 10). LGA-AFCase was measured in culture fluids from Experiment II of reference 10, and BPNase was measured in culture fluids from Experiment I of reference 10. Crusts were estimated to contribute 33% of the enzyme activity to culture fluids of 6-day lesions. The 33% is a mere guess but seems reasonable from the data presented in Results and from other experiments described in reference 10.

(Figure 2) and about the same level of BPNase per milligram of protein (Figure 4) that one- and six-day lesion culture fluids had. These facts may explain why higher LGA-AFCase levels were found in culture fluids from one-day lesion bases than in culture fluids from six-day lesion bases: One-day lesions were edematous and rich in extravasated serum (Table 2).

Conclusions. Our studies clearly suggest (see Table 3): [1] serum is the major source of both LGA-AFCase and BPNase in the extracellular fluids of normal skin and of both developing and healing SM lesions; [2] fibroblasts

may also be a major source of LGA-AFCase; [3] PMNs and MNs (because of their low numbers) released only small amounts of these two enzymes into the extracellular fluids; and [4] the dead (and live) cells (mostly PMNs) in the crust of healing lesions released appreciable amounts of both enzymes into culture fluids, but in vivo probably released much smaller amounts into the extracellular fluids within the lesions.

Evidence that LGA-AFCase and BPNase in Lesion Culture Fluids have Some Endopeptidase Activity

Endopeptidases are able to split the internal peptide bond in the "bait" region of α-macroglobulin proteinase inhibitors (αM) (25). Such splitting causes the αM to undergo a conformational change that "traps" the endopeptidase and inhibits its proteolytic activity (25). This property of αM can be used to distinguish endopeptidases from exopeptidases (25): By definition, exopeptidases cannot hydrolyze an internal peptide bond and therefore cannot cause the conformational change in αM or be inhibited by αM (see Discussion).

Experimental Procedures. First-day culture fluid from six-day SM lesions was applied uniformly along the top of a 7.5% polyacrylamide gel, containing 2% sodium dodecyl sulfate, and electrophoresed, both in the presence and absence of 5% mercaptoethanol. From a single gel, multiple identical strips were cut. Details of our procedures are presented in reference 9.

Some of the gel strips were incubated at 30°C for 20 h at pH 8.2 in the LGA-AFC substrate solution (described in Materials and Methods) and then gently rinsed in 0.9% NaCl solution. Several opalescent bands representing LGA-AFC hydrolysis were visible (Figure 5). These bands were more distinct when viewed under a black light lamp (Blak-Ray, long wave UVL-22, Ultraviolet Products, Inc., San Gabriel, California) because of their fluorescence.

Some of the strips were incubated at 30°C for 20 h at pH 6.5 in the benzoyl-phenylalanine-β-naphthyl ester (BPN) substrate solution (described in Materials and Methods). We found that postcoupling (of the released naphthol) with naphthylanil diazo blue B (NDBB) was not as effective as simultaneous coupling. Therefore, NDBB (dissolved in a drop of dimethylformamide) was mixed with the substrate solution, in a final concentration of 1.0 mg/ml, before the gels were added. Several dark blue-brown bands representing BPN hydrolysis were visible (Figure 5).

Still other gel strips were transblotted onto nitrocellulose and stained with specific antibody to rabbit αM by means of the immunoperoxidase technique (9) (Figure 5). The antibody, made in goats, specifically reacted with α_1- and α_2-macroglobulin inhibitors of rabbits. It was supplied by Dr. Katherine L.

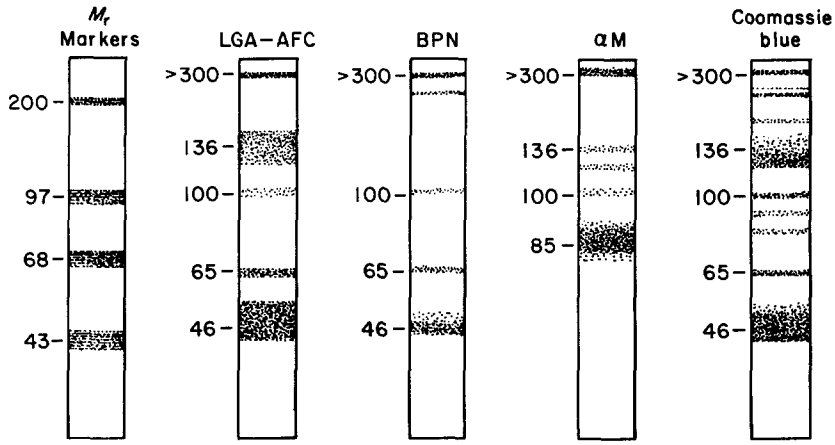

Fig. 5. A diagram representing the location of bands produced by the electrophoresis of 6-day SM lesion culture fluids on polyacrylamide gels containing sodium dodecyl sulfate. Details on these procedures were published in reference 9. After electrophoresis, the gels were cut into strips. One strip was incubated overnight in the LGA-AFC substrate solution described in Materials and Methods. Another strip was similarly incubated in the BPN substrate solution, containing the diazocoupler, naphthanil diazo blue B (see Results section). A third strip was transblotted onto nitrocellulose paper and stained with specific antibody to αM and the immunoperoxidase technique (see reference 9). For comparative purposes, a fourth strip was stained with the Coomassie blue stain for proteins. The figures listed represent the M_r of the bands in kilodaltons. LGA-AFCase and BPNase activities were present in the >300 and 100 αM staining bands, as well as in other bands that did not stain for αM. LGA-AFCase activity was also present in the 136 αM band (see Results and Discussion). Coomassie blue stained many more protein bands than did the other procedures.

Knight of the University of Illinois in Chicago. Bands containing αM and its fragments were stained blue–black by this technique.

With a metric ruler, we measured the distance of each band from the beginning of the running gel, so that bands showing the same degree of migration could be matched (Figure 5).

Results. Figure 5 depicts a gel that was representative of the five culture fluids that we evaluated. Each was from a six-day SM lesion on a different rabbit. A band of >300,000 M_r showed both LGA-AFCase and BPNase activities and also staining for the αM inhibitors. This >300,000 band consistently contained LGA-AFCase activity and often, but not always, contained BPNase activity. A 100,000 band also stained for the two enzymes and αM in the culture fluid from the rabbit represented in Figure 5, but culture fluids from other rabbits did not show this band. A 136,000 band stained for LGA-AFCase and αM, but not for BPNase. Other culture fluids showed bands of different M_rs that stained for one or both enzymes and αM.

Bands of 65,000 and 46,000 M_r stained for LGA-AFCase and BPNase,

but not αM. These two bands probably represent free LGA-AFCase and BPNase. They may have been dissociated from αM by the SDS or may never have been bound to it. It is likely that the LGA-AFC and BPN substrates are each hydrolyzed by more than one enzyme. The 85,000 band, stained for αM, in Figure 5 probably represents the 185,000 αM subunit that had been split in the "bait" region by proteinases (discussed in reference 9).

These results suggest that some of the proteases in the SM lesion culture fluids hydrolyzing LGA-AFC and BPN were endopeptidases. Others may have been exopeptidases, as these substrates can be hydrolyzed by a variety of proteases. Bands containing LGA-AFCase activity and αM were detected in the gels more often than bands containing BPNase activity and αM. Therefore, the endopeptidase–exopeptidase ratio of LGA-AFCase is probably higher than that of "BPNase." In fact, the BPNase preparation purified from beef lung (15) had no endopeptidase activity with hemoglobin or casein as its substrate.

Staining of the same gels for protein by Coomassie blue (9) revealed numerous bands (Figure 5), but only a few of these bands matched those stained for LGA-AFCase, BPNase, and αM. Thus, as expected, the proteins representing these two enzymes and αM represent only a small percentage of the total protein in the lesion culture fluids.

Protease Substrates Not Hydrolyzed by SM Lesion Culture Fluids

Two ^{14}C-labeled proteins and several synthetic peptide substrates were not appreciably hydrolyzed when incubated for 24 h with several first-day culture fluids from one-day and six-day SM lesions. The proteins [^{14}C]casein (9) and solubilized [^{14}C]elastin were incubated at 37°C, at pH 8.0 and 8.5, respectively, for 18 h (see Materials and Methods). Then, trichloroacetic acid or ammonium sulfate was added to precipitate the unhydrolyzed proteins and, after centrifugation and adding Econofluor (9), the supernatant fluids were read in a scintillation counter.

We measured the hydrolysis (by lesion culture fluids) of two synthetic peptide substrates for elastase: [1] methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valyl-anilide (aniline[^{14}C(U)]) (New England Nuclear Corp.) and [2] L-glutaryl-L-alanyl-L-alanyl-L-prolyl-L-valyl-4-methoxy-2-naphthylamine (Enzyme Systems Products, Livermore, California 94550). They were incubated with the culture fluids for 18 h at pH 7.6. A small amount of hydrolysis was sometimes found. The assay procedure for substrate [1] is described in Materials and Methods; for substrate [2], it was similar to the one used for BPN.

The hydrolysis (by the culture fluids) of [3] the synthetic substrate for cathepsin G, L-glutaryl-L-alanyl-L-alanyl-L-phenylalanyl-4-methoxy-2-naph-

thylamine (Enzyme Systems Products) and [4] the synthetic substrate for the trypsin-like Cathepsin B, *N*-benzoyl-D,L-arginine-β-naphthylamide (Sigma Chemical Co.) were measured under the same conditions as [2] above, except that cathepsin B was assayed at pH 5.6. Little or no hydrolysis of [3] and [4] was found. With [4], the presence of 0.01 M cysteine had little or no effect.

Elastase Inhibitors. [^{14}C]Suc(OMe)-Ala-Ala-Pro-Val-anilide was hydrolyzed by pancreatic elastase. Its hydrolysis was inhibited by culture fluids from peak (one- and two-day) SM lesions. Thus, inhibited elastase could be present in lesion culture fluids (see reference 9).

DISCUSSION

Proteolytic enzymes play major roles in the inflammatory process. The activation of the complement, kinin, plasmin, and clotting cascades in plasma involves the proteolytic cleavage of inactive proenzymes to produce the active enzyme (usually a protease) or mediator of the cascade (26). In addition, proteolytic enzymes (from epithelial cells, endothelial cells, fibroblasts and infiltrating leukocytes) play major roles in the breakdown of connective tissues during the inflammatory process and in the remodeling of connective tissues during repair (2–4, 27–29).

No free active proteinase (endopeptidase) was found in the organ-culture fluids of inflammatory skin lesions produced by sulfur mustard (SM). Therefore, the proteinases from the sources just listed probably acted locally where they were formed or released and then were inhibited—often by the serum proteinase inhibitors (9).

Detection of Inhibited Proteinases

Previously active proteinases may be detected in several ways: [1] by using antiserum to purified enzymes with immunoperoxidase techniques, [2] by measuring the products of their proteolytic action, [3] by removing or inactivating the inhibitor part of proteinase-inhibitor complexes, and [4] by using small synthetic peptides as substrates. Such peptides can enter the "cleft" in α-macroglobulin–proteinase complexes and become hydrolyzed. This report concerns the approach in [4]. Subsequent reports concern [2] and [3]. Purified rabbit proteinases to test [1] are not readily available, but our laboratory has used this method histochemically to demonstrate cathepsin D in macrophages found in chronic inflammatory lesions (30, 31).

The αMs are unique proteinase inhibitors (9, 25). They will bind a pro-

teinase only after it hydrolyzes an internal peptide bond in the αM. Such hydrolysis causes the αM to undergo molecular rearrangement, so that the αM surrounds the proteinase. The proteinase's catalytic site still remains active, but protein substrates can no longer be hydrolyzed: Proteins are usually too large to fit into the "cleft" in the αM, where the enzyme is now located. Small peptide substrates, however, readily reach the enzyme and become hydrolyzed, and the split-products then diffuse into the surrounding medium where they can be measured.

These principles can be used to distinguish exopeptidases from endopeptidases (25). If a protease is bound to αM, it must have hydrolyzed an internal bond in αM and therefore must be an endopeptidase.

Electrophoresis in acrylamide gels was used to separate proteinase–αM complexes from other proteins in SM lesion culture fluids. Bands containing these complexes were identified by their ability to [1] hydrolyze the fluorogenic peptide substrate, LGA-AFC, or the chromogenic peptide substrate, BPN, and [2] stain with a specific antibody to αM and the immunoperoxidase technique (Figure 5). Other electrophoretic bands either stained for the protease or for αM, but not for both. Thus, endopeptidases seem to be present among the various enzymes hydrolyzing LGA-AFC and BPN.

Source of LGA-AFCase and BPNase Activities in SM Lesions

Serum. This is a major source of both LGA-AFCase and BPNase (Tables 2 and 3). In serum, plasminogen activator, plasmin, α-thrombin, kallikrein, factor Xa and the C3- and C5-convertases hydrolyze LGA-AFC (Table 1 and references 23, 24), but specific enzymes in serum that hydrolyze BPN were not found in the literature. When two to four samples of both serum and plasma were compared, the levels of LGA-AFCase in plasma were found to be about twice those in serum, but the levels of BPNase were identical in each (unpublished experiments).

Fibroblasts. The fibroblast cell lines had high LGA-AFCase activity (Table 2), but the PMN and MN exudate cells had rather low activity.

The rapid clearance of locally formed LGA-AFCase–αM inhibitor complexes within the lesions probably explains why both serum and culture fluids from the fibroblast cell lines contained much higher LGA-AFCase activity per milligram of protein than did lesion culture fluids (Table 2).

BPN is an excellent substrate for cathepsin G (32), which resembles chymotrypsin. Homogenates of PMNs (from blood) and of whole spleens are rich sources of cathepsin G, but other sources of this enzyme have not been thoroughly evaluated (32).

Mast Cells. These cells (32–34), and probably basophils, hydrolyze BPN. From reference 33 and Table 2, we estimated that their extracts should contain roughly two to four times the BPNase activity of PMNs (per million cells). However, the number of mast cells and basophils within the SM lesions is small, approximately that of PMNs (excluding those in the crusts) (10). Since PMNs make such as small contribution to the BPNase in the extracellular fluids of these lesions (Table 3), mast cells and basophils should likewise make only a small contribution, even though the basophilic cells could release several times the BPNase that the PMNs could release. Basophils and mast cells remain close to the venules and do not seem to enter the crusts (5).

Epidermal Cells. These cells appear to have little or no LGA-AFCase and BPNase activity. At least, the mouse neonatal epidermal cells available to us showed no definite activity (unpublished data).

Crusts. Crusts contribute substantial amounts of both enzymes to culture fluids from healing lesions. The main sources of these enzymes seem to be the large amount of serum and dead PMNs that the crusts contain.

Plasma Protease Inhibitors

Plasma (and serum) contains α_1-proteinase inhibitor (formerly α_1-antitrypsin), αM, α_2-plasmin inhibitor, α_1-antichymotrypsin, antithrombin III, C1-inactivator, and inter-α-trypsin inhibitor (inter-α-globulin) (35). To our knowledge, the α_2-macroglobulins are the only inhibitors that do not inactivate the catalytic site of the bound proteinase, so that the hydrolysis of small peptide substrates still occurs.

The major inhibitor of plasmin is α_2-plasmin inhibitor, but the concentration of α_2M is higher (35). Plasmin is bound to both inhibitors (36) in proportions depending on the amount added to serum and on whether or not it was produced by urokinase or added directly as an active enzyme (36). The fact that some plasmin is bound to α_2M (in both serum and SM lesion culture fluids) does, however, explain why LGA-AFC is hydrolyzed and why its substrate, fibrin, is not (see Results).

Other Reports in This Series

Other reports in this series on inflammatory mediators and modulators in organ-culture fluids of dermal SM lesions concern [1] serum turnover (18), [2] α_1-proteinase inhibitor and the αM inhibitors (9), [3] chemotactic factors (37),

[4] lysosomal enzymes (10), [5] inhibited proteoglycanase and collagenase (38), and [6] products of hydrolase activity (before inhibition): glycosaminoglycans and hydroxyproline (39).

ADDENDUM

Two extensive reports on the trypsin-like and chymotrypsin-like enzymes found in normal skin were recently called to our attention (40, 41). They review the pertinent literature, list the activity of these enzymes on a variety of synthetic peptide substrates, and describe the effects of numerous synthetic and natural inhibitors on their hydrolytic activity.

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