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Pathogenesis and Treatment of Skin Lesions Caused by Sulfur Mustard:

Inflammatory Mediators and Modulators Released from Organ-Cultured Inflammatory Lesions Produced In Vivo in Rabbit Skin by Sulfur Mustard

ANNUAL/FINAL REPORT

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Our research program concerns the mediators and modulators that control the development and healing of dermal sulfur mustard (SM) lesions. SM injures the epithelial cells and some cells in the deeper tissues. An acute inflammatory response follows, specifically, extravasation of serum, granulocyte and monocyte infiltration, and fibroblast activation. The epithelium dies and a crust over the ulcer forms. In human beings, blister formation is prominent. Then healing takes place. The epithelium grows in from the periphery and from the uninjured cells of the hair follicles. Fibroblasts lay down new collagen, elastin and ground substance. Finally, extensive remodeling takes place, so that the injured tissues eventually return to normal.

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18. SUBJECT TERMS (continuation of DD FORM 1473)

trypsin-inhibitory capacity, α_1-proteinase inhibitor (α_1-antitrypsin); α_1- and α_2-macroglobulin proteinase inhibitors, chemotaxis of granulocytes and macrophages, lysosomal enzymes of skin, granulocytes, macrophages and fibroblasts, acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme (lactic dehydrogenase), trypsin-like and chymotrypsin-like enzymes, proteoglycanase, glycosaminoglycans, collagenase, hydroxyproline-containing peptides.

19. ABSTRACT (continuation of DD FORM 1473)

This process of injury, inflammation and repair is controlled by numerous mediators and modulators, produced by every viable cell participating in the reaction. The literature is replete with lists of such mediators, and new mediators, or new functions of old ones, are discovered each year. For the development of new therapeutic regimens, one needs to know which of many possible mediators exert the major control of each stage of the inflammatory process. Only when such information is available, can one select or develop appropriate pharmaceutical agents and know the time during the course of the lesion when such agents should be applied.

Therapeutic agents could affect some sources of mediators and modulators more than others. For example, biomedically engineered cell modulators are now becoming available, e.g., gamma-interferon, Interleukin 1, and epidermal and fibroblast growth factors. Soon, we shall be able to use such products to stimulate specific cell types. Knowledge of the mediators produced by each cell type and of their role in the progression and healing of SM lesions could guide the selection and timing of these new therapeutic agents.

We established an organ culture system to collect (in the culture fluids) the mediators and modulators at each stage of the SM reaction. Then these mediators were correlated with the histological and histochemical changes observed in the lesions. Our results have changed our concept of the whole inflammatory process:

1. Extravasated serum (observed grossly as edema) is more beneficial than harmful. It brings to the local site (a) albumin (major neutralizer of toxic substances and pH buffer), immunoglobulins (containing antibodies which protect against infection), (c) antioxidants against reactive oxygen intermediates, e.g. H_2O_2 and O_2^- released from phagocytes, and (d) protease inhibitors which protect against the numerous proteases activated during the inflammatory process. We measured these components in culture fluids from SM lesions (antioxidants excepted).

2. Extravasated serum (edema fluid) is not static, waiting to be absorbed into the lymphatics. It turns over constantly: In peak SM lesions, about 3 times in 24 hours; and in healing lesions, about once in 24 hours. Thus, extravasated serum will continue to supply fresh mediators and modulators throughout the course of SM injury and repair.

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3. The extravascular protein (serum) has a somewhat different composition from that of serum obtained from the blood. In normal skin and healing lesions, the fraction of culture fluid protein containing gamma-globulin (antibodies) is higher than the same fraction in blood. Perhaps, gamma-globulin is preferentially absorbed by ground substance when it is in its normal gel-like state. In healing SM lesions, the α_1-globulin fraction is lower than in peak lesions and normal skin, a finding consistent with the formation of proteinase-inhibitor complexes and their subsequent elimination. The albumin fraction in the lesions did not change throughout lesion development and healing.

4. A variety of potentially damaging proteases are released extracellularly in SM lesions. This damage, however, is confined to areas near the cells producing these proteases, because of the presence of extravasated α_1 proteinase inhibitor (formerly called α_1-antitrypsin) and α_1- and α_2-macroglobulin inhibitors. With gel electrophoresis, Western blots and specific antibodies to these major inhibitors, we were able to show that these two inhibitors were mostly complexed with proteinases. Sufficent amounts of free inhibitors remained, however, in reserve.

5. A variety of substrates and methodologies were used to identify the types of proteases in the SM lesions. Proteoglycanase and collagenase were present, complexed with inhibitors. We could recover the active enzymes from such complexes by inactivating the inhibitors. Additional evidence for their existence came from finding hydrolytic products of ground substance (glycosaminoglycans) and collagen (hydroxyproline-containing peptides) in the SM lesion culture fluids. Plasmin and plasminogen activator seem to be present, but we had to use small peptide substrates to detect them. Such substrates can reach the catalytic site of proteinases complexed with α-macroglobulin, become hydrolyzed, and then be released as split products. Another small peptide substrate was used to detect the presence of a chymotrypsin-like hydrolytic enzyme.

6. The sources of several lysosomal enzymes were determined by quantitative histological and histochemical procedures on tissue sections of lesions and on cell suspensions of granulocytes, macrophages and fibroblasts. Biochemical procedures were used to identify these same hydrolases in the SM lesion culture fluids and in serum. To our surprise, we found that granulocytes were a minor source of lysosomal enzymes, compared to macrophages in peak lesions and fibroblasts in healing lesions. Serum was also a major source of some of these enzymes.

7. Finally, we have begun an extensive investigation on chemotactic factors present in SM lesions. Factors attracting both granulocytes and macrophages were present in culture fluids from both developing and healing lesions. Leukotriene B_4, the complement component, C5a, and Interleukin 1 all seem to be involved, but many more studies must be performed to confirm these pilot experiments.

These and future studies (of the mediators and modulators at each stage of SM lesion development and healing) should be of help in selecting and developing new therapeutic agents (especially biomedically engineered cell modulators) for the treatment of sulfur mustard injury (see above).

FINAL REPORT -- SUMMARY

Our research program concerns the mediators and modulators that control the development and healing of dermal sulfur mustard (SM) lesions. SM injures the epithelial cells and some cells in the deeper tissues. An acute inflammatory response follows, specifically, extravasation of serum, granulocyte and monocyte infiltration, and fibroblast activation. The epithelium dies and a crust over the ulcer forms. In human beings, blister formation is prominent. Then healing takes place. The epithelium grows in from the periphery and from the uninjured cells of the hair follicles. Fibroblasts lay down new collagen, elastin and ground substance. Finally, extensive remodeling takes place, so that the injured tissues eventually return to normal.

This process of injury, inflammation and repair is controlled by numerous mediators and modulators, both from the cells and from the extravasated plasma in the reaction. The literature is replete with lists of such mediatorsμ and new mediators, or new functions of old ones, are discovered each year. For the development of new therapeutic regimens, one needs to know which of many possible mediators exert the major control over each stage of the inflammatory process. Only when such information is on hand can one select or develop appropriate pharmaceutical agents and know the time during the course of the lesion when such agents should be applied.

Therapeutic agents could affect some sources of mediators and modulators more than others. For example, biomedically engineered cell modulators are now becoming available, e.g., gamma-interferon, Interleukin 1, and epidermal and fibroblast growth factors. Soon we shall be able to use such products to stimulate specific cell types. Knowledge of the mediators produced by each cell type and of their role in the progression and healing of SM lesions could guide the selection and timing of these new therapeutic agents.

We established an organ culture system to collect (in the culture fluids) the mediators and modulators at each stage of the SM reaction. Then these mediators were correlated with the histological and histochemical changes observed in the lesions. Our results have changed the textbook concept of the whole inflammatory process:

1. Extravasated serum (observed grossly as edema) seems to be more beneficial than harmful. It brings to the local site (a) albumin (major neutralizer of toxic substances and pH buffer), immunoglobulins (containing antibodies which protect against infection), (b) both chemotaxins and chemotaxis inhibitors, (c) antioxidants against reactive oxygen intermediates, e.g., H_2O_2 and O_2^- released from phagocytes, and (d) proteinase inhibitors which protect against the numerous proteinases activated during the inflammatory process. We measured these components (except for chemotaxis inhibitors and antioxidants) in culture fluids from 1.0 cm^2 SM lesion explants.

2. Extravasated serum (edema fluid) is not static, waiting to be absorbed into the lymphatics. It turns over constantly: in peak SM lesions, about 3 times in 24 hours, and in healing lesions, about once in 24 hours. Thus, extravasated serum continues to supply fresh mediators and modulators throughout the course of SM injury and repair. It is therefore a major, constantly replenished regulator of the inflammatory process.

3. The extravascular protein (serum) has a somewhat different composition from that of serum obtained from the blood. In normal skin and healing lesions, the fraction of culture fluid protein containing gamma-globulin (antibodies) is higher than the same fraction in blood. Perhaps gamma-globulin is preferentially absorbed by ground substance when it is in its normal gel-like state. In healing SM lesions, the α_1-globulin fraction is lower than in peak lesions and normal skin, a finding consistent with the formation of proteinase-inhibitor complexes and their subsequent elimination. The albumin fraction in the lesions does not change throughout lesion development and healing.

4. A variety of potentially damaging proteinases are released extracellularly in SM lesions. This damage, however, is confined to areas near the cells producing these proteinases, because of the presence of extravasated α_1-proteinase inhibitor (formerly called α_1-antitrypsin) and α_1- and α_2-macroglobulin inhibitors. With gel electrophoresis, Western blots and specific antibodies to these major inhibitors, we were able to show that these two inhibitors were mostly complexed with proteinases. Sufficent amounts of free inhibitors remained, however, in reserve. Thus proteinases are present, but their activities are local and short-lived.

5. A variety of substrates and methodologies were used to identify the types of proteases in the SM lesions. Proteoglycanase and collagenase were present, complexed with inhibitors. We could recover the active enzymes from such complexes by inactivating the inhibitors. Additional evidence for their existence came from finding hydrolytic products of ground substance (glycosaminoglycans) and collagen (hydroxyproline-containing peptides) in the SM lesion culture fluids. Plasmin and plasminogen activator seemed to be present, but we had to use small peptide substrates to detect them. Such substrates can reach the catalytic site of proteinases complexed with α-macroglobulin, become hydrolyzed, and then be released as split products. Another small peptide substrate was used to detect the presence of a chymotrypsin-like hydrolytic enzyme. Proteinases are a cause of blister formation, both in human beings and in animals.

6. We began an extensive investigation of the chemotactic factors present in SM lesions. Factors attracting both granulocytes and macrophages were present in culture fluids from both developing and healing lesions. Leukotriene B_4, the complement component C5a, and Interleukin 1 all seem to be involved, but more studies must be performed to confirm these pilot experiments.

7. The sources of several lysosomal enzymes within SM lesions were determined by quantitative histological and histochemical procedures on tissue sections of lesions and on cell suspensions of granulocytes, macrophages and fibroblasts. Biochemical procedures were used to identify these same hydrolases in the SM lesion culture fluids and in serum. To our surprise, we found that granulocytes were a minor source of lysosomal enzymes when compared to the macrophages within peak lesions and the fibroblasts within healing lesions. Serum, and the crusts of healing lesions were major sources of these enzymes, and probably play important roles in lesion development and healing.

These and future studies (of the mediators and modulators at each stage of SM lesion development and healing) should be of help in selecting and developing new therapeutic agents, including biomedically engineered cell modulators (see above). For this purpose, we strongly recommend more interaction between the clinicians, who set the guidelines for the treatment of SM casualties, and medically trained research pathologists (such as ourselves), who have thoroughly studied the SM lesion. Only by such interaction can basic research studies be maximally utilized to improve the therapy of SM injury in human beings.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

ACKNOWLEDGMENT

I wish to thank the many co-investigators in my laboratory, and the few elsewhere, for making this monograph possible. Their names will be included as co-authors when manuscripts resulting from this work are submitted for publication.

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CHAPTERS 1 AND 2

Chapter 1. Quantitative Histopathology: Polymorphonuclear Leukocyte, Basophil and Mononuclear Cell Survival; and Unbound (Serum) Protein Content of SM Lesions

Chapter 2: Evans Blue Dye Experiments Which Determined the Rate of Entry and Turnover of Serum Protein in Developing and Healing SM Lesions

ABSTRACT (BOTH CHAPTERS)

Our research program is a systematic study of the inflammatory mediators and modulators released from organ-cultured sulfur mustard (SM) lesions in various stages of development and healing. The organ culture fluids extract these mediators and modulators from the extracellular fluids within the SM lesions. This in vitro technique therefore enables monitoring the in vivo state of SM lesions as no previously employed technique has ever done.

Chapters 1 and 2 describe (a) the production in rabbits of dermal SM lesions, (b) their histopathology, (c) the organ culture techniques, and (d) the turnover of serum protein in these lesions.

When applied topically to the skin of rabbits in vivo, dilute SM (1.0% in methylene chloride) produced a slowly developing inflammatory response, which peaked in size at 1 and 2 days, ulcerated within 3 days, and re-epithelialized by 10 days. Histologically, basophils and polymorphonuclear leukocytes (PMN) were common in both early and late lesions, and the crust over the ulcers was composed of dead epidermal cells, fibrin and large numbers of PMN. Healing occurred under the crust by migration of epidermal cells from the margins of the lesions and from the hair follicles.

In organ culture, the lesion explants survived well, and even re-epithelialization took place. Their excellent survival enabled us to compare the life spans of the infiltrating leukocytes within an inflammatory site. PMN in the explants began disintegrating during the first day of culture, and almost all had disappeared by 3 days. In contrast, most of the basophils and mononuclear cells in the explants were still intact (and probably viable) after 3 days of culture.

Extravasated serum proteins and the fluids retained by them caused 1-day SM lesions to weigh 45% more than normal skin. This increased weight was largely retained in 2-, 3-, 6-, and 10-day lesions. However, when 1.0-cm^2 lesion biopsies were organ-cultured for 3 days, the 1-, 2-, and 3-day lesions lost weight, and the 6- and 10-day lesions (and normal skin) gained weight. These weight differences were not due to the amount of unbound (serum) protein extractable into the culture fluids because both the early lesions and the late lesions contained about the same amount of unbound (serum) protein. The most likely explanation for these weight differences is that the newly formed ground substances of late lesions absorbed culture fluid because the ground substance had changed from the sol state of acute inflammation (from which it was extractable) back to its normal gel state (from which it was not extractable).

The unbound (serum) protein extractable into the culture fluids averaged 1.9 mg for 1.0-cm^2 normal skin explants with a mean weight of 215 mg, and 6.4 mg for 1-day SM lesions with a mean weight of 313 mg. Since rabbit serum contains about 60 mg protein/ml, these figures indicate that normal skin contained about 15% (unbound) serum by weight and that 1-day lesions contained about 34% (unbound) serum by weight.

* * * * * * * *

The presence of such large amounts of serum in normal and inflamed skin suggests that serum could be a major source of mediators and modulators of the local inflammatory response. For this reason, we determined the rates of entry and turnover of extravasated serum protein in SM lesions.

Rabbits bearing 2-hr and 1-, 2-, 3-, 6-, and 10-day SM skin lesions were injected intravenously with Evans blue dye 2 hours before they were sacrificed. Then their skin lesions were excised and organ-cultured. The serum protein entering these lesions during the 2 hours before sacrifice had been labeled with Evans blue. By multiplying the amount of Evans blue contained in the lesions by a factor that converted micrograms of Evans blue into milligrams of serum protein, we could determine the 2-hr rate of entry of serum protein into these lesions.

This serum protein was both bound and unbound, and both Evans blue-labeled and unlabeled. The unbound serum protein was the protein that was extractable from the lesions into the culture fluids; and the unlabeled serum protein was the protein that had entered the lesions before the injection of Evans blue. The grossly edematous peak lesions (1 day of age) contained 7.8 mg of unbound serum protein per cm^2 of skin. Healing lesions (6 and 10 days of age) contained about 4.5 mg/cm^2, and normal skin about 1.7 mg/cm^2.

Lesions 1 day of age had the highest rate of serum protein entry, and about 36% of this Evans blue-labeled protein was unbound, i.e., extractable into the culture fluids. Lesions 3 and 6 days of age had a rate of serum protein entry that was roughly half that of 1-day lesions, and only about 13% of this entering protein was unbound. Normal skin had a very low rate of serum protein entry and only 8% of this entering protein was unbound.

The turnover rate of the unbound, extractable serum protein could be calculated from the 2-hr entry rate of the Evans blue-labeled protein and the total protein in the culture fluids. In 1-day lesions, about 25% of the serum protein in the culture fluids was protein entering during the last 2 hours, so that 100% of this unbound protein should have been replaced once in 8 hours. In contrast, in 3- and 6-day lesions, this unbound serum protein should have been replaced once in about 35 hours, and in normal skin once in 80 hours.

Evans blue-labeled serum albumin continously entered both the bound and unbound compartments of the SM lesions, even during the healing stages. The bound serum albumin was not extractable into the culture fluids because most of it was probably encapsulated by the now nonfunctional lymphatics within the explant and loculated within connective tissue compartments.

We conclude that the amount of serum protein in dermal inflammatory lesions produced by SM is rather high and that it has an unexpectedly rapid turnover rate, which enables it to be a major modulator of the inflammatory process.

Chapter 1: Quantitative Histopathology, Polymorphonuclear Leukocyte, Basophil and Mononuclear Cell Survival, and Unbound (Serum) Protein Content of SM Lesions

Chapter 2: Evans Blue Dye Experiments which Determined the Rates of Entry and Turnover of Serum Protein in Developing and Healing SM Lesions

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INTRODUCTION TO CHAPTERS 1 AND 2

One of the major challenges in research on inflammation is to determine which of many inflammatory mediators and modulators play major roles at each stage of the inflammatory process. An opportunity to respond to this challenge is provided by short-term organ cultures of developing and healing inflammatory lesions. Inflammatory mediators and modulators in the culture fluids can often be identified and assayed, and correlations can be made with the types and number of cells observed in histological sections of the lesions.

This approach has not been used extensively in the literature. The most notable examples have measured various cytokines produced in culture by isolated, intact schistosome (or similar) granulomas (1-3), specifically, migration inhibitory factor(s) (4), chemotactic factor(s) for macrophages (5), eosinophil-stimulation promoter(s) (6), and fibroblast-stimulating factor(s) (7, see 8). Fell and her colleagues at Strangeways Laboratory (see 9) developed the organ culture technique, and it was used to identify various proteases (10) and their activators (11-15) and inhibitors (11,16-18) in synovial, articular and other tissues. Skin explants were used to study the role of anti-cell-surface antibodies from pemphigus patients in producing the acantholysis present in the lesions of this disease (19,20).

Following the topical application of the radiomimetic, alkylating agent, sulfur mustard (SM), epidermal cells slowly die during the first day (because of damage to their DNA), and extravasation of serum and immigration of leukocytes occur (21, and Section I of this report). A crust-covered ulcer develops in 2 to 3 days, and healing is nearly complete in 10 days. No abscesses form, and overt infection is usually avoided because of the crust.

The first chapter of this Final Report describes the survival of epidermal cells, PMN, basophils and mononuclear cells in organ-cultured SM lesion explants of various ages. It also describes the amount of unbound serum protein extracted from these lesions by the culture fluids. A previous report from our laboratory (21) described the histopathology of only early (15 min to 24 hr) SM lesions: vascular permeability, ultrastructural changes in resident cells, and basophil and PMN infiltration. The higher concentration of SM employed in this previous study (usually 2.5% instead of 1.0%) caused the lesions to ulcerate sooner and PMN to infiltrate more rapidly than in the present study, but both studies revealed unexpectedly high numbers of basophils in the early stages of lesion development.

The second chapter of this Final Report describes the turnover of serum protein in developing and healing SM lesions. Most of the studies in the literature have concerned the leakage of serum protein from the circulation into areas of inflammation (see 30-34), or the removal of serum protein from these areas by the lymphatics (see 35,36), but not the local turnover of serum protein in the inflammatory lesion itself. Such measurements were made possible by organ-culturing SM lesion biopsies and assaying the unbound serum protein extracted into the culture fluids. Evans blue dye was used to determine the rates of entry and turnover of serum protein in the SM lesions.

Introduction (continued)

We found that extravasated serum was present in sulfur mustard lesions in rather large amounts and that it had a rather rapid turnover. Such serum could modulate the inflammatory process by limiting the damage caused by the proteases and oxygen-radicals released by the infiltrating leukocytes.

CHAPTER 1

Quantitative Histopathology; Polymorphonuclear Leukocyte, Basophil and Mononuclear Cell Survival of SM Lesions; and Unbound (Serum) Protein Content

MATERIALS AND METHODS

Production and Organ Culture of Sulfur Mustard Lesions

SM (1% in methylene chloride) was received from the U.S. Army Medical Research Institute of Chemical Defense at Aberdeen Proving Ground, MD 21010-5425. The SM (7.5 ul) was applied topically with a Hamilton liquid 100-ul syringe (Series 710 NCH, Cat. No. 71002, Pierce, Box 117, Rockford, IL 61105) to many sites on the clipped flanks and backs of 3.5 kg female New Zealand White rabbits, so that 2-hr and 1-, 2-, 3-, 6-, and 10-day SM lesions were present simultaneously at the time the rabbits were sacrificed. Each of the 6 to 8 rabbits had 7 similar skin lesions at each time period, or 42 lesions in all. When autopsied, the rabbits were free of disease.

In general, SM produced rather uniform lesions. Individual SM lesions on the same rabbit almost always resembled each other, and individual lesions on different rabbits usually resembled each other, but occasional variations in size and cell infiltration occurred.

We repeatedly applied 7.5 ul of methylene chloride to the skin of rabbits. This small dose evaporated so rapidly that no erythema, edema, nor cell infiltration was observed at the site of methylene chloride application. For this reason, we believe that explants of normal skin were satisfactory controls in these studies.

After blood was collected from an ear vein to obtain 1 or 2 ml of serum, each rabbit was killed by an intravenous injection of 1.5-2.0 ml of sodium pentobarbital (65 mg/ml) or 0.8 ml of euthanasia solution (T-61, Taylor Pharmacal Co., Decatur, IL 62525, distributed by American Hoechst Corp., Somerville, NJ 08876). After the rabbit became unconscious, we exsanguinated it from the femoral vessels in order to reduce the amount of blood in the biopsy specimens. At death, the area of the skin containing the lesions was lightly wiped with 70% alcohol and allowed to dry. Then the entire skin of the back and flanks was removed surgically in one piece and placed on a sterile plastic sheet, made wet with Hanks balanced saline solution. One-cm^2 center sections of the SM lesions were excised with heavy surgical scissors, blotted with gauze to remove excess moisture, and weighed.

Each of these biopsies was immediately remoistened and then washed 3 times with Hanks solution. They were cultured individually in small, sterile, plastic Petri dishes (35 x 10 mm, Falcon Plastics, Division of Becton Dickinson Co., Oxnard, CA 93030). Each Petri dish contained 2.5 ml of RPMI 1640 culture medium with glutamine (Gibco Laboratories, Grand Island, NY 14072, Cat. No. 320-1875), supplemented with penicillin (100 units/ml), streptomycin (100 ug/ml) and additional glutamine (2 mM).

Three small Petri dishes, each containing a 1.0-cm^2 lesion explant (or 1.0 cm^2 of normal skin), were placed in one large plastic Petri dish (100 x 15 mm, Falcon Plastics), and the large Petri dishes stacked in a heavy plastic vacuum jar (Oxoid U.S.A., Columbia, MD 21045). The jar was then gassed with a 95% O_2-5% CO_2 mixture at 1.1-1.2 atmospheres of pressure and sealed. It was rocked at 6 times per min in an incubator at 37 C. The tops of the 1.0-cm^2 skin explants were not covered by the culture medium but were directly exposed to the gaseous O_2-CO_2 mixture. The bottoms of the explants were submerged in the culture medium but were freely movable as the dish rocked back and forth. Originally we used Fell's stainless steel screen supports (22), which allowed only the bottom surface of the explant to contact the culture medium. We found, however, that such supports were not necessary for organ-culturing skin explants.

The culture fluids were collected and replaced daily for 3 days. On each day of culture (including day 0), a single explant was removed from the 7 identical explants and prepared for histological examination. The culture fluids from the 4-6 remaining identical explants were pooled and divided into small, capped test tubes, which were stored at -70 C until assayed for inflammatory mediators and modulators. These fluids were clear, and the occasional agar plate that we streaked was negative for bacteria.

Preparation of Histological Sections

Glycol methacrylate-embedded tissue sections were prepared from a center cut through the SM lesions and stained with Giemsa (26). Sections from 0 hr and 1-, 2-, and 3-day cultured skin specimens were made and evaluated microscopically for epidermal cell death and leukocyte infiltration. The amount of epidermal cell death was estimated linearly along the 1.0 cm top surface of the tissue section (see footnote a of Table 1). Dead keratinocytes have pyknotic or karyolytic nuclei. The total number of leukocytes in the 1.0-cm, full-thickness tissue section was determined with a 40 X objective lens and a manual hematological cell counter.

Histological studies showed that skin biopsies survived well in organ culture for at least 3 days. There was no morphological evidence of epidermal cell death in the normal skin explants, and the epidermal cell death seen in the lesion explants seemed to be due to the toxicity of the SM, not to the culturing procedure.

The quantitations of leukocytes and epidermal cell death in the histological sections were not made blindly. While counting, the investigator was aware of the age of the lesion, but not aware of what the cell counts should be nor of the results of his previous counts. We have found that a knowledge of what an investigator is evaluating enables the discovery of histopathological events that would have been missed otherwise.

Weighing and Quantitation of the Skin Explants

Each explant was made 1.0 cm^2 by cutting out the central portion of the SM lesion with strong scissors and measuring with a ruler. Normal skin and biopsies of 2-hr and 1-, 2-, 3-, 6-, and 10-day SM lesions (each with 1.0 cm^2 of surface area) had average weights varying from 0.22 g to 0.31 g because of differences in edema and leukocyte content and in normal skin thickness among the rabbits. In culture, larger quantities of each inflam-

matory mediator or modulator should be released by explants of edematous, leukocyte-infiltrated lesions than by normal skin explants. Therefore, the use of skin surface area (1.0 cm^2), rather than weight, as the basis for our quantitation was most appropriate.

Protein Determination

We used Bradford's Coomassie Blue procedure, as modified by Bio-Rad Laboratories (Richmond, CA 94804). This test is based on a shift in absorbance from 465 to 595 nm when Coomassie Brilliant Blue G-250 binds with protein (24). It is as sensitive as the Lowry procedure (28), and easier to use.

Specifically, 5.0 ml of the diluted Bio-Rad Dye Reagent was added to 0.10 ml of each culture fluid. (One part concentrated reagent [Cat. No. 500-0006] was diluted with 4 parts distilled water and filtered to make the diluted reagent.) The mixture was vortexed and its optical density (OD) read at 595 nm in about 20 min (any time between 5 min and 1 hr is satisfactory). The OD of the reagent blank, containing 0.10 ml of RPMI 1640 culture medium, was subtracted from the OD readings of the culture fluid sample. A standard curve with Pentex bovine (serum) albumin (BSA) (Fraction V Powder [Code 81-003], Miles Laboratories, Inc., Kankakee, IL 60901) was made: 0.01-0.08 mg in 0.10 ml showed OD readings of 0.100-0.900 in a straight-line relationship after 5.0 ml of the diluted Bio-Rad reagent was added.

Statistics

Except when specified differently, the one-tailed Student's *t* test was used. The data were first examined to determine whether they were normally distributed. In all instances they were, and outliers were rare. In the figures and tables, standard errors of the mean are shown.

CHAPTER 1

RESULTS

Gross Pathology

The rabbit SM lesions were grossly edematous from 2 hr to over a week. One-day lesions showed the most edema, but 2- and 3-day lesions had nearly as much. (Fluid even leaked out as the central cut was made to prepare a representative lesion for fixation.) Six- and 10-day lesions were reduced in thickness and more indurated.

Central blanching of the lesions occurred in 1 day. This area seemed to be identical with the area directly exposed to the SM solution when it was spread on the skin surface. The same area proceeded to necrosis by 2 days and formed a crust by 3 days. On day 6, healing was apparent and was almost complete by day 10.

Histopathology

Rabbit epidermis is rather thin, consisting of 1-3 viable basal cells covered by 1-3 keratinized cells, depending on the individual rabbit and the area of skin examined (Figure 1).

One day after the application of SM, the epidermal cells showed (a) pyknosis or karyolysis of their nuclei (Figure 2), or (b) spreading and flattening (covering the space left by shed dead cells) (Figure 2). At 2 days, and occasionally at 1 day, ulceration was present, and crusts were forming (Figure 3). At 3 days, the crusts contained many PMN.

By 6 days, healing had begun. Epidermal cells had spread under the crust. They originated from uninjured hair follicles and normal skin at the edge of the lesions. By 10 days, the ulcers were usually completely covered by thin, flat epidermal cells, and the crusts were less adherent (Figure 4). These results are summarized semiquantitatively in Table 1.

From the onset of the lesions to their healing, the numbers of both PMN and basophils in the interstitial tissues were fairly constant (Table 1). Although rabbit blood contained about 5% basophils and about 50% PMN (unpublished data from our laboratory), the lesions contained almost equal numbers of basophils and PMN in the interstitial spaces. This observation is deceptive, however, because PMN continuously entered the lesions en route to the injured epithelium (Figure 3), where they accumulated and formed a major component of the crust (Table 1). The basophils, on the other hand, seemed to enter mainly during the early stages of the lesions and to remain in the interstitial tissues close to the venules from which they emigrated (21). In addition, the life span of PMN in the tissues is only 1-3 days, and although the life span of basophils in the tissues is unknown, it seems to be longer than that of PMN, at least in organ culture (see below).

The macrophages in tissue sections of these lesions were hard to differentiate from large lymphocytes, activated fibroblasts, and other mesenchymal cells. A rough estimate of this mononuclear group is presented in Table 1: On days 1 through 10, the number of mononuclears found in the SM lesions was about twice that found in normal skin.

Most of the PMN in the lesions accumulated at the surface and eventually became part of the crust. Such surface accumulation was sometimes present on day 2 and usually present on day 3. In the 1.0 cm tissue sections, the median numbers of surface PMN were 3,000, 1,200 and 16,000 for 3-, 6- and 10-day lesions, respectively (Table 1). Over 90% of these PMN were dead and had lost their granules.

Normal rabbit skin contained about 20 intact mast cells in each 1.0-cm tissue section. Their numbers varied considerably among the rabbits, and possibly among different areas in the skin of the same rabbit. SM lesions 1, 2, 3, 6, and 10 days of age contained, on the average, about half as many mast cells as did normal skin (Table 1), but some of the lesions showed no such decrease. The apparent disappearance of mast cells might be explained by a complete discharge and dissolution of their granules, which caused them to resemble macrophages and to be unrecognizable in the tissue sections. A few of the mast cells had ruptured and scattered their granular contents nearby (as had some of the basophils in Figure 2). Most of the mast cells, however, seemed intact and showed no evidence of degranulation. Probably the mast cell population is heterogeneous: some cells discharging their vasoactive amines and contributing to the inflammatory process, and others not participating.

Occasional eosinophils were present (Figure 4). Although the eosinophils usually have larger and more tightly packed granules than PMN have, rabbit eosinophils cannot always be differentiated from PMN because in this species, both cell types are equally eosinophilic. (PMN are called heterophils in the rabbit, since they are not neutrophilic.) Eosinophils were not seen in normal rabbit skin, and only 0-10 eosinophils (with a mean of 2) were seen in developing and healing SM lesions.

Survival of Cells in Organ-cultured SM Lesions

Explants of normal skin survived beautifully in organ culture. Little or no change in the epidermis was observed histologically after 3 days of culture. Even the few specimens cultured for 6 days appeared in rather good condition.

We used histological criteria to determine whether the cells in the tissue sections had died. The appearance of pyknotic or karyolytic nuclei in epidermal cells was the first sign of cell death. Ulceration, i.e., absence of the epithelial covering, was a late sign of cell death. The death of granulocytes and macrophages was measured histologically by the disappearance of these cells from the explant. Since many cell types are in the explants, no other criteria of cell death, e.g., dye uptake or oxygen consumption, are applicable.

Epidermal cell survival. The SM-injured epidermal cells continued to die in culture at approximately the same rate as they did in lesions on the intact animal (Table 2). For example, the number of dead basal epithelial cells in 2-hr SM lesions cultured for 1 day was the same as the number in SM lesions left on the animal for 1 day. In vitro and in vivo epithelial cell death at other times could not be compared because almost all of the SM-injured epithelial cells died during the first day. (SM rapidly reacts with tissue components [including DNA] and no longer exists in its free form after the first few minutes [26]. For this reason, SM does not continue to kill the epithelial cells.)

The healing of the SM lesion explants also continued in culture. Viable cells from the hair follicles and from the edges of the lesions migrated under the crust in vitro in the same manner as they migrated in vivo. Such healing was most strikingly observed in 3-day SM lesions cultured for 3 days (Table 2). In this case, the re-epithelialization of the ulcer bed had increased about 4-fold, similar to lesions left on the animal for 6 days. Likewise, 6-day lesions cultured for 3 days showed re-epithelialization similar to that in 10-day in vivo lesions (Table 2). In each case, however, the in vivo regenerating epithelium was thicker and more differentiated than the in vitro regenerating epithelium.

The size of the ulcer was reduced in culture in inverse proportion to the epithelial regrowth (Table 2). The size of the crust was also reduced in culture (Table 2), possibly because the moist conditions and the rocking of the explants loosened adherence of the crust to the underlying tissues, and because the entry of blood-borne PMN into the crust was eliminated.

Leukocyte survival. Organ culture enabled us to measure directly the survival of the various infiltrating leukocytes in this acute inflammatory model (Table 3): Additional leukocytes did not enter because the lesion was removed from the circulation. The number of PMN in SM lesions cultured for 1 day was about half that in nonincubated lesions. The number of PMN in lesions cultured for 2 days was about a third, and by 3 days, most of the PMN had disappeared. This trend was similar in both progressing and regressing lesions. (Some of the surviving PMN were possibly eosinophils, as these two cell types usually could not be distinguished from each other in Giemsa-stained rabbit tissue sections.)

In areas of acute inflammation, basophils as well as mononuclears and fibroblasts seem to be relatively long-lived cells, compared to PMN. The number of basophils in the SM lesion explants remained more or less constant during the 3 days of culture (Table 3), and the number of cells in the mononuclear-fibroblast group did likewise. Some lesions of certain ages showed a slight decrease during culture in the number of cells in the basophil and mononuclear-fibroblast groups. However, other lesions of the same ages did not show this decrease.

Weights of 1.0-cm^2 Biopsies of Rabbit Sulfur Mustard Lesions of Various Ages

Figure 5 shows that 1.0-cm^2 biopsies of SM lesions weighed more than similar biopsies of normal skin. They reached peak size at 1 day and remained close to this weight even in their healing stages. The initial weight increase was mainly due to edema: the leakage of serum proteins, which osmotically caused fluid retention. Leukocyte infiltration contributed to this weight increase and probably was a major reason for the slow decline toward normal weight after the gross edema had subsided. The mononuclear cell infiltration, the proliferation of fibroblasts, and the production of new ground substance and collagen fibers also contributed to the weight of the lesions during healing.

Figure 6 lists the changes in weight of the biopsies after 3 days in culture. Normal skin biopsies gained nearly 18% of their original weight. One-, 2- and 3-day SM lesions lost 19%, 7% and 4% of their initial weight, respectively, and the healing lesions (6 and 10 days) showed a slight gain (7% and 6%) during this time. These results suggest that in culture: (a)

Normal skin explants may absorb some fluids and become slightly edematous; (b) peak SM lesions lose some of their serum proteins and the fluids retained by these proteins; and (c) healing SM lesions have less serum protein to lose and/or have protein bound more firmly (perhaps to a more stabilized ground substance). These conclusions are supported by the measurements of protein in the culture fluids presented in the following section.

Unbound Protein in the Culture Fluids of Rabbit Sulfur Mustard Lesions of Various Ages

The culture fluids contained the freely diffusible substances in extracellular spaces of the lesion biopsies. When the culture medium was changed daily, more proteins were extracted into day 1 culture fluids than into day 2 culture fluids, and the least amount was extracted into day 3 culture fluids (Figure 7). The unbound proteins apparently diffused out of the biopsies during culture and were not replaced.

The 1-day SM lesions contained the highest amount of unbound protein (Figure 7). Two-, 3-, 6-, and 10-day lesions contained about 15% less. The lesions showed no change in unbound protein content as they healed.

Assuming that almost all of the unbound protein in the lesions was serum protein (preliminary studies), we can calculate serum content of the lesions as a percentage of their weight. Normal skin averaged 215 mg per 1.0-cm^2 biopsy (Figure 5) and contained 1.9 mg of unbound protein [(0.52 + 0.16 + 0.07 mg) x 2.5 ml] (see Figure 7). The serum of these animals contained 60.6 mg of protein in 1.0 ml (which is about 1000 mg by weight). If the 215 mg explant were entirely serum, it would contain about 13 mg of protein. Since the normal skin contained 1.9 mg unbound protein, it contained about 15% serum. Similarly, 1-day SM lesions weighed about 313 mg (Figure 5) and contained 6.4 mg of unbound protein [(1.72 + 0.57 + 0.25 mg) x 2.5 ml]. If the 313 mg explant were entirely serum, it would contain about 19 mg of protein; therefore, 1-day SM lesions contained about 34% serum. (For the sake of simplicity, the specific gravity of serum, about 1.026, was not used in these calculations. Its use would have changed the 34% to 35%.)

Unbound Protein in the Culture Fluids of Normal Rabbit Skin

Each 1.0-cm^2 biopsy of normal skin from the rabbits, bearing multiple SM lesions of various ages, contained 1.9 mg of unbound protein (see previous paragraph). Since the presence of inflammatory lesions in other parts of the skin may have altered the serum protein content of the normal skin between these lesions, 6 similar rabbits without skin lesions were sacrificed, and biopsies of their skin were organ-cultured under the same conditions as those described for the 6 rabbits represented in Figure 7. The day 1 culture fluids contained 0.36 $\pm$ 0.04 mg of protein per ml; the day 2 culture fluids, 0.13 $\pm$ 0.02 mg/ml; and the day 3 culture fluids, 0.05 $\pm$ 0.01 mg/ml. The sum of these values x 2.5 ml = 1.35 mg of unbound protein per 1.0-cm^2 biopsy, compared to the 1.9 mg found for the normal skin of rabbits bearing SM lesions ($P = 0.03$).

This reduced content of unbound protein of completely normal skin was, however, not reproducible: In another study (Section II), 1.0-cm biopsies from 4 rabbits (used to assess the entry of Evans blue-labeled serum protein into completely normal skin) contained 1.9 $\pm$0.2 mg of unbound protein. This figure was the same as the 1.9 mg found for normal skin between the SM lesions (see above).

We conclude, therefore, that the unbound serum protein content of normal skin is subject to physiological variations, although the amount of this serum protein is always much lower than that found in inflammatory lesions. Skin is a major heat regulatory organ, so its content of blood fluctuates throughout the day. Therefore, it is not surprising that variations also occurred in the leakage of serum protein from these heat-regulating vessels.

CHAPTER 2

Evans Blue Dye Experiments which Determined the Rates of Entry and Turnover of Serum Protein in Developing and Healing SM Lesions

[The precise definitions of the terms used in this section appear in the Glossary on page 56.]

MATERIALS AND METHODS

Production of Sulfur Mustard Lesions

Dermal SM lesions of various ages were produced in rabbits (21, and Chapter 1 of this report). After the animal was sacrificed, the lesions were removed and 1.0-cm^2 central biopsies were made and organ-cultured (Chapter 1). Giemsa-stained glycol methacrylate-embedded tissue sections were prepared for histological evaluation from both cultured and uncultured biopsies (Chapter 1).

Evans Blue Dye Injections

Evans blue dye (C.I. 23860) was purchased from J.T. Baker Chemical Co., Phillipsburg, N.J. 08865, Lot No. 213505. It has been used for years in estimating vascular leakage (see 30-33). When it is injected intravenously, it binds firmly to serum proteins, especially albumin.

In our experiments, a 1.0% solution of Evans blue in 0.9% NaCl was injected, at a dose of 20 mg/kg, into the ear veins of rabbits bearing dermal SM lesions 2 hr and 1, 2, 3, 6, and 10 days of age. Two hr later, the animals were sacrificed by the intravenous injection of 1.5-2.0 ml of sodium pentobarbital (65 mg/ml). After they lost consciousness, we exsanguinated them by cutting the femoral blood vessels to reduce the amount of intravascular blood in the lesion biopsies.

Different lots of Evans blue varied considerably in dye content per milligram of powder. We therefore used the same lot of dye in all of our Evans blue experiments. We selected 2 hr for the period during which the Evans blue-labeled serum protein was allowed to extravasate into the SM lesions, because it was long enough to minimize technical variations and short enough to enable the completion of our experimental procedures during the working day.

Evans Blue Extractions

Before portions of the blue-stained lesions were removed from the pelts, lesion diameters were measured with calipers (see Table 4). Then central 1.0-cm^2 biopsies were excised and weighed. Areas (1.0 cm^2) of normal skin from the same rabbit served as controls (see Table 4 and Figure 8). The Evans blue dye was extracted from each biopsy in 4.0 ml of formamide at 60 C for 72 hr. The amount of dye in the extracts was determined by measuring the optical density at 620 nm in a spectrophotometer (21,37).

Evans blue-dyed BSA was used as a standard. To small test tubes containing 2.5-50 ug of Evans blue dye in 0.5 ml of culture medium RPMI 1640, 0.5 ml of BSA (4 mg/ml of RPMI 1640) was added and thoroughly mixed. (The BSA, Catalog No. A-9647, was purchased from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.) The mixtures were precipitated with 0.14 ml of 40% trichloroacetic acid (TCA), centrifuged at 2,500 rpm for 15 min, decanted, dried overnight at 45 C and extracted at 60 C for 3 days with 2.0 ml of formamide. In general, the ratio of Evans blue dye (extracted) to OD units (at 620 nm) was fairly constant over a range of 0-10 ug of dye and 0.000-0.500 OD units.

Evans Blue Binding to Proteins

Part I. We investigated whether the Evans blue dye was irreversibly bound to serum proteins at the concentrations used in our experiments, namely, 20 mg/kg, or about 1 mg/ml of serum for a 3-kg rabbit (which contains about 60 ml of serum).

Two experiments were performed: (1) 0.10 ml of Evans blue (10 mg/ml of 0.9% NaCl) was added to 0.90 ml of BSA (50 mg/ml of 0.9% NaCl); (2) 0.10 ml of this Evans blue solution was added to 0.90 ml of normal rabbit serum, which also contained about 50 mg of protein per ml (Section I of this report). The test tubes were vortexed and left 10 min at room temperature. Then, 1.0 ml of 10% TCA was added, and the resulting precipitate was centrifuged at 2,500 rpm for 15 min. The supernates contained no visible blue color, a result confirmed by spectroscopy at 620 nm. Thus the binding of Evans blue by serum proteins is both rapid and complete at these concentrations.

To ascertain that Evans blue was not precipitated by TCA, we mixed 1.0 ml of Evans blue (25 ug/ml of saline) with 1.0 ml of 10% TCA. No precipitate was formed, and the OD was identical to that of the saline solution diluted with an equal quantity of water (if the pH was readjusted to neutrality).

Part II. In a similar experiment, performed to determine which serum proteins bound the Evans blue, the same concentrations of Evans blue, BSA and normal rabbit serum as in Part I were incubated for 10 min at 37 C (instead of at room temperature). The Evans blue solutions were then electrophoresed on two identical slabs of agarose gel. One of the slabs was stained with Coomassie blue to identify the various serum proteins; the other slab was left unstained so that the location of the Evans blue dye could be matched with the bands on the Coomassie blue-stained preparation. Most of the Evans blue was bound to the albumin fraction of serum, but a trace was bound to the β-globulin fraction. No other serum fraction was dyed with Evans blue.

Part III. A third experiment was performed to determine stability of the Evans blue-protein binding. Once bound to one protein, could Evans blue leave and bind to another protein? Evans blue-serum and Evans blue-BSA complexes were prepared at 37 C, as described in Part II. Then an equal quantity of undyed BSA was added to the Evans blue-serum complexes and an equal quantity of undyed rabbit serum was added to the Evans blue-BSA complexes and the samples were further incubated at 37 C. Aliquots were removed at 1, 8, and 24 hr and electrophoresed on agarose slabs, as described in Part II. The unlabeled BSA did not remove Evans blue from the β-globulin

fraction of labeled serum, and the β-globulin fraction of unlabeled serum did not remove Evans blue dye from the labeled BSA. In other words, the binding of Evans blue to these serum proteins was irreversible, at least under these conditions.

Estimate of the Amount of Evans Blue in the Entire Sulfur Mustard Lesion

From the amount of Evans blue in the central 1.0-cm^2 biopsy, the average diameter of the lesion, and the formula πr^2, we calculated the amount of Evans blue in the entire lesion (Table 4). The 2-hr Evans blue lesions were slighly smaller than the 1.0-cm^2 biopsies, and the 1- and 2-day lesions were somewhat larger, especially the 1-day lesions (which were markedly edematous). The 3-, 6-, and 10-day lesions were approximately the same size as the 1.0-cm^2 biopsy.

We used 1.0-cm^2 explants of the SM lesions as the basis of quantitation throughout this report. The 1.0 cm^2 represents a standard unit area of inflammation. If the reader is interested in values for the entire SM lesion, Table 4 can be used to make corrections in the tables and figures.

CHAPTER 2

RESULTS

Entry of Serum Protein into Sulfur Mustard Lesions

Evans blue dye was injected into rabbits bearing dermal SM lesions of various ages, and the animals were sacrificed 2 hr later. The Evans blue immediately bound to the circulating serum albumin (see Materials and Methods). Thus, only labeled serum protein entered the lesions during this 2-hr period, and the amount that entered was proportional to the amount of dye that could be extracted from the lesions.

The greatest accumulation of Evans blue dye occurred in 2-hr, 1-day and 2-day lesions (Figure 8). A reduced accumulation (about 40% of peak values) occurred in 3- and 6-day lesions. The slight rise in 10-day lesions was not statistically significant or reproducible in a similar but smaller experiment.

Unbound Serum Protein in Sulfur Mustard Lesions of Various Ages

The unbound serum protein in the organ-cultured dermal SM lesions was extracted by the culture fluids. This unbound protein falls into two categories: (1) Evans blue-labeled protein (which entered the lesions during the 2-hr period between the injection of Evans blue and the sacrifice of the animal) and (2) unlabeled protein (which was in the lesions prior to the injection of Evans blue).

(a) Evans blue-labeled protein. In SM lesions, Evans blue is always attached to unbound and bound serum protein and is never free. By measuring the Evans blue extracted by the culture fluids and the Evans blue remaining in the lesion explants after 3 days of culture, we could determine the relative amounts of unbound and bound serum protein in these lesions (see Comments in the Footnotes on Table 6 for a more complete discussion).

Our results are presented in Table 5. The culture fluids extracted 23-36% of the total (bound plus unbound) Evans blue from 2-hr, 1-day and 2-day lesions, but extracted only 11-16% from 3-, 6- and 10-day lesions. In other words, a greater percentage of Evans blue-labeled serum protein was unbound when the lesions were largest and most edematous and contained the most serum protein. Conversely, a greater percentage was bound in healing lesions, which contained less serum protein; and the greatest percentage was bound in normal skin, which contained the least (serum) protein. Thus the binding sites for such protein in the dermis seemed to be saturable, because a greater percentage of this protein was bound (and not extracted by culture fluids) when only a small amount had leaked into the lesions (Table 5, Column F). Also, changes in the gel-sol state of the ground substance may have profoundly influenced the binding of extravasated serum protein (see Discussion).

Table 5 also compares the amount of Evans blue-labeled protein extracted from the lesions by day 1, 2, and 3 culture fluids. A mean of 85% was extracted by day-1 culture fluids and most of the remainder by day-2 culture fluids. This unbound serum protein should therefore be free to diffuse into most parts of the inflammatory lesion.

(b) Unlabeled, unbound protein. By measuring the total protein in the culture fluids and subtracting the Evans blue-labeled protein, we could calculate the amount of unlabeled, unbound protein. (Evans blue-labeled protein contained 0.54 mg of protein for every 1.00 ug of Evans blue [Table 6].)

The concentrations of both total protein and Evans blue-labeled protein were highest in the culture fluids of 1-day lesions (Table 6 and Figure 9). Subsequently the total unbound protein dropped slowly to 55% of peak values, and the Evans blue-labeled, unbound protein dropped rapidly to 15% of peak values.

Thus after the day 1 peak, fresh serum protein entered at a decreased rate and, therefore, older serum protein constituted a larger percentage of the serum protein in the lesions (Table 6, Column D).

The slight rise in the leakage of serum protein in 10-day lesions was not always present, nor was it statistically significant. Perhaps this rise was due to the scratching of these healing lesions by certain rabbits and not others.

Turnover of Unbound Serum Protein in Sulfur Mustard Lesions

The data just described suggest that early lesions have a rather high serum protein turnover. Evans blue-labeled protein constituted nearly all of the serum protein that had entered during the life of a 2-hr lesion. (Such lesions were started 10 min before the intravenous Evans blue injection.)* In contrast, a 24-hr lesion had 22 hr of unlabeled serum protein and only 2 hr of Evans blue-labeled protein entering during its life. These findings can be utilized to estimate the turnover of unbound serum protein in SM lesions of various ages (Table 6). If we assume that the 2-hr entry rate of Evans blue into 1-day SM lesions continued for 24 hr, these 1-day lesions turned over their unbound serum protein about 2.9 times in 24 hr. Similarly, 2-day lesions turned over their unbound serum protein about 1.5 times in 24 hr; 3- and 6-day lesions, about 0.7 times; and 10-day lesions, about 1.4 times (Table 6, Column F). As mentioned above, the rise at the 10-day figure was not always present. The differences in these turnover rates between 1-day lesions and 3- or 6-day lesions were highly significant ($P < 0.001$).

These results can also be expressed as the time required for one complete turnover of the serum proteins in the lesions (Table 6, Column G). One-day lesions turned over their protein once in about 8 hr; 2-day lesions, once in about 16 hr; 3- and 6-day lesions, once in about 35 hr. This decrease in the turnover rate of serum protein in the healing lesions was mainly due to the decrease in the extravasation of fresh, Evans blue-labeled serum protein, because the total unbound serum protein in these lesions decreased relatively little during the healing process (Figure 9).

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* With a 2-hr lesion, a negligible amount of protein leakage is believed to occur during the 10 min before the Evans blue injection. SM injury tends to be delayed, so the leakage is thought not to start immediately. At most, the first 10 min would account for approximately 8% of the total leakage into a 2-hr lesion (assuming a constant rate of leakage). (Such lesions were really 2 hr and 10 min old.)

Turnover of Serum Protein in Normal Skin

Normal skin also contained unbound serum protein (see Section I). Specifically, in these experiments 1.7 ±0.3 mg of such protein was extracted from 1.0-cm^2 biopsies of the normal skin between the SM lesions (Table 6). The turnover of this unbound serum protein was slow compared to that in acute inflammatory lesions, i.e., once every 80 hr in contrast to once every 8 hr in the 1-day inflammatory lesions (Table 6).

These experiments were repeated with normal skin from 4 rabbits without SM lesions. Compared to normal skin on SM rabbits, skin from rabbits without lesions contained about 3 times the amount of Evans blue (per 1.0 cm^2) (Table 5, Column A); about 10 times the amount of unbound Evans blue extracted into the culture fluids (Table 6, Column A); and about the same amount of total unbound protein.

We could not, however, compute the unbound serum protein turnover rate in the skin of these normal rabbits (as in Table 6). Such turnover rates were based on a conversion factor derived from the Evans blue entry into the 2-hr SM lesions, but these normal rabbits had no SM lesions.

Thus the normal skin of rabbits showed considerable variation in Evans blue content and in the Evans blue extracted by the culture fluids in different experiments. One would also expect variations in the turnover rates of serum protein in normal skin. Such rates might be affected by skin thickness, hair density, room temperature, humidity, time of feeding, the animal's activity, and its age (see Discussion). For this reason, we believe that *normal skin controls should be obtained from each rabbit for comparison with* lesions on the skin of that rabbit.

DISCUSSION OF CHAPTERS 1 AND 2

The mediators of the inflammatory process are produced or activated locally, and are usually inactivated locally before they enter the systemic circulation. Otherwise, a state of shock would result. Many of these mediators are not stored in the cells producing them, but are synthesized and released immediately. Organ culture is one of the best ways to collect mediators from inflammatory lesions, since it leaves the architecture of the tissue intact and cell-cell interactions relatively undisturbed (9).

The cells in organ-cultured lesions are in a more natural environment than free cells sieved out of such lesions. Nonetheless, these cells are not in the same environment as they are in the living host, where the circulation continuously instills fresh serum (and fresh leukocytes) into the site. Organ culture cannot duplicate this dynamic in vivo process, but culture fluids from inflammatory lesions in various stages of progression and regression can be assayed for serum content and for various inflammatory mediators and modulators, and their leukocyte content can be determined histologically. In addition, organ culture enables an assessment of the intralesional life spans of the various leukocytes, since new cells from the circulation no longer enter the population.

* * * * * *

The first chapter of this Final Report describes the histology of developing and healing inflammatory lesions produced in the skin of rabbits by SM, the vesicant used in the First World War. SM does not cause blisters in common laboratory animals, possibly because animal epidermis is anchored by many hair follicles. This chemical irritant does, however, cause vasodilation and edema, epidermal cell death followed by ulceration, and a leukocyte response that in the early stages consists primarily of basophils and PMN (heterophils in the rabbit), and soon includes many mononuclear cells. Recent work in our laboratory (27) suggests that the early basophil response was due to the slow cell death caused by SM and the mildness of the injury it causes. Many PMN accumulate, probably because of leukotaxins produced directly or indirectly by dead epidermal cells, disintegrating PMN and/or contaminating bacteria.

Activated macrophages and activated fibroblasts are quite prominent in the lesions from day 1 on (Figures 1 and 4). These are large (often polygonal) cells, which cannot always be distinguished from each other. The macrophages are probably ingesting and digesting extravasated serum proteins, cellular debris, and ground substance. They also secrete hydrolases that may break down these substances extracellularly (28). The fibroblasts participate in some of these functions (13) as well as in the synthesis of new ground substances, collagen and elastin, which are an important part of the repair process.

The histological response varies considerably among rabbits, largely because of differences in skin thickness and hair follicle density. Ranges and standard errors of the mean are provided in the tables. Additional irritation may be caused by scratching (most often during the healing stages), but overt infection did not occur. In spite of these variations, the histological pattern described in this report seems to be an accurate representation of the development and regression of SM skin injury in this species of animal.

The changes in weight of 1.0-cm^2 lesion biopsies after 3 days in culture has not, to our knowledge, been mentioned in the literature on inflammation. Such changes are due to the absorption of culture fluid by normal skin and healing lesions and the loss of interstitial fluid from the early edematous lesions.

Ground substance holds large quantities of fluid. Its gel-like state is characteristically altered in inflammation, where it may be transformed to a sol, or fluid, state (29). (This transformation enables leukocytes to reach their targets more easily.) The weight loss of the peak lesions in culture is perhaps due to a sol state, in which proteins and glycoproteins are free in solution and easily extractable into the culture fluids. The weight gain of normal skin and healing lesions in culture is perhaps due to a gel state, where the glycoproteins and glycosaminoglycans absorb water and salts from the culture fluids.

The proteins extractable from the lesions into the culture fluids will be the subject of our next annual report. Their electrophoretic patterns on acrylamide gels and their precipitin bands in immunodiffusion plates clearly suggest that they were almost entirely of serum origin. Two-directional electrophoresis on acrylamide gel showed that the majority of protein spots in the culture fluids were also present in serum itself.

* * * * * *

The second chapter of this Final Report describes the turnover of extravasated serum in SM lesions of various ages. Serum contains potentially pro-inflammatory factors (38,39), e.g., complement components, kininogen and plasminogen, and anti-inflammatory factors, e.g., C1-esterase inactivator inhibitors of chemotaxis (40,41), antiproteases (α_1-antitrypsin, α_2-macroglobulin, and $\alpha_2$antiplasmin), ceruloplasmin (an antioxidant) (44), and kininase (38). Since extravasated serum is such an important modulator of the inflammatory response, we measured the rates of entry and turnover of serum protein in developing and healing SM skin lesions before we measured some of the mediators that were present.

Two hours before rabbits (bearing dermal SM lesions of various ages) were sacrificed, Evans blue dye was injected intravenously. This dye immediately labeled the circulating serum albumin (see Materials and Methods). This labeled albumin was used as a measure of the 2-hr rate of serum protein entry into the SM lesions. Within the lesions, 64 to 89% of the entering labeled albumin was bound (Table 5), but the remainder was unbound and could be extracted by the culture fluids along with unbound, unlabeled serum protein, much of which had previously entered the lesions.

This model allows some definite conclusions to be made on the entry, distribution and turnover of serum albumin in developing and healing SM lesions, and allows some tentative conclusions for the other serum proteins that accompanied this serum albumin.

Definite conclusions. (a) The leakage of serum albumin into peak lesions (2 hr to 1 day) was about twice that into healing lesions (3 to 10 days) (Table 4 and Fig. 8). (b) The leakage into healing lesions was about 3 times that into normal skin. (c) Within the lesions, an average of 20% of the extravasated serum albumin was unbound, i.e., extractable into the culture fluids (Table 5). (d) This unbound serum albumin had an unexpectedly high

turnover rate: once every 4 to 8 hr in peak lesions, and once every 30 to 40 hr in healing lesions (Table 6). Even during the healing stages of these acute inflammatory lesions, appreciable amounts of serum protein still entered and left the site (Table 6). Therefore, serum protein probably plays a major modulating role in all stages of the inflammatory process.

Tentative conclusions. (a) The serum albumin to globulin ratio in the culture fluids was about the same as in serum itself (preliminary studies). Therefore, the turnover of the unbound serum albumin might reflect the turnover of the unbound serum protein in general. (b) The bound Evans blue-labeled serum albumin (and probably other serum proteins) also had an appreciable turnover rate. These two tentative conclusions warrant further discussion.

(a) Unbound serum protein. Were we justified in using the entry rate of Evans blue-labeled serum albumin in the lesions as a rough measure of the turnover rate of serum protein in general? The answer is probably yes, as far as unbound serum protein is concerned, because serum and culture fluids showed similar two-dimensional gel electrophoretic patterns (preliminary studies).

The constancy of the composition of the unbound proteins in the lesions does not, however, ensure identical turnover rates for each electrophoretic fraction: Globulin is larger than albumin and may enter and leave the lesions more slowly than albumin. In other words, the levels of each fraction in the extractable fluids may remain relatively constant and yet may have different turnover times. We believe, however, that the turnover time of serum albumin measured in this report is in the general range of the turnover time of the serum globulins. They both enter inflammatory sites because of vascular leakage and both leave primarily because of drainage via the lymphatics. Such leakage and drainage should be affected only slightly by the differences in the size of their protein molecules.

(b) Bound serum protein. Serum protein (albumin) continually entered and was bound in SM lesions of all ages, but the lesions did not continually grow in size. Only the 1- and 2-day lesions showed gross edema. Therefore, from the third day on, the amount of serum protein that entered and bound was probably equivalent to the amount of bound serum protein that disappeared from the lesions. In other words, the bound serum protein probably turned over at an appreciable rate. Quantitation of this rate was impossible: We could not measure the size of the bound serum protein pool in the lesions because many nonserum proteins were present.

Most of the bound (nonextractable) serum protein in the explants was probably encapsulated within nonfunctional lymphatics (see below). Some was probably loculated within connective tissue compartments or 'capsules' (45). Some was probably bound to the ground substance; and a small amount was probably adsorbed onto collagen (and elastic) fibers.

Nevertheless, the high rate of entry of labeled serum protein (albumin) into the bound serum protein pool leads to several interesting speculations. Bound serum proteins (at least the bound albumin) are not in equilibrium with the unbound serum proteins extractable into the culture fluids. Only 11 to 36% of the Evans blue-labeled serum albumin in the SM lesions was extracted into culture fluids over 3 successive days (Table 5). Therefore, 64-89% of the serum albumin seemed to be tightly bound.

How, then, did these bound serum proteins turn over? We believe that they left via the lymphatics and also were endocytosed and digested by cells in the inflammatory lesions, especially the macrophages and fibroblasts. Evidently the protein in the lymphatics was not free to diffuse into the culture fluids but was sealed in place (i.e., bound) by the lymphatic endothelium. In vivo, the contents of the lymphatics are propelled along valved channels by external movement (and contractions of the larger lymphatics) (35,36). In vitro, no such movement occurs locally, and the larger lymphatics have been cut, so that they could not create suction on the smaller lymphatics.

The unbound serum protein (the turnover of which is described in this report) probably plays a more functional role in inflammation than the bound serum protein. The unbound protein is freely diffusable and therefore available to provide local inflammatory mediators (or inhibitors) throughout the lesion. In contrast, most of the bound serum protein may formerly have been unbound serum protein that had already played its role and then left the site via the lymphatics.

Finally, normal skin contained appreciable amounts of unbound and bound serum protein (Tables 5 and 6). The amount of this protein (and probably its turnover) varied from experiment to experiment, probably because of skin thickness, hair density, room temperature, humidity, time of feeding, and the animal's age and activity. Extravasated serum protein probably serves an important function in the physiology of normal skin. In fact, the distribution and turnover of extravascular serum proteins (especially albumin) have been extensively studied in experimental animals (35,46,47,48), and even in man (see 49,50). In the body as a whole, the amount of serum albumin found extravascularly is 4 to 5 times that found intravascularly (46). Of all the tissues, skin and muscle contain the most extravascular albumin (46,47).

TABLES AND FIGURES

(Chapters 1 and 2)

Table 1: Histological Composition of Sulfur Mustard Lesions during their Growth and Regression

Characteristics of the 1.0-cm central tissue section	Normal Skin	Age of SM Lesions					
		2 hours	1 day	2 days	3 days	6 days	10 days
Dead epidermal cells (90% of surface = ++++)	0	0 (0 to ±)[c]	+++½ (3+ to 4+)	+++ (2+ to 4+)	++½ (1+ to 3½+)	+½ (0 to 3+)	½ (0 to 2+)
Ulcer (90% of surface = ++++)	0	0	0	+ (0 to 2½+)	++ (0 to 3+)	+ (0 to 3+)	+ (0 to 4+)
Crust: a coagulum containing dead and dying PMN (90% of surface = ++++)	0	0	0	± (0 to 2+)	++ (0 to 4+)	++½ (0 to 4+)	+++ (2+ to 4+)
PMN in crust (median) (rough estimate)[a]	0	0	0	0 (0 to 300)	3,000 (0 to 50,000)	1,200 (0 to 18,000)	16,000 (600 to 36,000)
PMN (within tissues: those in crust not included)[a]	3 ±2	49 ±14	146 ±34	98 ±15	119 ±30	72 ±15	125 ±52
Basophils[a]	11 ±5	55 ±19	80 ±20	89 ±31	135 ±28	108 ±20	120 ±42
Mast cells[a]	23 ±4	26 ±6	9 ±3	11 ±3	8 ±2	10 ±4	8 ±5
Mononuclears, fibroblasts, etc.[a,b]	140 ±40	110 ±30	250 ±40	390 ±60	380 ±90	270 ±70	260 ±40

Table 1: Histological Composition of Sulfur Mustard Lesions during their Growth and Regression (continued)

Footnotes:

[a] Number of PMN, basophils, mast cells or mononuclears in a central 1.0-cm cross section of the dermal SM lesion. Different lots of rabbits may vary considerably in the amount of cell infiltration present in the lesions. The means and their standard errors (or ranges) are listed. There were 8 rabbits in this experiment. Another experiment, containing 3 rabbits, showed similar results.

[b] When activated, mononuclear cells (macrophages, large lymphocytes) and fibroblasts cannot be distinguished with certainty in histological sections. Small lymphocytes were rare. Nonactivated fibroblasts were present only in normal skin. They enlarged considerably during the inflammatory process, as did the endothelial cells of blood vessels.

[c] $\pm$ means that the effect is definitely present, but slight, i.e., less than 6%.

Table 2: Death and Regeneration of the Epidermis in Organ-cultured Sulfur Mustard Lesions[a]

Age of SM Lesion	State of Epidermis	Time in Organ Culture			
		none[c]	1 day	2 days	3 days
Normal Skin	Dead	0	0	0	0
	Regen.[b]	0	0	0	0
	Ulcer	0	0	0	0
	Crust	0	0	0	0
2 hours	Dead	0 (0 - ±)	+++ (0 - 4+)	+½ (0 - 4+)	+++ (1+ - 4+)
	Regen.	0	0	0	0
	Ulcer	0	0	0	0
	Crust	0	0	0	0
1 day	Dead	+++½ (3+ - 4+)	++++ (3½+ - 4+)	+++ + (3½+ - 4+)	++++ (3½+ - 4+)
	Regen.	0	0	0	0
	Ulcer	0	± (0 - 1½+)	± (0 - 1½+)	+ (0 - 3+)
	Crust	0	0	0	0
2 days	Dead	+++ (2+ - 4+)	+++ (0 - 4+)	+++ (2+ - 4+)	+++ (3+ - 4+)
	Regen.	0	± (0 - 1+)	½ (0 - 1+)	+ (½ - 2+)
	Ulcer	+ (0 - 2½+)	+ (0 - 3+)	½ (0 - 1½+)	+ (0 - 2+)
	Crust	½ (0 - 2+)	+ (0 - 2+)	0	0
3 days	Dead	+++½ (1+ - 3½+)	++½ (0 - 4+)	++½ (½ - 4+)	+½ (0 - 3½+)
	Regen.	½ (0 - 2+)	++½ (0 - 3+)	+ (0 - 3+)	++ (0 - 4+)
	Ulcer	++ (0 - 3+)	+ (0 - 2½+)	+ (0 - 4+)	+ (0 - 2+)
	Crust	++ (0 - 4+)	+ (0 - 4+)	+ (0 - 3+)	+ (0 - 3+)
6 days	Dead	+½ (0 - 3+)	+ (0 - 3+)	+ (0 - 2+)	½ (0 - 2+)
	Regen.	+++½ (0 - 4+)	+++ (0 - 4+)	+++ (2+ - 4+)	++++½ (3+ - 4+)
	Ulcer	+ (0 - 3+)	± (0 - 1+)	± (0 - ½)	0
	Crust	+++½ (0 - 4+)	+ (0 - 4+)	+ (0 - 3+)	0 (0 - 1+)
10 days	Dead	½ (0 - 2+)	0	0	0 (0 - 1+)
	Regen.	++++½ (3+ - 4+)	++++ (3+ - 4+)	++++ (3½+ - 4+)	++++ (3½+ - 4+)
	Ulcer	+ (0 - 4+)	½ (0 - 2+)	+ (0 - 2½+)	± (0 - 1½+)
	Crust	+++ (2+ - 4+)	+ (0 - 4+)	+ (0 - 4+)	± (0 - 1+)

Table 2: Death and Regeneration of the Epidermis in Organ-cultured Sulfur Mustard Lesions[a] (continued)

Footnotes:

[a] Code: 1/2 means about 12% of the 9.0-mm lesion length, or 1 1 mm; + means about 25%, or 2.2 mm; ++ means about 50%, or 4.5 mm; +++ means about 75%, or 6.8 mm; ++++ means about 100%, or 9.0 mm. The SM spread on the skin surface only about 9.0 mm and, therefore, epidermal injury never extended to the full 10-mm biopsy length. ± means that the effect is definitely present, but slight, i.e., less than 6%. The means are presented and the ranges are given in parentheses. The results from only 6 rabbits are listed, as the histological sections from 2 of the 8 rabbits were too poor in quality to be studied adequately.

[b] Regen. = regenerating

[c] Not cultured.

Table 3: Survival of Cells in Organ-cultured Sulfur Mustard Lesions[a]

	Age of SM Lesion	Time in Organ Culture			
		none[b]	1 day	2 days	3 days
PMN	Normal skin	± (0 - ½)	± (0 - ½)	± (0 - ½)	½ (0 - ½)
	2 hours	++ (½ - 3½+)	+ (0 - 2+)	± (0 - ½)	± (0 - 1+)
	1 day	+++½ (½ - 4+)	+½ (± - 3½+)	+ (0 - 2+)	½ (0 - 1+)
	2 days	+++ (2+ - 3½+)	+½ (± - 4+)	+ (0 - 1½+)	± (0 - ½)
	3 days	+++ (½ - 4+)	+½ (± - 2½+)	+ (± - 2½+)	± (0 - 1+)
	6 days	+++½ (½ - 3+)	+ (0 - 1½+)	+ (± - 2+)	± (0 - 1+)
	10 days	+++ (1+ - 4+)	+ (± - 2+)	± (0 - ½)	0 (0 - ±)
Basophils[c]	Normal skin	+½ (0 - 2+)	+ (1+ - 2+)	+½ (1+ - 2+)	+ (2+ - 3+)
	2 hours	++ (½ - 3½+)	++ (1+ - 2½+)	++ (1+ - 2+)	+½ (½ - 2½+)
	1 day	+++½ (½ - 3½+)	++ (1+ - 3+)	++ (1+ - 3+)	++ (½ - 2½+)
	2 days	+++ (½ - 4+)	++ (1+ - 3+)	++ (1½+ - 2½+)	+ (½ - 1½+)
	3 days	+++½ (1½+ - 4+)	++ (½ - 3+)	++ (½ - 4+)	++ (1+ - 4+)
	6 days	+++ (1+ - 4+)	++ (½ - 4+)	++ (½ - 3½+)	++ (1+ - 3+)
	10 days	+++ (1+ - 4+)	++ (0 - 3+)	+++½ (1+ - 3+)	+½ (0 - 2+)
Mononuclear-Fibroblast Group[c]	Normal skin	+ (½ - 3+)	+ (0 - 2+)	+ (½ - 1½+)	+ (1+ - 1½+)
	2 hours	+ (½ - 3+)	+ (½ - 2+)	+ (½ - 1+)	½ (½ - 1+)
	1 day	+++½ (1+ - 4+)	+++½ (2+ - 4+)	+++ (1½+ - 4+)	+ + (2+ - 4+)
	2 days	+++ (1½+ - 4+)	+++ (1½+ - 4+)	+++½ (2½+ - 4+)	+++ (2½+ - 4+)
	3 days	+++ (1+ - 4+)	+++½ (2½+ - 4+)	++++ (2½+ - 4+)	+++½ (2½+ - 4+)
	6 days	+++½ (½ - 4+)	+++ (2+ - 3½+)	++ (1½+ - 2½+)	++ (1+ - 2+)
	10 days	+++½ (1½+ - 3½+)	++ (1+ - 3+)	++ (1+ - 2½+)	++ (1+ - 2+)

Table 3: Survival of Cells in Organ-cultured Sulfur Mustard Lesions[a] (continued)

Footnotes:

[a] Code: For PMN and basophils, 1/2 means about 12 cells in the 1.0-cm tissue section; + means about 25 cells; ++ means about 50 cells; +++ means about 100 cells; ++++ means 200 or more cells. For the mononuclear-fibroblast group, 1/2 means about 200 cells: + means about 400 cells; ++ means about 800 cells; +++ means about 1200 cells; and ++++ means 1600 or more cells. Wherever used, ± means only a rare cell was found (fewer than 5 per tissue section). The means are presented and the ranges are given in parentheses. The results from only 6 rabbits are listed (see footnote a in Table 2).

[b] Not cultured.

[c] In some SM lesions of certain ages, the numbers of cells in the basophil and mononuclear-fibroblast groups decreased slightly during the 3 days of culture. In other lesions of the same age group, no such decrease occurred.

Table 4: The amount of Evans Blue in the Entire Sulfur Mustard Lesion at Various Times after its Inception

Age of SM lesion	A Diameter of entire lesion mm	B Weight of the central 1.0 cm^2 biopsy mg	C Amount of Evans blue in the central 1.0 cm^2 biopsy[b] µg	D Amount of Evans blue in entire lesion calculated from surface area[a,c] ($C \times \pi r^2$) µg
Normal skin	--	200±20	1.7±0.2	—
2 hr	10.3±0.2	190±10	11.6±2.4	9.8±2.0
1 day	13.3±0.7	320±20	13.7±1.4	18.7±1.1
2 days	12.0±0.4	250±10	7.7±0.8	8.7±0.9
3 days	11.7±0.3	230±20	5.1±0.6	5.4±0.4
6 days	11.9±0.9	190±10	5.1±0.5	5.7±0.7
10 days	11.6±0.7	190±10	6.2±0.4	6.6±0.8

Table 4: The Amount of Evans Blue in the Entire Sulfur Mustard Lesion at Various Times after its Inception (continued)

Footnotes:

[a] The Evans blue content of the entire lesion was calculated by multiplying the surface area of the lesion (using the formula: πr^2, where r is the radius) by the amount of Evans blue in the 1.0-cm^2 biopsy. For these calculations, we assumed that the lesion was a flat cylinder instead of a flat disc. The means and their standard errors are listed.

[b] These figures are plotted as the top curve of Figure 8.

[c] The figures in Column D are slightly different from those that would be obtained using the means in Columns A and C, because in Column D, the values for each of the 6 rabbits were calculated individually and then averaged.

Table 5: Amount of Bound and Unbound Evans Blue-labeled Serum Protein in Sulfur Mustard Lesions during their Development and Healing[a]

Age of SM lesion	A	B		C		D		E	F	G	
	Amount of Evans blue in the entire 1 cm^2 lesion[b]	Amount of Evans blue in day 1 culture fluids		in day 2 culture fluids		in day 3 culture fluids		Total amount of Evans blue in all culture fluids (extractable)	Unextractable Evans blue (bound in lesions)[c] A-E	Percent of unbound and bound Evans blue-labeled serum protein in the SM lesions[a,d]	
	ug	ug	B/E[e]	ug	C/E[e]	ug	D/E[e]	ug	ug	unbound E/A[e]	bound F/A[e]
Normal[f] Skin	0.87±0.36	0.05±0.04	71%	0.02±0.02	29%	0	0	0.07±0.06	0.81±0.31	8%	93%[c]
2 hr	8.13±1.04	2.02±0.44	81%	0.37±0.09	15%	0.11±0.06	4%	2.49±0.51	5.63±0.68	31%	69%
1 day	9.85±0.94	2.77±0.25	79%	0.61±0.11	18%	0.12±0.07	3%	3.50±0.33	6.34±0.66	36%	64%
2 days	6.20±0.86	1.19±0.20	83%	0.20±0.07	14%	0.04±0.03	3%	1.43±0.19	4.77±0.71	23%	77%
3 days	4.67±0.83	0.46±0.07	88%	0.04±0.03	8%	0.02±0.02	4%	0.53±0.09	4.14±0.77	11%	89%
6 days	4.00±0.36	0.51±0.11	88%	0.05±0.03	9%	0.02±0.01	3%	0.57±0.13	3.44±0.38	14%	86%
10 days	5.91±0.37	0.81±0.22	88%	0.10±0.03	11%	0.01±0.01	1%	0.92±0.22	4.99±0.29	16%	84%
Average for lesions:			85%		12%		3%			22%	78%

Table 5: Amount of Bound and Unbound Evans Blue-labeled Serum Protein in Sulfur Mustard Lesions during their Development and Healing[a] (continued)

Footnotes:

[a] After intravenous injection, Evans blue attaches to and therefore labels serum protein, mainly serum albumin (see Materials and Methods). In these experiments, each microgram of Evans blue represented 0.54 mg of Evans blue-labeled serum protein (see Table 6).

[b] The amount of Evans blue in the 10-cm^2 lesion explant after 3 days in culture plus the amount of Evans blue in the day 1, day 2, and day 3 culture fluids. These values are plotted as the bottom curve in Figure 8.

[c] The values in Column F are slightly different from those that would be obtained using the means in Columns A and E because in Column F, the values for each of the 6 rabbits were listed individually and then averaged. In column G, 93% rather than 92% appears for the same reason.

[d] In these experiments, the protein extractable into the culture fluids was called unbound serum protein. Our interpretations have assumed that the ratio of unbound to bound Evans blue-labeled serum protein in the lesions was the same as the ratio of unbound to bound unlabeled serum protein in the lesions. The P values for the percentages in unbound Evans blue-labeled serum protein (listed in Column G) are as follows: normal skin vs. 1- or 2-day lesions: $P < 0.001$; normal skin vs. 3-, 6- or 10-day lesions: $P < 0.03$; 1-day lesions vs. 2-, 3-, 6-, or 10-day lesions: $P < 0.001$; 2-day lesions vs. 3-, 6- or 10-day lesions: $P < 0.01$.

[e] X 100

[f] Normal skin from the 6 rabbits with SM lesions used in this experiment.

Table 6: Serum Protein Turnover in Sulfur Mustard Lesions

Age of SM lesions ($1.0\ cm^2$ explants)	A Evans blue extracted into day 1, day 2 and day 3 culture fluids ug	B Total protein in day 1, day 2 and day 3 culture fluids mg	C Evans blue-labeled protein in these culture fluids $\frac{A \times 0.54 \times 10^3}{1000}$ mg	D 2 hr serum protein (C) entry as percent of total protein (B) in these culture fluids C/B X 100	E Total extractable serum protein entering the lesions in 24 hr at 2 hr rate C X 12 mg	F Turnover in 24 hr: Total extractable protein entering the lesions (E) divided by total extractable protein present (B)[c] E/B	G Time required to turn over once the extracted serum protein in the lesions $\frac{24\ hr}{F}$
Normal skin[d]	0.07±0.06	1.7±0.3[e]	0.04±0.03	2.4%	0.5±0.4	0.3 X	80 hr
2 hr	2.49±0.51	3.0±0.5[e]	1.34±0.28	44.7%	16.1±3.4	5.4 X	4 hr
1 day	3.50±0.33	7.8±1.0	1.89±0.18	24.2%	22.7±2.2	2.9 X	8 hr
2 days	1.43±0.19	6.0±0.7	0.77±0.10	12.8%	9.2±1.2	1.5 X	16 hr
3 days	0.53±0.09	5.4±0.6	0.29±0.05	5.4%	3.5±0.6	0.6 X	40 hr
6 days	0.57±0.13	4.7±0.4	0.31±0.07	6.6%	3.7±0.8	0.8 X	30 hr
10 days	0.92±0.22	4.3±0.4	0.50±0.12	11.6%	6.0±1.4	1.4 X	17 hr

Table 6: Serum Protein Turnover in Sulfur Mustard Lesions (continued)

Footnotes:

[a] The 6 rabbits used in this experiment were different rabbits from those used in the histopathology studies reported in Section I.

[b] For this table, we have assumed that the protein extracted into the culture fluids represented the unbound serum protein in the lesions, that the Evans blue-labeled serum protein was a measure of the serum protein entering the lesions during the 2 hr prior to the sacrifice of the animal, and that the protein turnover in the lesions was mostly due to the entry and exit (or metabolism) of serum protein. [A complete discussion of this table follows below.]

[c] This is the 24-hr turnover rate if the 2-hr rate were maintained (see Footnote re. Column E in Comments, below). The P values for the number of turnovers in 24 hours (listed in Column F) are as follows: normal skin vs. 1-day lesions: $P < 0.001$; normal skin vs. 2-, 3-, 6- or 10-day lesions: $P < 0.02$; 1-day lesions vs. 3- or 6-day lesions; $P < 0.001$: 1-day lesions vs. 2-day or 10-day lesions: $P < 0.01$; 2-day lesions vs. 3-day lesions: $P < 0.001$; 2-day lesions vs. 6-day lesions: $P < 0.01$.

[d] Normal skin from the 6 rabbits with SM lesions used in this experiment.

[e] The 1.7 and 3.0 figures were really 1.73 and 3.03.

Comments on Table 6:

Column A lists, in micrograms, the amount of Evans blue dye found in day 1, day 2 and day 3 culture fluids (added together) from 1.0-cm^2 SM lesion explants of various ages. The procedure for precipitating the proteins from the culture fluids and determining their Evans blue content is described in Section II, Materials and Methods.

Column B lists, in milligrams, the amount of serum protein found in day 1, day 2, and day 3 culture fluids (added together) from these explants, determined by direct measurement with the Bio-Rad reagent. Some of this extractable protein could have been released from lesion components other than serum, but over 80% was probably of serum origin.

Column C lists the protein equivalent to the Evans blue label, namely, the total amount of Evans blue-labeled protein in day 1, day 2, and day 3 culture fluids. These results were derived by multiplying the micrograms of Evans blue (Column A) by 0.54×10^3 and changing micrograms to milligrams (dividing by 1000).

The factor 0.54×10^3 was derived as follows: Ten minutes after the application of SM to the skin, Evans blue was injected intravenously. It bound to the serum protein immediately, so that almost all of the serum protein leaking into the 2-hr lesions was labeled with Evans blue. The amount of protein extracted into the culture fluids of 1.0-cm^2 normal and 2-hr SM skin explants was 1.7 and 3.0 mg, respectively, by direct measurement

(Column B) with the Bio-Rad reagent (see Section II, Materials and Methods). Thus 2.49 ug Evans blue found in the 2-hr lesions (Column A) was equivalent to 1.34 mg of unbound Evans blue-labeled protein [really 3.03 minus (1.73-0.04)]. (The "0.04 mg" is the unbound Evans blue-labeled protein in normal skin, which must be subtracted from the 1.73 mg of total unbound protein in normal skin to obtain a value for the unlabeled unbound protein in normal skin.) The factor 0.54 (i.e., 1.34 mg of Evans blue-labeled unbound protein divided by 2.49 ug of Evans blue) converts micrograms of Evans blue into milligrams of Evans blue-labeled protein.

This factor was then used in Column C to obtain the mg of protein in the culture fluids for the 1-, 2-, 3-, 6-, and 10-day SM lesion explants. Into these lesions, Evans blue-labeled serum protein had extravasated for only 2 hr, and unlabeled serum protein had previously extravasated for the rest of each lesion's life, i.e., for 22, 46, 70, 142, and 238 hr, respectively. (The factor was similarly used for the normal skin explants.)

Column D lists the extractable serum protein (unbound Evans blue-labeled protein) entering each lesion for the 2-hr period as a percent of the total extractable protein (total unbound protein) in each lesion.

Column E lists the total extractable serum protein (total Evans blue-labeled unbound protein) that would have entered the culture fluids, assuming that the rate of entry at 2 hr continued for 24 hr. (We realize that this 2-hr rate of serum protein entry was decreasing each day. Thus the turnover rates listed apply only to time when the lesions were removed from the animal and are not to be considered rates that continued for 24 hr.)

Column F compares the total extractable serum protein (total unbound Evans blue-labeled protein) entering in 24 hr (at the 2-hr rate, shown in E) with the total extractable serum protein (shown in B) found in each lesion. Thus it indicates the 24-hr turnover of extractable serum protein in the SM lesions. For example, there was 7.8 mg of extractable (unbound) serum protein in the 1-day lesions (Column B), but 22.7 mg entered in 24 hr (Column E). Therefore, the unbound protein existing in the lesions was replaced 2.9 times in 24 hr, if one assumes an unchanged turnover rate.

Column G lists the time required (in hours) for one turnover of the unbound serum protein in the lesions. For example, if the unbound serum protein in the 1-day lesions is replaced 2.9 times in 24 hours (Column F) it would take about 8 hours (24 hr divided by 2.9) to replace it once.

Figure 1 - Rabbit skin adjacent to the exact area to which sulfur mustard was applied 2 days previously. The epidermis appears normal. It has a basal layer, 1 to 2 cells thick, and a keratinized layer, approximately 2 cells thick. Mononuclear cells and fibroblasts are common in the dermis. They seem to be larger and more active than those in normal skin that is not adjacent to lesions. The dark cell in the lower right corner is a mast cell full of granules. The collagen fibers of the dermis are intact, but slightly more separated than normal because of the presence of edema fluid. (Glycol methacrylate-embedded tissue section, stained with Giemsa; X 650)

Normal skin shows no microscopically apparent changes during the several days of culture, except for a slight bit of edema.

Figure 2 - A 1-day rabbit dermal sulfur mustard lesion. The epidermis on the left is dead, and the nuclei of the basal cells show karyolysis. The epidermis on the right is flattened, and some of the nuclei are pyknotic. The trough in between is made by a hair follicle located just outside of the plane of the section. Every granulocyte in the photograph is a basophil, and some have discharged their granules. The separation of the collagen bundles seen in the right side of the photograph is histologic proof of the marked edema observed grossly. (Glycol methacrylate-embedded tissue section, stained with Giemsa; X 650)

Figure 3 - Beginning ulceration and crust formation in 1-day rabbit dermal sulfur mustard lesion. Many PMN have infiltrated below the thin, and probably dead, epidermis. The PMN on top (left of center) are dead and degranulated; next are degranulating PMN, and below them are still intact PMN with their full complement of granules (which are recent arrivals). Ulceration is associated with a large influx of PMN from the bloodstream, through the connective tissues, and into the bed of the ulcer. The few dark cells at the base of the photograph are basophils, which do not migrate into the crust. (This 1-day lesion is more advanced than the average 1-day lesion and resembles many of the 2-day lesions.) (Glycol methacrylate-embedded tissue section, stained with Giemsa; X 750)

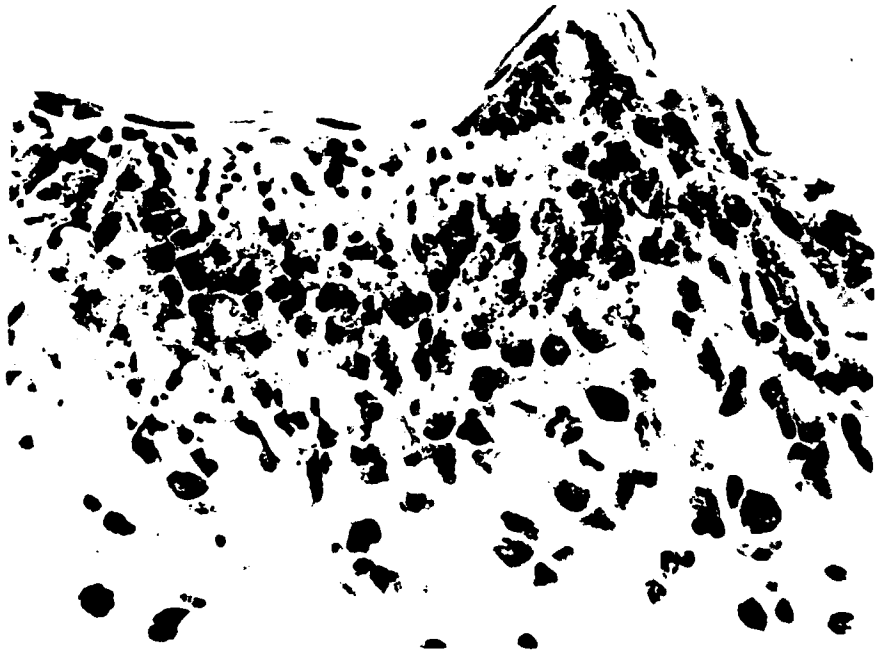


Figure 4 - A healed rabbit dermal sulfur mustard lesion, 10 days after its onset. The crust of disintegrated PMN has been undermined by migrating epithelial cells, which have differentiated into a basal layer, a keratinized layer, and a cornified layer. The increased thickness of the epidermis is probably a response to the increased blood supply associated with the inflammatory process. One eosinophil (near the left edge, just under the basal cell layer) and a few mononuclear cells and basophils can be seen in the dermis. (Glycol methacrylate-embedded tissue section, stained with Giemsa; X 500)

Figure 5 - Initial weights of 1.0-cm^2 biopsies of rabbit dermal sulfur mustard lesions before organ culture The means and their standard errors from 24 biopsies (6 from each of 4 rabbits) are depicted. One-, 2-, 3-, 6-, and 10-day SM biopsies were significantly heavier than normal skin biopsies ($P < 0.001$).

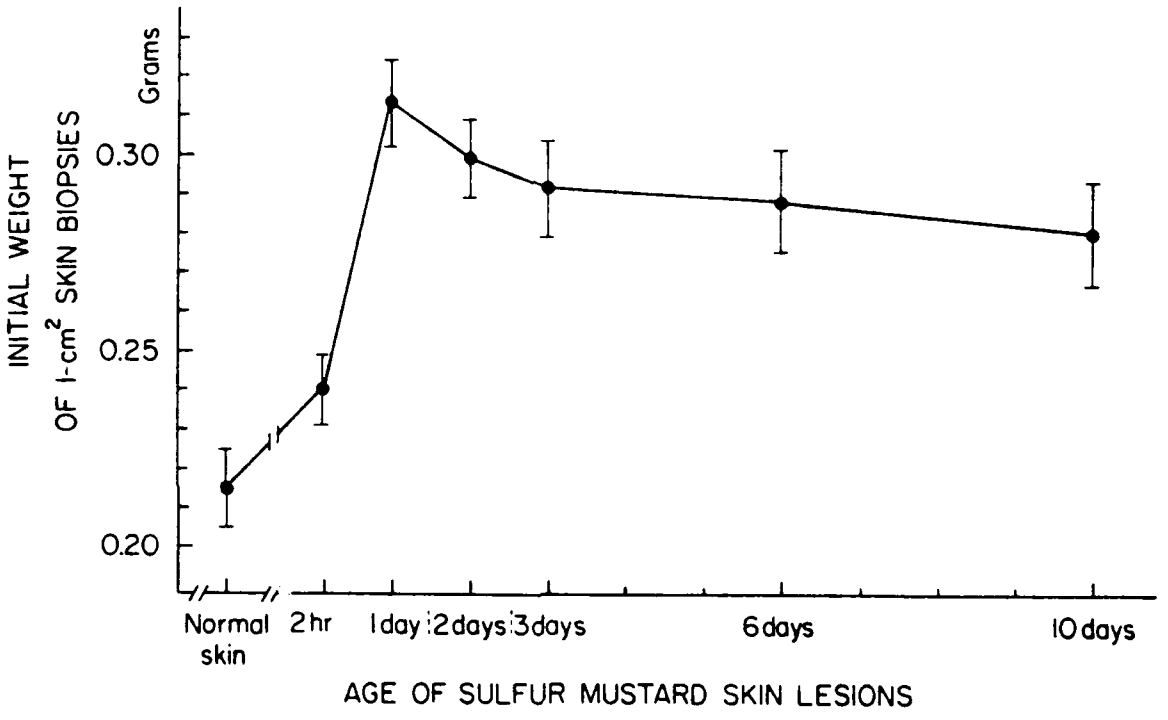


Figure 6 - Changes in weight of 1.0-cm^2 biopsies of rabbit dermal sulfur mustard lesions. The means and standard errors (of the same 24 biopsies as in Figure 5) are depicted. After the 3 days in organ culture, the changes in weight of 1-, 2- and 3-day SM biopsies (which lost weight) were significantly different from the changes in weight of normal skin and 6- and 10-day SM biopsies (which gained weight) ($P < 0.001$). The 2-hr, 6-day and 10-day SM lesion weight changes were also significantly different from those of normal skin (P's < 0.001, $= 0.002$, and < 0.001, respectively, with the two-tailed Student's t test).

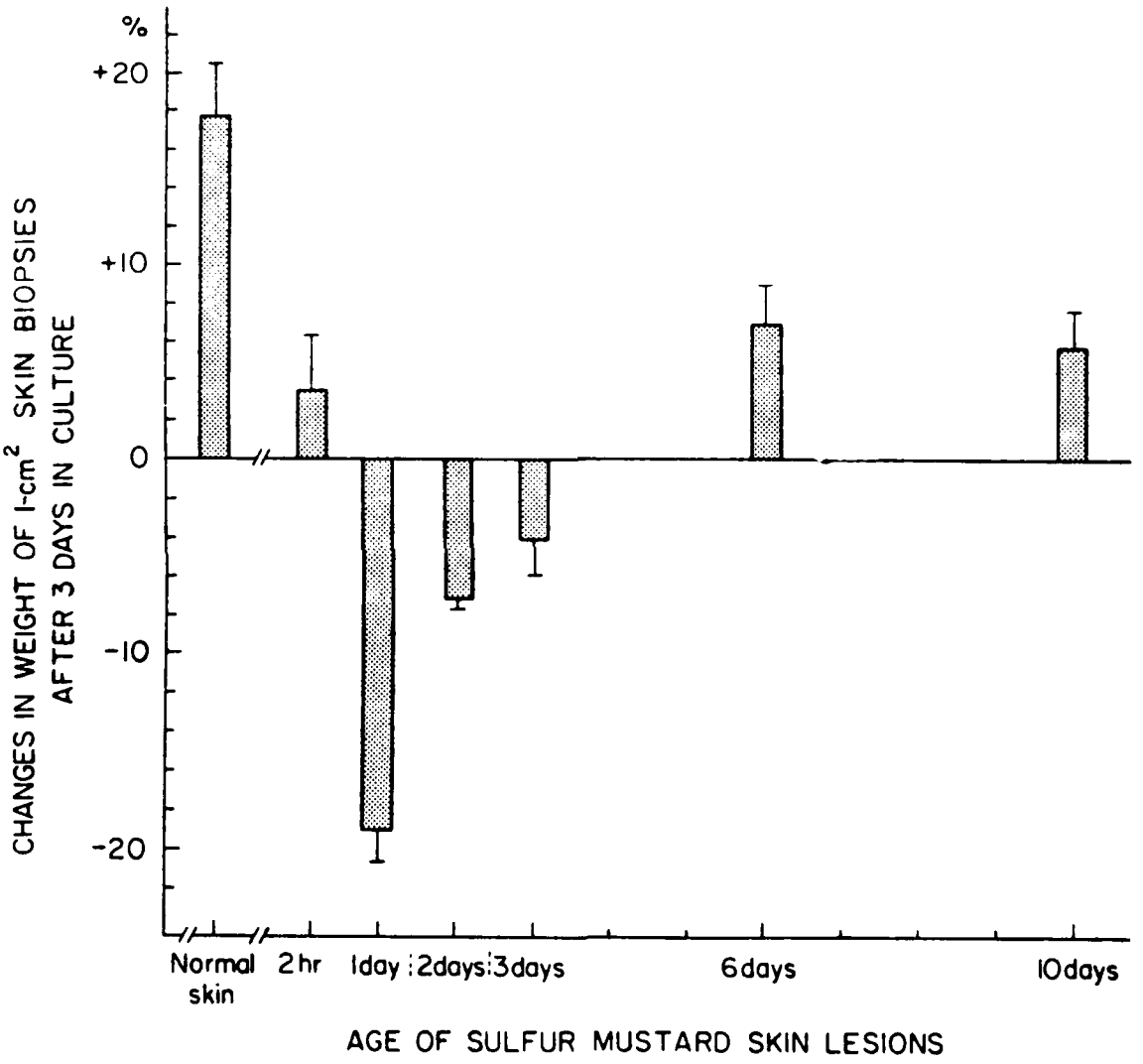


Figure 7 - Protein concentration in day 1, day 2, and day 3 organ culture fluids from 1.0-cm^2 rabbit dermal sulfur mustard lesions of various ages. The means and their standard errors (from 6 rabbits) are depicted. The protein concentrations in culture fluids from 1-, 2-, 3-, 6-, and 10-day SM lesions were significantly higher than those from normal skin on all 3 days of culture ($P < 0.001$). In first-day culture fluids, the protein concentration of 1-day lesions was significantly different from that of 3-day lesions ($P = 0.012$). The sera of these rabbits had a mean protein concentration of 60.6 $\pm$1.1 mg/ml. Therefore, a protein concentration of 1.5 mg/ml was equivalent to 25 ul of serum in 1.0 ml of culture fluid (i.e., a 1 40 dilution of serum).

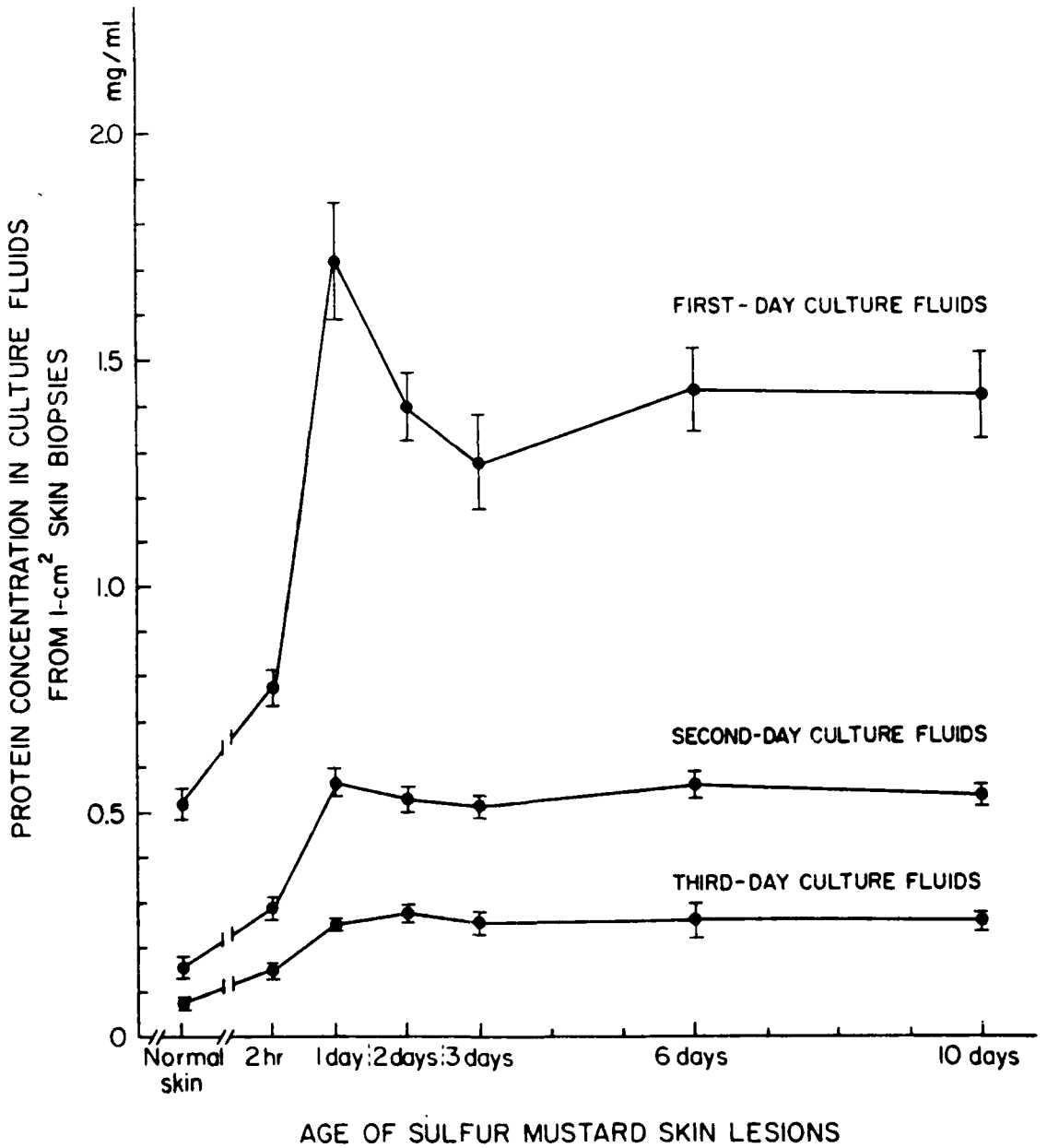


Figure 8 - Evans blue content in 1.0-cm^2 central biopsies of rabbit dermal sulfur mustard lesions of various ages. The Evans blue is bound to (and is a measure of) the serum protein that entered the lesions during the 2 hr before the animal was sacrificed. The top graph depicts micrograms of Evans blue per 1.0-cm^2 explant, extracted by formamide from 12 lesions at each time period (2 lesions from each of 6 rabbits). The bottom graph depicts (a) micrograms of Evans blue per 1.0-cm^2 explant, extracted by formamide from 18 organ-cultured lesions at each time period (three lesions from each of 6 rabbits after 3 days in culture), plus (b) the Evans blue extracted by formamide from protein (in the first-, second- and third-day culture fluids) precipitated by 5% TCA. The slight discrepancy was probably due to some hydrolysis of Evans blue-bound serum protein by tissue proteases during the 3 days of culture. Such hydrolysis could have produced TCA-soluble Evans blue-bound peptides. (At this concentration, Evans blue itself was not precipitated by TCA.)

This figure clearly shows that the early SM lesions (2 hr, 1 day and 2 days of age) had more extravasation of Evans blue-labeled serum protein than did healing lesions (3 and 6 days of age). (For the "not-cultured" biopsies, using the 1-tailed, paired-sample Student's t test, $P < 0.001$ for 1-day lesions vs. 3- or 6-day lesions, and $P \leq 0.025$ for 2-hr or 2-day lesions vs. 3- or 6-day lesions. The means and their standard errors are depicted. The slight rise at 10 days occurred only with some rabbits and was not statistically significant.

Figure 8 - Evans blue content in 1.0-cm^2 central biopsies of rabbit dermal sulfur mustard lesions of various ages.

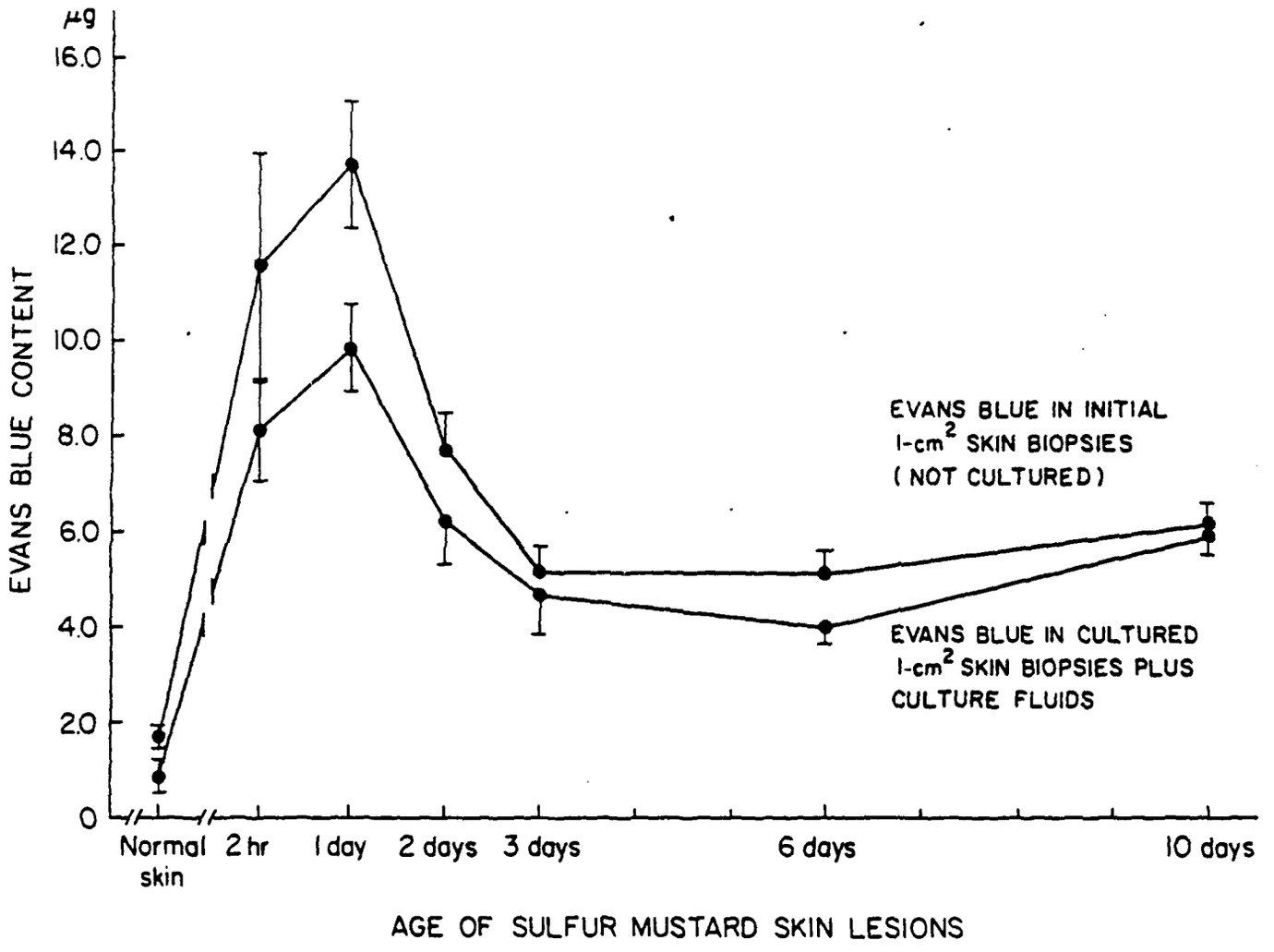


Figure 9 - Amount of protein (Figure 9a) and Evans blue (Figure 9b) extracted by culture fluids from 1.0-cm^2 central biopsies of rabbit dermal sulfur mustard lesions of various ages. As stated in Figure 1, the Evans blue is bound to (and is a measure of) the serum protein that entered the lesions during the 2 hr before the animal was sacrificed. Each 1.0-cm^2 biopsy was cultured in 2.5 mlμ the day 1, 2, and 3 culture fluids were assayed for protein and Evans blue, and the totals from all 3 days of culture are depicted in the graphs. Note that the protein extracted from the lesions by the culture fluids decreased only slightly as the lesions healed. (With the 1-tailed, paired-sample Student's t test, for 1-day SM lesions vs. 3- and 6-day SM lesions, $P < 0.02$). Note also that the Evans blue extracted from the lesions by the culture fluids decreased markedly as the lesions healed. (With the one-tailed paired-sample t test, for 1-day SM lesions vs. 3- or 6-day SM lesions, $P < 0.001$). During the healing process, not only did less Evans blue-labeled (serum) protein enter (see Figure 8), but that which did enter was evidently more firmly bound (Table 5). The 6 rabbits used in this experiment were different from those used in the histopathology study (Section I).

Figure 9a

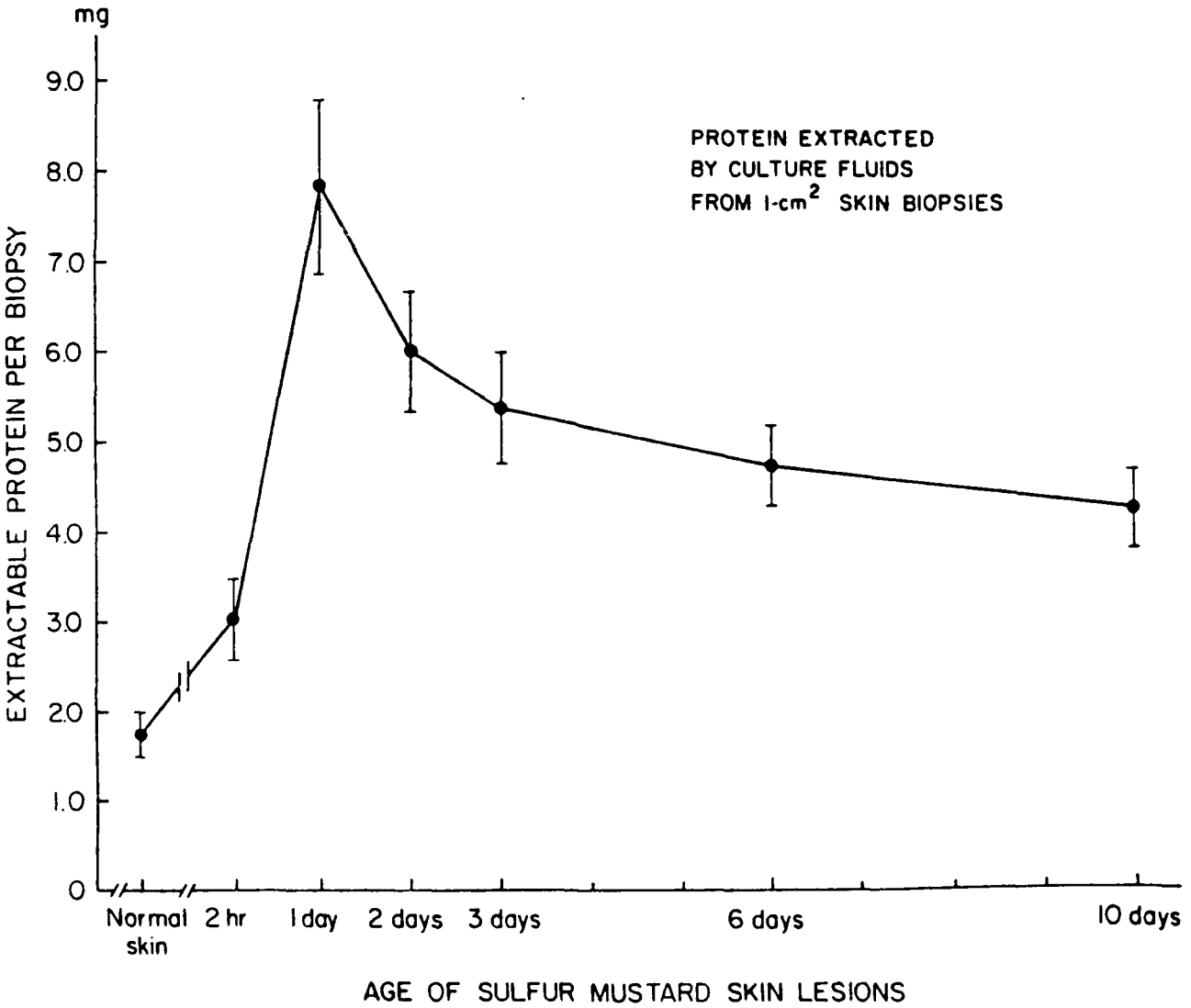
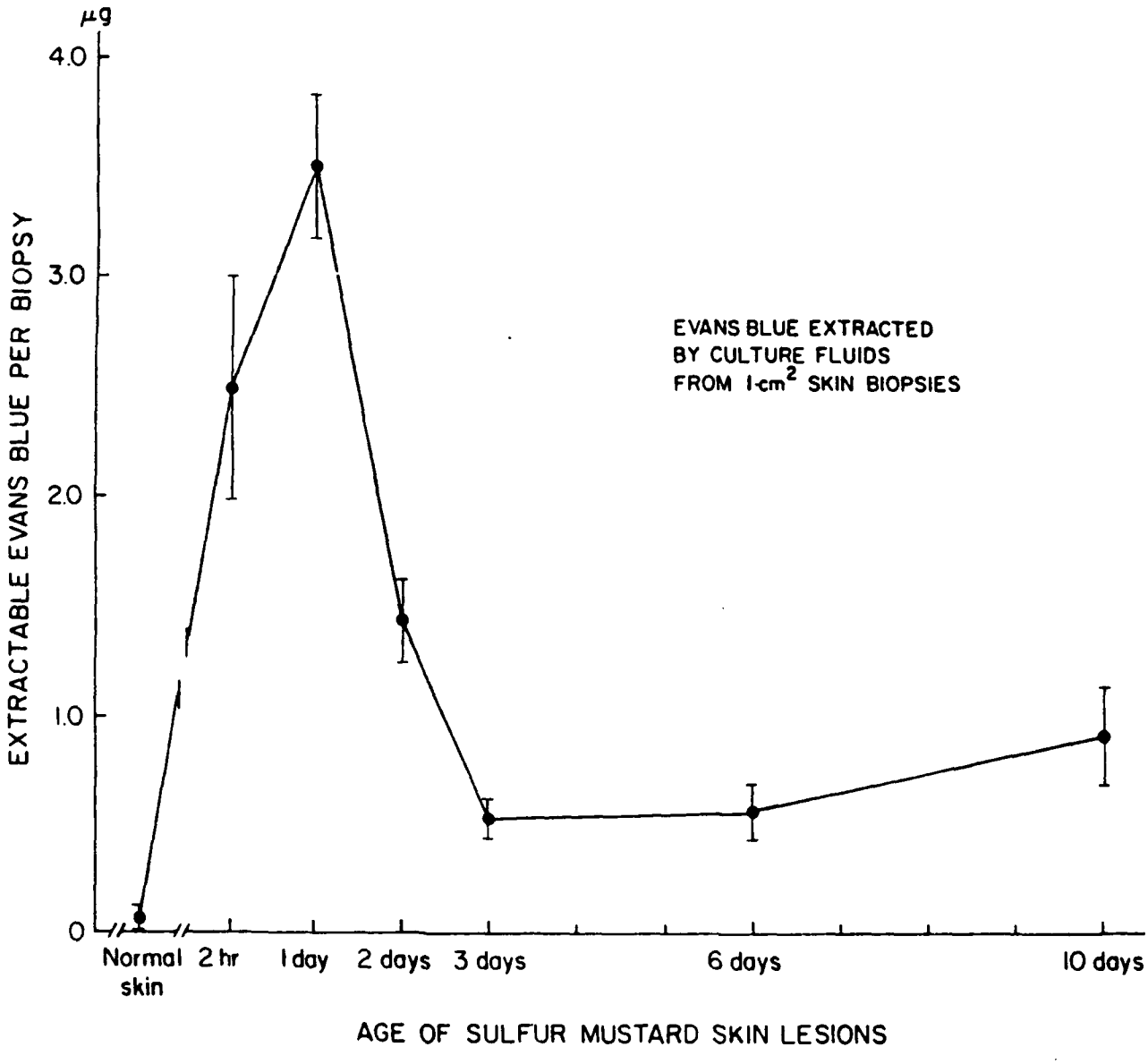


Figure 9 - Amount of protein (Figure 9a) and Evans blue (Figure 9b) extracted by culture fluids from 1.0-cm^2 central biopsies of rabbit dermal sulfur mustard lesions of various ages.

Figure 9b



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GLOSSARY

Serum Protein--In the lesions, all Evans blue-labeled protein (bound and unbound) is serum protein, mainly serum albumin. Evans blue was given intravenously for the purpose of labeling serum protein. It binds quickly and firmly to such protein (see Materials and Methods). In this report, the protein content of the lesions refers to their serum protein content and usually to the unbound protein extracted by the culture fluids. Preliminary immunodiffusion and gel electrophoresis studies indicate that this extractable protein was almost entirely serum protein.

Unbound and Extractable are used interchangeably. Protein in the culture fluids is by definition unbound and extractable. Unexpectedly, normal skin contains an appreciable amount of unbound serum protein, which turns over slowly.

Bound Protein is protein that is not extractable into the culture fluids. It is probably rather tightly bound to the ground substance and possibly collagen fibers, because relatively little is extracted by third-day culture fluids.

Labeled protein is Evans blue-labeled protein, which may be bound or unbound (extractable).

Unlabeled protein is serum protein in the lesions that was not labeled by Evans blue. (It was in the lesions before Evans blue was administered.) We could not measure the bound unlabeled serum protein in the lesions; we could only measure the unbound (extractable) unlabeled serum protein. Perhaps the ratio of unlabeled bound to unlabeled unbound serum protein in the lesions is the same as the ratio of the Evans blue-labeled bound to Evans blue-labeled unbound serum protein (Table 2).

ACKNOWLEDGMENTS

We are grateful to Dame Honor Fell (Strangeways Laboratory Cambridge, England), for teaching us the organ culture techniques; to Dr. Helen Abbey, of our Biostatistics Department, for advice on the statistical treatment of our data; to Chester F. Reather, for the photomicrographs; to Ilse M. Harrop, for typing and editing the manuscript; and Dr. Bruno Papirmeister (U.S. Army Medical Research Institute of Chemical Defense), for his advice and guidance during these studies.

Electrophoretic Protein Fractions, Trypsin-inhibitory Capacity, α_1-Antiproteinase, and α_1- and α_2-Macroglobulin Proteinase Inhibitors in SM Lesion Culture Fluids and in Serum

ABSTRACT

In this chapter, we describe the electrophoretic protein fractions and trypsin-inhibitory capacities (TICs) of the SM lesion culture fluids, and the levels of α_1-antiproteinase and α-macroglobulin proteinase inhibitors in the culture fluids. These studies showed that extravasated serum is probably a major regulator of the course of SM lesions, and that the serum proteinase inhibitors inactivated all of the potentially damaging proteinases in the extracellular fluids in SM lesions. In other words, these proteinase inhibitors probably prevented SM blistering in rabbits and probably limit the blistering in SM lesions of man.

With one-dimensional electrophoresis, the albumin and β-globulin fractions of protein in culture fluids varied little with the development and healing of the SM lesions. These fractions proportionally resembled the corresponding fractions found in serum. The α_1-globulin fraction was proportionally smaller than the corresponding fractions of serum as the lesions healed. The α_2-globulin fraction was proportionally smaller than the corresponding fraction of serum at all stages of lesion development and healing. The gamma-globulin fraction was proportionally larger as the lesions healed.

With two-dimensional electrophoresis, about 68%, 46% and 35% of the protein spots in culture fluids from representative 1-day and 6-day SM lesions and normal skin, respectively, matched those from serum. In each case, the large, diffuse, serum albumin spot represented about two thirds of the protein present. Thus, gravimetrically, in normal skin and in both developing and healing lesions, the extracellular proteins were 80% to 90% of serum origin.

The trypsin-inhibitory capacity (TIC) per milligram of protein in the culture fluids of healing lesions was markedly less than the TIC per milligram of protein in the fluids of peak lesions. This decrease correlates well with the decrease found in the α_1-globulin fraction, which contains α_1-antiproteinase (α_1PI) (and α_1-macroglobulin (α_1M) in rabbits). The α_1PI and the α_1M-α_2M proteinase inhibitors were identified in the culture fluids by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blots, specific antibodies, and the immunoperoxidase technique. The levels of both free and proteinase-complexed α_1PI and α-macroglobulin (αM) inhibitors in the culture fluids decreased as the lesions healed. In both developing and healing lesions, at least half of the α_1PI and αM inhibitors seemed to be complexed with proteinases.

Thus serum seems to be a major source of unbound extracellular protein within acute inflammatory lesions, and serum proteinase inhibitors seem to be the host's major defense against local damage by proteinases from serum, infiltrating leukocytes and activated fibroblasts.

CHAPTER 3

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FIGURES

INTRODUCTION

Many of the mediators and modulators of inflammation are released at the local site and are inactivated or inhibited by plasma (or serum) components. Short-term organ culture of inflammatory lesions in serum-free medium provides an opportunity to collect and identify some of these regulators, and to correlate their presence with the various cells identified histologically in the lesions.

The control of proteinases in sites of inflammation is especially important. Proteinases play major roles in activating (and modulating) the complement, clotting, kinin, and plasmin systems (1,2). Proteinases may also hydrolyze cellular and tissue debris, and even fibrin, collagen and elastin.

In culture fluids from dermal sulfur mustard lesions of various ages, we have not been able to find active proteinase activity with ^{14}C-casein as the substrate, apparently because the culture fluids were rich in serum (3,4), which contains a variety of proteinase inhibitors (5,6), e.g., α_1-proteinase inhibitor (α_1PI) (formerly called α_1-antitrypsin), α_1- and α_2-macroglobulin (the αMs), C-1 inactivator, α_2-antiplasmin, antithrombin-III, α_1-antichymotrypsin, inter-α-trypsin inhibitor, and inter-α-globulin. Alpha-1-proteinase inhibitor and α_1- and α_2-macroglobulins (α_1M and α_2M) are the major proteinase inhibitors in serum. [α_1M is the rabbit equivalent of the human α_2M proteinase inhibitor, but rabbits also have an α_2M proteinase inhibitor (see Discussion)].

The experiments reported herein show that the major electrophoretic fractions of serum are present in organ-culture fluids of developing and healing sulfur mustard (SM) lesions (produced in vivo in the skin of rabbits). These experiments also identify α_1PI and the αMs as the major sources of the trypsin-inhibitory capacity (TIC) of these culture fluids.

MATERIALS AND METHODS

Production and Organ Culture of Sulfur Mustard Lesions

Sulfur mustard (SM) (1% in methylene chloride) was received from the U.S. Army Medical Research Institute of Chemical Defense at Aberdeen Proving Ground, MD 21010. With a Hamilton syringe, 7.5 ul of the SM solution was applied, at various times, to many sites on the clipped back and flanks of rabbits (3), so that 2-hr, and 1-, 2-, 3-, 6- and 10-day SM lesions were present when the rabbit was sacrificed. Shortly before then, blood was withdrawn from an ear vein, and the serum was separated by centrifugation.

Procedures (a) for obtaining 1.0-cm^2 biopsies of these SM lesions, (b) for organ-culturing them in serum-free medium RPMI 1640, and (c) for preparing glycol methacrylate-embedded tissue sections (for histological studies) were described in reference 3 and Chapter 1 of this report.

One-dimensional Gel Electrophoresis

(a) Agarose gel electrophoresis (to measure the albumin, α_1-, α_2-, and gamma-globulins in the culture fluids). The composition of the proteins in the culture fluids was determined by slab-type gel electrophoresis (7). One ml of each sample was concentrated 10- to 20-fold by ultrafiltration with an Amicon CS-15 filter (Amicon Corporation, Lexington, MA 02173). Then, 3 to 4 ul of the sample was placed on a thin sheet of agarose gel (Corning Agarose Universal Gel Films, Cat. No. AC470100, Fisher Scientific, Pittsburgh, PA 15219), and electrophoresed in the Corning Electrophoresis Starter System (Cat. No. 09-529-201C, Fisher) for 25 min at 100 volts in a 0.05 M sodium barbital buffer (pH 8.6) (Cat. No. AC470180, Fisher). The gels were stained for 20 min in 1% Amido Black 10 B (Cat. No. AC470120, Fisher) in 5% acetic acid. They were then dried and decolorized in 5% acetic acid until the background was clear. The proportion of albumin and various classes of globulin in each sample was determined quantitatively at 525 nm with a scanning densitometer (Helena Scientific, Beaumont, TX 77704), and the results are represented in Figure 1.

(b) SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Undiluted SM lesion culture fluids (8, 4, 2 ul) or rabbit serum (1 ul of a 1:40 dilution) were mixed with 15 ul of a 25 mM Tris buffer (pH 6.8), containing 10% glycerol, 3.0% SDS, 5% 2-mercaptoethanol and 0.005% bromophenol blue. They were incubated for 1 hr at 37 C and then electrophoresed with a 25 mA current for 45 min at 23 C in a Mini-Vertical Slab Cell (Cat. No. 165-1700, Bio-Rad Laboratories, Richmond, CA 94804) that contained 10% polyacrylamide gel, 25 mM Tris-HCl (pH 8.6), and 200 mM glycine (8). The gels were run in duplicate, one for staining and one for Western blots. The gels to be stained were fixed for 1 hr at room temperature in an aqueous solution containing 10% acetic acid and 20% methanol. Then they were stained with 1% Coomassie blue (7). The gels for Western blots were incubated for 30 min in 25 mM Tris buffer (pH 8.6), 200 mM glycine, 100 mM NaCl in 40% methanol before being electrotransferred to nitrocellulose (see below). For comparative purposes, diluted sera from the same rabbits were electrophoresed in a similar manner.

The SDS treatment allows the proteins in solution to be separated at a uniform charge, with a distribution based on their molecular weights. The addition of mercaptoethanol allows still further fractionation by reducing the S-S bonds between peptide chains in the proteins. Thus SDS-PAGE gives better characterization of proteins than does the agarose gel electrophoresis described above.

Two-dimensional Gel Electrophoresis

Two-dimensional slab gel electrophoresis was performed by the method originally described by O'Farrell (9), as modified and improved by the Andersons (10-13). One-ml samples of culture fluids from SM lesions, and of serum (diluted 1:4), were centrifuged at 5000 X g for 10 min. One part of supernate was mixed with three parts of diluent. The diluent contained 2% sodium dodecyl sulfate, 1% dithioerythritol and 10% glycerol in 0.05 M 2-(cyclohexylamino)ethanesulfonic acid (pH 9.5). These mixtures were heated at 95 C for 5 min, allowed to cool, and then concentrated about 10-fold in a Centricon-10 microconcentrator with a 10,000 dalton cut-off membrane (YM-10) (Cat. No. 4205, Amicon Corporation, Danvers, Mass. 01923). The retentate was mixed with 1.0 ml of a solution containing 9 M urea, 2% Ampholines (ISO-DALT, 2% grade 3-10 Servalyte, Serva Biochemical, Garden City Park, NY 11040), and 5% dithioerythritol. This solution was again ultrafiltered, and the retentate collected, with 1.0 ml of the same urea-mix.

The samples were assayed for total protein with the Bio-Rad Protein Assay Kit (see 3), and 20.0 ug of protein was applied to the first-dimension of the acrylamide gel (about 4 ul for the serum samples, and 10-50 ul for the culture samples). Isoelectric focusing was conducted at 700 volts for 17 hr. The second dimension of the acrylamide gel was electrophoresed at constant 0.06 ampere for 3.5 to 4.0 hr. Molecular weight and charge calibrators were included in each gel. Ultrasensitive silver staining was performed with a slight modification of the method of Oakley (14).

The VISAGE system (Bio-Image Corporation, Ann Arbor, Mich. 48106) was used to digitize the gels at high resolution, and for scanning, analyzing, and comparing them.

Western Blots

The electrotransfer of the proteins from the one-dimensional acrylamide gels to nitrocellulose (Western blots) was carried out at 4 C with a current of 0.3 ampere for 3 hr in a transfer chamber (Cat. No. TE 42, Hoefer Scientific Instruments, San Francisco, CA 94107) containing 25 mM Tris buffer (pH 8.6), 200 mM glycine and 100 mM NaCl (15,16). The blots were dried at 23 C and stored at 4 C.

The electrotransfer of proteins from the two-dimensional acrylamide gels to nitrocellulose was done in a Bio-Rad Trans-Blot cell (Cat. No. 170-3910, Bio-Rad Laboratories) in 192 mM glycine, 25 mM Tris (pH 8.3), and 20% methanol at 0 C. Up to 2 gels were transblotted simultaneously at 60 volts for 16 hr. The transfer appeared to be over 95% complete, when silver-stained Western blots were compared to duplicate silver-stained nonblotted gels.

Specific antisera

Polyclonal antiserum to purified rabbit α_1-proteinase inhibitor (α_1PI) was produced in goats with purified α_1PI in complete Freund's adjuvant (17). This antibody binds specifically to rabbit α_1PI.

Polyclonal antiserum to purified rabbit α-macroglobulin (α_1M-α_2M) was supplied by Drs. Katherine L. Knight and Doina Ganea, Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL. It, too, was produced in goats, by using complete Freund's adjuvant (18). The antibody is specific for the two rabbit macroglobulin proteinase inhibitors, α_1M and α_2M (19) (which are closely related, but distinct, glycoproteins [20]). This antibody will not, however, distinguish between the two α-macroglobulins.

Immunoperoxidase Staining of the Western Blots (16)

The blots were placed in a 15 mM phosphate-buffered saline solution (pH 7.2), containing 0.1% Tween-20 (PBS-T20), until they were thoroughly wet. The PBS-T20 was replaced with a blocking solution of 2% normal goat serum in PBS-T20, and the blots were incubated for 1 hr at 37 C on a rocking platform. Then they were similarly incubated 1 to 4 hr at 37 C in goat antisera against α_1PI (17) or α_1M-α_2M (18), which had been previously diluted to the appropriate concentration in blocking solution. The blots were washed with five changes of PBS-T20.

Peroxidase-conjugated IgG fraction of rabbit anti-goat IgG (Fc fragment, gamma-chain specific) (Cat. No. 3206-0122, Cappel Laboratories, West Chester, PA 19380) was diluted 1:100 in blocking solution and incubated with the appropriate blots for 1 hr at 37 C. The blots were then washed with five changes of PBS-T20 and then with five changes of PBS without Tween 20.

The peroxidase substrate was prepared by dissolving 10 mg of 4-chloro-1-naphthol in 4 ml of methanol and 16 ml of PBS. The substrate solution was activated by adding 20 ul of 30% H_2O_2, and the color reaction was allowed to develop for 15 min at room temperature. The reactions were terminated by removing the substrate solution, washing the blots with PBS, and drying them at 23 C.

Proteinase Inhibitor Determination (Trypsin-inhibitory Capacity)

Reagents

^{14}C-casein working substrate: ^{14}C-Methylated α-casein (Cat. No. NEC-735, New England Nuclear Corp., Boston, Mass. 02118) had a specific activity of 2.3 uCi/mg and a concentration of 0.005 mCi and 2.16 mg in 1.0 ml of 0.01 M sodium phosphate buffer (pH 7.2). One part of this ^{14}C-casein was diluted with 24 parts of unlabeled casein (20.7 mg/ml in the same phosphate buffer) to produce the working substrate. The unlabeled casein was α-casein (Cat. No. C-7891, Sigma Chemical Co.).

Trypsin solution: Lyophilized trypsin from Worthington (Millipore Corp., Freehold, NJ 07728), was made up to 2.5 ug/ml in distilled water. The trypsin activity showed a straight-line relationship with trypsin concentration, up to about 0.15 ug/ml. A final concentration of 0.10 ug/ml was used with the lesion culture fluids.

Procedure

The culture fluids were diluted with RPMI 1640 to make a protein concentration of 30 ug/ml, and 100 ul was placed in a 12- x 75-mm capped, plastic test tube. (The first-day culture fluids had higher protein concentrations than the second-day and third-day fluids, and required more dilution [see 3].) Buffer (355 ul of 0.056 M Tris, pH 8.0) and trypsin solution (20 ul) were added and mixed with the culture fluids. After 10 min at room temperature, the tubes were placed in an ice bath, and ^{14}C-casein (25 ul of working substrate) was added. Then the solutions were incubated by shaking for 90 min at 37 C in a water bath. (This concentration of trypsin showed a straight-line relationship with time of incubation for as long as 2 hr.) Trichloroacetic acid (7%, 1.2 ml) was then added to precipitate the unhydrolyzed casein, and after their contents were mixed, the tubes were centrifuged at 2000 rpm for 15 min. The supernate (1.2 ml) was removed and placed into a screw-capped scintillation counting bottle. Then 3.8 ml of scintillation fluid (AQUASOL-2 [Universal L.S.C. Cocktail], Cat. No. NEF-952, New England Nuclear Corp.) was added and the mixture was counted for 2 min for ^{14}C radioactivity in a Beckman LS 7500 liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA 92713).

The proteinase inhibitor titer was calculated by subtracting the ^{14}C counts of trypsin mixed with SM lesion culture fluids from the ^{14}C counts of trypsin alone (after allowance was made for controls containing only RPMI 1640). The trypsin-inhibitory capacity was listed as the micrograms of trypsin inhibited by 1.0 ml of culture fluid from 1.0 cm^2 skin biopsies cultured in 2.5 ml.

The calculations were made according to these formulas:

$$\text{Fraction of trypsin inhibited} = \frac{\left[\begin{array}{c}\text{}^{14}\text{C counts}\\ \text{with trypsin}\\ \text{alone}\end{array}\right] - \left[\begin{array}{c}\text{}^{14}\text{C counts with trypsin}\\ \text{plus SM lesion culture}\\ \text{fluid}\end{array}\right]}{[\text{}^{14}\text{C counts with trypsin alone}]}$$

$$\text{Amount of trypsin inhibited in } \mu g = \left[\begin{array}{c}0.05\ \mu g \text{ of}\\ \text{trypsin}\end{array}\right] \times \left[\begin{array}{c}\text{fraction of}\\ \text{trypsin}\\ \text{inhibited}\end{array}\right] \times \left[\begin{array}{c}10 \text{ (to convert}\\ 100\ \mu l \text{ of cul-}\\ \text{ture fluid to}\\ 1.0 \text{ ml)}\end{array}\right] \times \left[\begin{array}{c}\text{the dilution of}\\ \text{the culture}\\ \text{fluid used}\end{array}\right]$$

A straight-line relationship existed between 20% and 80% inhibition of the 0.05 ug of trypsin used. Our experiments were performed in this range. When 0.078 ul of rabbit serum was diluted to 100 ul with RPMI 1640, 0.05 ug of trypsin was inhibited by 50%. This amount of serum (0.078 ul) was the mean serum value for the 6 rabbits used for Figures 6 and 7.

Statistics

Except when specified differently, the one-tailed, paired-sample Student's *t* test was used. The data were first examined to determine whether they were normally distributed: In all instances, they were, and outliers were rare. In the figures and tables, the means and their standard errors are shown.

RESULTS

One-dimensional Electrophoretic Fractions (in Agarose) of Proteins in Culture Fluids from Rabbit Sulfur Mustard Lesions of Various Ages and from Normal Skin

The major fractions of serum protein -- albumin, α_1-globulin, α_2-globulin, β-globulin and gamma-globulin -- were identified in the culture fluids by agarose gel electrophoresis. When compared to normal skin, the peak (1-day) lesions showed a 2.8- to 4.8-fold increase in the concentration of protein in each fraction (Table 1).

When compared to culture fluids of peak lesions, the fluids of healing (6-day) lesions showed a decrease of about 45% in the α_1- and α_2-globulin content and a decrease of about 28% in the albumin content (Table 1). The decreases in total protein and in the β-globulin fraction were not statistically significant, and the gamma-globulin fraction tended to increase, not decrease, with healing. These results reflect both a decrease in the extravasated serum in healing lesions and the changes that occurred in the composition of the protein extracted by the culture fluids.

The changes in the unbound proteins within the lesions are best understood when the composition of the various culture fluids is compared with that of serum itself. In Figure 1, each fraction of the total protein in a given set of culture fluids was expressed as a percentage of the corresponding fraction of serum protein. For example, in culture fluids from 6-day lesions, the α_1-globulin fraction was 3.0% of the total protein. In serum, the α_1-globulin fraction was 4.7% of the total protein. Therefore, the α_1-globulin fraction of protein in these culture fluids was 64% (3.0 ÷ 4.7 X 100%) of the α_1-globulin fraction of serum protein. Such percentages for first-day culture fluids were plotted in Figure 1. The percentages for second-day culture fluids were quite similar. Third-day culture fluids could not be evaluated, because the amount of each protein fraction (with the exception of albumin) was too small to measure quantitatively.

The albumin and β-globulin fractions of the protein in culture fluids from SM lesions of all ages were not statistically different from the corresponding fractions of serum protein (Figure 1). The α_1-globulin fraction of the protein in culture fluids from 3-, 6- and 10-day SM lesions was significantly smaller than the corresponding fraction of serum protein. A major component of this fraction is α_1-proteinase inhibitor (see Discussion).

The α_2-globulin fraction of the protein in culture fluids from SM lesions of all ages was also significantly smaller than the corresponding serum protein fraction (Figure 1). α_2-Macroglobulin is the major proteinase inhibitor in the serum α_2-globulin fraction (see Discussion).

In contrast, the gamma-globulin fraction of the protein in culture fluids from normal skin and from 3-, 6- and 10-day SM lesions were significantly larger than the corresponding fraction of serum protein (Figure 1). However, during the acute phases of the lesions (at 2 hr and on days 1 and 2) this gamma-globulin fraction was about equal to that found in serum. At this time, there was a large influx of serum, and the ground substance was in a "sol" state (see Discussion and Reference 3).

Two-dimensional Acrylamide Gel Electrophoretic Fractions of the Proteins in Culture Fluids from SM Lesions and from Normal Skin

In order to more accurately assess the protein composition of the culture fluids, we prepared two-dimensional gels from the serum and from the 1-day culture supernates of (a) normal skin, (b) a 1-day SM lesion (Figure 2) and (c) a 6-day SM lesion -- from each of 3 rabbits. Because of the relatively low amount of protein loaded onto the gel (2.0 ug), most of the silver-stained protein could be resolved as distinct spots, 300 to 500 per gel. For all samples, about two thirds of the stained protein was due to serum albumin (see Table 1). This albumin was polydispersed and not included in the spot counts.

On these two-dimensional gels, the sera and the culture fluids of 1-day SM lesions (Figure 2) showed a similar distribution of spots. Culture fluids of both normal skin and 6-day lesions also showed a similar distribution of spots. However, these culture fluids of normal skin and 6-day SM lesions showed many faint spots of tissue origin (rather than serum origin) that were not visible in gels containing sera or in those containing culture fluids of 1-day lesions. Since 2.0 ug of protein was applied in each case and since the culture fluids of 1-day lesions contained the highest levels of extravasated serum protein (3,4), these faint spots (found in the gels of normal skin and 6-day lesions) were probably diluted out in the gels of 1-day lesions.

Computer-generated Analysis of the Two-dimensional Gels

The VISAGE gel comparison software system was used to make a detailed study of four gels from a representative rabbit. Two digitized gel images, displayed as overlays on a video screen, were compared at one time (Figure 2). The spots from one gel were depicted as red, the spots from the other gel, as green, and the overlapping spots, as yellow. These images were evaluated to determine how many spots were shared.

Of the 586 spots enumerated in the culture fluid from the 1-day SM lesion, 68% were identical to those of serum. In contrast, of the 427 spots enumerated in the culture fluid from normal skin, only 35% of the spots were identical to those of serum. The culture fluid from the 6-day lesion had intermediate values: Forty-six percent of the 302 spots enumerated were identical to those of serum. A qualitative survey showed that most of the culture fluid spots that did not match the serum spots were relatively small and light when compared with those that did match the serum spots.

No attempt was made to enumerate the family of spots representing polydispersed albumin because their protein concentration was too high. In all culture fluids, the pattern of the albumin spots resembled that found in serum. About two thirds of the total protein in every culture fluid was such albumin (Table 1), and the nonalbumin spots of serum origin were at least 35% to 68% of the remaining one third of the protein (see above). These results indicate that 80% to 90% of the protein in the culture fluids was of serum origin.

Western Blots to Identify α_1-Proteinase Inhibitor and the α-Macroglobulins in the Culture Fluids and in Serum

The proteins on one-dimensional acrylamide gel electrophoresis slabs (Figure 3) were transferred to nitrocellulose sheets and stained by the immunoperoxidase technique with an antibody specific for rabbit α_1-proteinase inhibitor (α_1PI) and with an antibody specific for the two rabbit α-macroglobulins (αMs) (see Materials and Methods). In the rabbit, both α_1M and α_2M are proteinase inhibitors (see Discussion).

(a) Culture fluids. In blots from one-dimensional SDS-PAGE gels, three major bands were stained by specific antiserum to α_1PI and the immunoperoxidase technique (Figure 4). The 55,000 M_r band probably represents free α_1PI (21). The 71,000 and 88,000 M_r bands, probably represent α_1PI-proteinase complexes (see 22). (M_r stands for relative molecular mass.) All three α_1PI bands were most intense in gels prepared from peak lesions, less intense in gels prepared from healing lesions, and still less intense in gels prepared from normal skin. Their ratio was approximately 9:3:1, respectively (Figure 4). In culture fluids from 1-day SM lesions, roughly one third of the α_1PI seemed to be free, and roughly two thirds seemed to be complexed with proteinases (Figure 4). In culture fluids from 6-day SM lesions, a slightly greater proportion of the total α_1PI seemed to be complexed with proteinases (Figure 4). In our hands, densitometer readings on the Western blots did not provide as accute a quantitative assessment as did the visual comparison of densities of the two-fold dilutions of the various culture fluids.

In blots from one-dimensional SDS-PAGE gels prepared from SM lesion culture fluids, the major area stained by specific antiserum to α_1M-α_2M (and the immunoperoxidase technique) was a band of about 85,000 M_r (Figure 5). The next major area stained by the αM antiserum consisted of multiple bands of lower M_r. A 185 M_r band was present in the culture fluids from 1-day SM lesions. Human α_2M is known to break down into 85,000 and 185,000 M_r components when treated with SDS under reducing conditions (21,23,24).

A simplified (but reasonable) interpretation of our results is (a) that the 85,000 M_r band contained αM that had bound (or complexed with) proteinases, (b) that the 185,000 M_r band contained αM that had not bound proteinases, or (c) that the bands of lower M_r contained hydrolytic products of both free αM and proteinase-complexed αM (see Discussion). The ratio of 85,000 M_r αM bands in culture fluids from 1-day SM lesions, to those from 6-day SM lesions and to those from normal skin was about 9:3:1, similar to α_1PI. However, the bands (from such culture fluids) containing hydrolytic products of αM had somewhat different ratios (Figure 5), probably because of the increased proteinases in the healing 6-day lesions (see 25,26) associated with the remodeling of connective tissue. In all culture fluids at least half of the αM was present in the gels mainly as the 85 K band. In other words, considerable amounts of αM were complexed with proteinases (see Discussion).

Blots of the two-dimensional gels were immunostained only for αM (and not for α_1PI). The αM spots are shown by the arrow in Figure 2.

(b) Sera. We studied serum rather than plasma, because plasma rapidly clots as soon as it leaves the bloodstream and only serum exists in the

extracellular fluids of the tissues. When plasma clots to form serum, proteinases, such as thrombin, kallikrein, plasmin, are known to be activated from their proenzymes (2). For this reason, plasma (not serum) is generally used for the isolation of the free inhibitors (20,21).

As we expected, one-dimensional gels of rabbit serum, immunostained for α_1PI and αM (Figures 4 and 5) showed essentially the same band pattern as culture fluids from 1-day SM lesion, which are known to contain large amounts of serum (3,4) (see Discussion).

Trypsin-inhibitory Capacity of First-day Culture Fluids of Rabbit Sulfur Mustard Lesions of Various Ages

The TIC of first-day culture fluids from 1-day SM lesions had five times the inhibitory capacity of culture fluids from normal skin (Figure 6). The TIC of first-day culture fluids from healing (6- and 10-day) lesions was about half that of culture fluids from peak (1-day) lesions (Figure 6).

The TICs per milligram of protein for rabbit sera and for the organ culture fluids are depicted in Figure 7. The TIC per milligram of protein in culture fluids from healing lesions was somewhat reduced, probably because of the increased proteolytic activity in the healing lesions (25,26).

Trypsin-inhibitory Capacities of Second- and Third-day Culture Fluids of Rabbit Sulfur Mustard Lesions of Various Ages

The second- and third-day fluids were evaluated in order to determine whether the explants themselves produced trypsin-inhibitory substances. Most of the unbound serum inhibitors are extracted by the first-day culture fluids. An increase in inhibitor levels per milligram of protein in the second- and third-day fluids would suggest a preferential synthesis of these inhibitors over other protein constituents.

Second- and third-day culture fluids had a reduced total protein content (see 3) and also reduced trypsin inhibitory capacities (Figure 6). The TIC/mg of protein patterns in the second- and third-day culture fluids resembled the pattern in first-day culture fluids (shown in Figure 7). That That is, the second- and third-day culture fluids of peak lesions showed the same TIC per mg of protein as serum, and culture fluids from 6- and 10-day lesions showed less, not more. TIC per mg of protein (data not shown).

These findings suggest (a) that the cells in these inflammatory lesions did not synthesize and release large amounts of proteinase inhibitor(s), and (b) that serum was the major source of the extracellular inhibitor(s) present.

Trypsin-inhibitory Capacity of Rabbit Serum

An average of 31 ul of serum from these rabbits inhibited 10 ug of trypsin in 1.0 ml of RPMI 1640. Serum had a mean protein concentration of 60.6 ±1.1 mg/ml. The first-day culture fluids from 1-day SM lesions had a protein concentration of about 1.5 mgml (3), which is 1/40th of the protein concentration of undiluted serum. Serum had approximately the same TIC per milligram of protein as first-day culture fluids from the majority of the SM lesions (Figure 7).

DISCUSSION

Developing and healing rabbit dermal sulfur mustard (SM) lesions were organ-cultured. The culture fluids extracted from these lesions the unbound extracellular inflammatory mediators and modulators. The electrophoretic fractionation of the proteins in the organ culture fluids showed that 80% to 90% of the proteins were similar to those in serum (Figures 1 and 2). Serum albumin was the main component.

The α_1-proteinase inhibitor (α_1PI) and the α_1- and α_2-macroglobulins (α_1M and α_2M) in these culture fluids were measured by means of Western blots, specific antibodies and the immunoperoxidase technique. The amounts of these inhibitors (per milligram of protein) in the lesion culture fluids were less than the amount found in serum (Figures 4 and 5), probably because of complexing with local proteinases and subsequent clearance. The remaining free α_1PI and αM inhibitors (Figures 4 and 5) still could provide most of the TIC present in the culture fluids.

Serum Protein Fractions in the Organ Culture Fluids of Normal Skin and Dermal SM Lesions

The extravascular distribution of any plasma protein fraction is complex and incompletely understood, even in normal skin (27,28). The rate of entering and leaving the extravascular compartment (see 4), the gel-sol state of the ground substance, molecular sieving, connective tissue pockets, and adherence to connective tissue fibers all seem to play a contributing role in the distribution of each fraction (27,28).

(a) Normal skin. In the extracellular fluids (i.e., culture fluids) of normal rabbit skin, the albumin, α_1-globulin and β-globulin fractions were similar to those found in serum (Figure 1). The α_2-globulin fraction was less than that of serum, and the gamma-globulin fraction was more than that of serum (Figure 1).

(b) Dermal SM lesions. Greater amounts of each serum protein fraction were present in the extracellular fluids of developing and peak lesions than in normal skin and healing lesions (see 3 and 4 and Table 1). In peak lesions, a large proportion of each fraction was evidently unbound and therefore extracted into the culture fluids with its composition unchanged. The albumin, α_2-globulin and β-globulin fractions varied little as the lesion developed and healed. The α_1-globulin fraction decreased with healing, and the gamma-globulin fraction increased (Figure 1). In humans, the protease inhibitors, α_1-proteinase inhibitor (α_1PI) (formerly called α_1-antitrypsin) and α_2-macroglobulin (α_2M), make up a major portion of the α_1-globulin and α_2-globulin electrophoretic fractions of serum, respectively (5,21,22,29-32).

The α_1-globulin fraction of the culture fluids decreased with the healing of the SM lesions. This was probably because the α_1PI-proteinase complexes left the α_1-globulin fraction, possibly entering the β-globulin fraction (see 33).

The α_2-globulin fraction of the lesion culture fluids was consistently lower than the corresponding serum fraction (Figure 1). The αMs, which have a molecular weight (M_r) of 175,000 daltons, probably did not extravasate into the lesions (or normal skin) as readily as did α_1PI, a 55,000-dalton protein (21).

(c) Gamma-globulin is more positively charged than the other serum fractions (29) and therefore may bind more firmly to the negatively charged hyaluronic acid and chondroitin sulfate of the ground substance. In normal skin and healing SM lesions, this ground substance seemed to be in the gel state (see 3). In developing and peak lesions, this ground substance seemed to be in a sol state (see 3). When electrophoresed, the gamma-globulin that was eluted from the fixed gel state by the culture fluids should migrate normally, but the gamma-globulin that was bound to unfixed sol state ground substance should appear in the culture fluids as a complex. This complex should not migrate with the gamma-globulin fraction, but migrate more slowly (perhaps with the albumin fraction, where its low percentage would not be noticed).

The host's serological defense (antibodies) against infectious agents resides almost entirely within the circulating gamma-globulin fraction. The preferential local accumulation of this fraction in normal skin and healing inflammatory lesions should help the host prevent or control infection by microbial agents.

Trypsin-inhibitory Capacity and the α_1PI and αM Proteinase Inhibitors in the Culture Fluids

In the plasma of normal rabbits, α_1PI accounts for about 86% of the TIC (34, see 17). Both α_1PI (31) and α_1M (35,36) are acute phase reactants. Rabbits also have an α_2M, which is closely related to their α_1M (see 35,37). The ratio of α_1M to α_2M in normal rabbit plasma is 2:3 (38). Human α_2M is evidently not an acute phase reactant (see 35 and 39).

Sera from normal rabbits and sera from rabbits bearing multiple dermal SM lesions had the same TIC per milligram of protein: 10.2 $\pm$0.8 and 9.1 $\pm$0.6, respectively, (unpublished results from our laboratory). Thus if the serum levels of α_1PI and α_1M had increased as acute phase reactants (shortly after the six 10-day SM lesions were begun), these inhibitor levels had returned to normal by the time of sacrifice, when the serum was collected (see 36).

In culture fluids from SM lesions, the total TIC and TIC per mg of protein decreased as the lesions healed (Figures 6 and 7). This decrease seems to be correlated with decreases in α_1PI and the αM levels (Figures 4 and 5). The proteinase inhibitor levels in the culture fluids depend on numerous factors, such as the amounts of inhibitor entering the lesions from the circulation, the amounts bound by the ground substance, the amounts leaving via the lymphatics, and the amounts combining with local proteinases and subsequently ingested by macrophages. The drop in the culture fluid TIC associated with the healing of the lesions seemed to be mainly due to the decreased entry of inhibitors into the lesions from the blood (see 4) and partly due to combining with proteinases associated with the remodeling part of healing (see 25,26)) and their subsequent clearance.

The α-Macroglobulin Proteinase Inhibitors

SDS-PAGE under reducing conditions causes αM-proteinase complexes to be split into fragments, among which several laboratories (20,23,24,40) have identified the 85,000 M_r fragment that apparently results from proteinases splitting a peptide bond in the 'bait' region of the 185,000 M_r subunits.[a],[b] The 85,000 M_r fragment was a major component of our SM lesion culture fluids (Figure 5). Thus a large amount of the αM in the lesions must have been complexed with proteinases (see 23,24,40).

Bands of lower molecular weight were also present in the gels stained with the αM antiserum (see Figure 5). The most prominent band had an M_r of about 68,000. These lower M_r bands were probably fragments of free and proteinase-complexed αM derived from spontaneous autolytic cleavage (23,41, 42), probably occurring both in vivo in the blood and lesions and in vitro in the skin explants and during SDS-PAGE procedures.

The existence of these lower M_r bands in the SDS gels make it impossible to estimate the exact proportion of free and proteinase-bound αM in the lesion culture fluids, but the large band of 85,000 M_r suggests that a substantial amount of the αM was present as αM-proteinase complexes (Figure 5).

(a) α_2-Macroglobulin Inhibitor

A conformational change occurs when the 'bait' region of αM's 185,000 M_r subunit is split by one of a variety of proteinases (20,43,44), so that the proteinase becomes almost completely surrounded by the αM molecule and is no longer able to hydrolyze substrates of large molecular weight. (The proteinase will still hydrolyze small peptide substrates up to 8-10,000 M_r (20,45) and the terminal regions of larger proteins, such as trypsinogen, plasminogen and fibrinogen (21).) A percentage (8 to 61% depending on the proteinase (42)) also becomes irreversibly bound, when an αM internal gamma-glutamyl thioester of cysteine reacts non-enzymatically with a lysyl side chain of the proteinase to form a covalent bond (see 46-48). Inhibition of the proteinase by αM occurs irrespective of whether or not the proteinase is covalently bound (42). Covalent binding does not apparently, inhibit the catalytic action of the bound proteinase on small peptide substrates (21,23).

(b) α_1-Proteinase Inhibitor

A major molecular change also occurs when α_1PI inhibits serine proteinases. (It does not inhibit thioproteinases (49).) In this case, the proteinase splits a 6000 to 8000 M_r peptide from native α_1PI, which subsequently undergoes rearrangement and (covalent) acyl bond formation with the hydroxyl group of the serine in the proteinase's active site (see 50). The α_1PI-proteinase complex is not dissociated by SDS under reducing conditions (49). Methionine is present in α_1PI's active site (49), and oxidation of methionine (by leukocyte oxidases, cigarette smoke, ozone and other oxidative air pollutants) destroys the ability of α_1PI to inhibit proteinases (49,51). α_1PI seems to be the body's major inhibitor for leukocyte elastase, which is involved in the development of emphysema (52).

α_1PI and αM Proteinase Inhibitors in Serum

With SDS gels, Western blots and immunocytochemical techniques (Figures 4 and 5), we found that rabbit serum (and plasma) apparently contained more α_1PI-proteinase complexes (and less free α_1PI) than we had reason to expect from published studies, employing somewhat different techniques (see 53,54).

Our results with αM-proteinase complexes are, however, in agreement with unpublished results of experiments by Drs. Doina Ganea and Katherine L. Knight of the University of Illinois. Both of our laboratories found larger amounts of αM-proteinase complexes in rabbit serum and plasma than were reported for other plasmas (see 53,54). These results may represent either species differences or differences in the technical procedures employed.

Sources and Fate of Extracellular Proteinases and Proteinase Inhibitors in SM Lesions

Serum contained a predominance of proteinase-complexed α_1PI and αM (see Results) over free α_1PI and αM. The plasma that extravasates into sites of inflammation must therefore clot rapidly, activating the proenzymes of several plasma proteinases, including thrombin, kallikrein, plasmin and certain complement components (1,2), and releasing platelet and leukocyte proteinases (21). Then these now active proteinases must rapidly combine with the plasma proteinase inhibitors that simultaneously extravasate into the tissue spaces (see 20,40). In inflammatory lesions, the quantity of the α_1PI and αM inhibitors seems to be ample; and these inhibitors are apparently sufficient not only to inhibit the activated plasma proteinases but also to inhibit the proteinases released from the granulocytes (55,56), macrophages, (57-59) and fibroblasts (59-61). The levels of the αMs and α_1PI in such lesions are probably not entirely dependent on the extravasation of serum, because these inhibitors are also synthesized and secreted by macrophages (48,62-66) and fibroblasts (20,48,67).

Assays of the culture fluids from the SM lesions show that the trypsin-inhibitory capacities (Figures 6 and 7) and the levels of the free (and proteinase-bound inhibitors (Figures 4 and 5) decreased as the lesions healed. This decrease is probably due to a diminution in the extravasation of plasma into healing SM lesions (3,4) and also due to an increase in the local production of proteoglycanase (25,26) and collagenase (25,26) (and perhaps other proteinases), both of which are associated with the remodeling of connective tissues.

In the tissues, the α_1PI-proteinase and αM-proteinase complexes are endocytosed by macrophages and fibroblasts (48,68-71). The αM-proteinase complexes are removed quite rapidly (20,53), because these cells have receptors for the the rearranged macromolecule (20,70,71). The α_1PI-proteinase complexes are probably removed at a slower rate.

The αMs bind proteinases with high affinity, so that some proteinases that are complexed with α_1PI become transferred to the αMs, when both α_1PI and αM are present (20,33,53). In fact, the intravenous injection of α_1PI-trypsin complexes into dogs is harmless until the amount given causes the circulating αMs to be depleted (by transfer of trypsin to the αMs and subsequent clearance of the new complexes by the reticuloendothelial system). Then the dog goes into irreversible shock (52-54). The transfer of protein-

ases to the αMs might partly explain why we found more free immunoreactive α_1PI than free αMs in culture fluids from SM lesions (Figures 4 and 5).

Thus the proteinases, proteinase inhibitors and proteinase-inhibitor complexes in the extracellular fluids of the SM lesions have more than one source and may undergo a variety of fates.

The Content and Turnover of Serum Protein in SM Lesions

Peak SM lesions (1 day of age) contained much extractable serum protein (about 35% by weight), which turned over three times each day (3,4). Normal skin contained about 15% extractable serum protein, which turned over only once every 3 days. Healing SM lesions contained intermediate amounts of extractable serum protein, which had intermediate turnover rates. Thus the extravasated serum in acute inflammatory lesions was not static, but was constantly replenished by a fresh supply of serum protein from the blood vessels.

This report and our two previous reports (3,4) indicate that within inflammatory lesions, extravasated serum proteinase inhibitors were continuously replaced and therefore provided constant protection against damage by local proteinases.

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Table 1

Concentrations of the Major Electrophoretic Fractions of First-Day Culture Fluids from Normal Skin and from Peak and Healing Sulfur Mustard Skin Lesions[a]

Electrophoretic fraction	Source of Culture Fluid: Normal skin ug/ml	Peak (1-day) lesions ug/ml	Healing (6-day) lesions ug/ml	Peak lesions concentr./ normal skin concentr.	Healing lesions concentr./ peak lesion concentr.
Albumin	348 ±44 (65%)	1272 ±50 (70%)	912 ±107 (65%)	3.7 ±0.7	0.72 ±0.10
α_1-globulin	25 ±5	73 ±7	40 ±4	2.9 ±0.6	0.55 ±0.03
α_2-globulin	27 ±4	104 ±8	58 ±4	3.9 ±0.7	0.56 ±0.06
β-globulin	56 ±12	192 ±18	157 ±21	3.4 ±1.6	0.82 ±0.10
gamma-globulin	76 ±17	182 ±24	229 ±38	2.4 ±0.4	1.26 ±0.37
Total	532 ±40	1823 ±130	1396 ±90		
Average				3.4 ±0.4	0.77 ±0.37

[a]The skin explants, 1.0 cm^2 in size, were cultured in 2.5 ml of supplemented RPMI 1640 (see 3). The culture fluids were electrophoresed to agarose gels without SDS and without mercaptoethanol. The percentage of albumin is listed in parentheses. The protein in each fraction was determined by densitometry of the Amido Black-stained gels.

The P values for 1 day vs. 6 days for albumin, α_1-globulin and α_2-globulin were 0.012, 0.002 and 0.001, respectively. Although suggestive, the trends in the β- and gamma-globulin fractions were not statistically significant. Similar comparisons between normal skin and 1-day or 6-day SM lesions were all highly significant ($P < 0.001$). The two-tailed paired-sample Student's t test was used.

Figure 1. Electrophoretic fractions of first-day culture fluids from 1.0-cm^2 dermal sulfur mustard skin lesions of various ages from six rabbits. On the left, the albumin, α_1-globulin, α_2-globulin, β-globulin, and gamma-globulin fractions of the protein in first-day culture fluids are expressed as percentages of the corresponding fraction of the serum protein from the same rabbits (see Results). On the right the electrophoretic fractions of this rabbit serum are expressed as a percentage of total serum protein. The shaded areas represent mean serum values and their standard errors. The means for the culture fluids and their standard errors are represented by the line graphs.

The albumin and β-globulin fractions of the protein in culture fluids from lesions of all ages resembled the corresponding fractions of serum protein. The α_1-globulin fractions of the protein in culture fluids from 3-, 6- and 10-day SM lesions were smaller than the corresponding serum fraction (P = 0.001 0.012 and 0.028, respectively), and α_1-globulin fractions of 6-day lesions were smaller than those of 1-day lesions, but not to a statistically significant degree. The α_2-globulin fractions of the protein in culture fluids from normal skin and from 1-, 2-, 3-, and 6-day SM lesions were also smaller than the corresponding serum fraction (P = 0.001, 0.026, 0.003, 0.022 and 0.002, respectively). The gamma-globulin fractions of the protein in culture fluids from normal skin and from 3-, 6- and 10-day SM lesions were larger than the corresponding serum fractions (P = 0.026, = 0 014, <0.001 and <0.001, respectively). The two-tailed, paired-sample Student's t test was used.

Figure 1

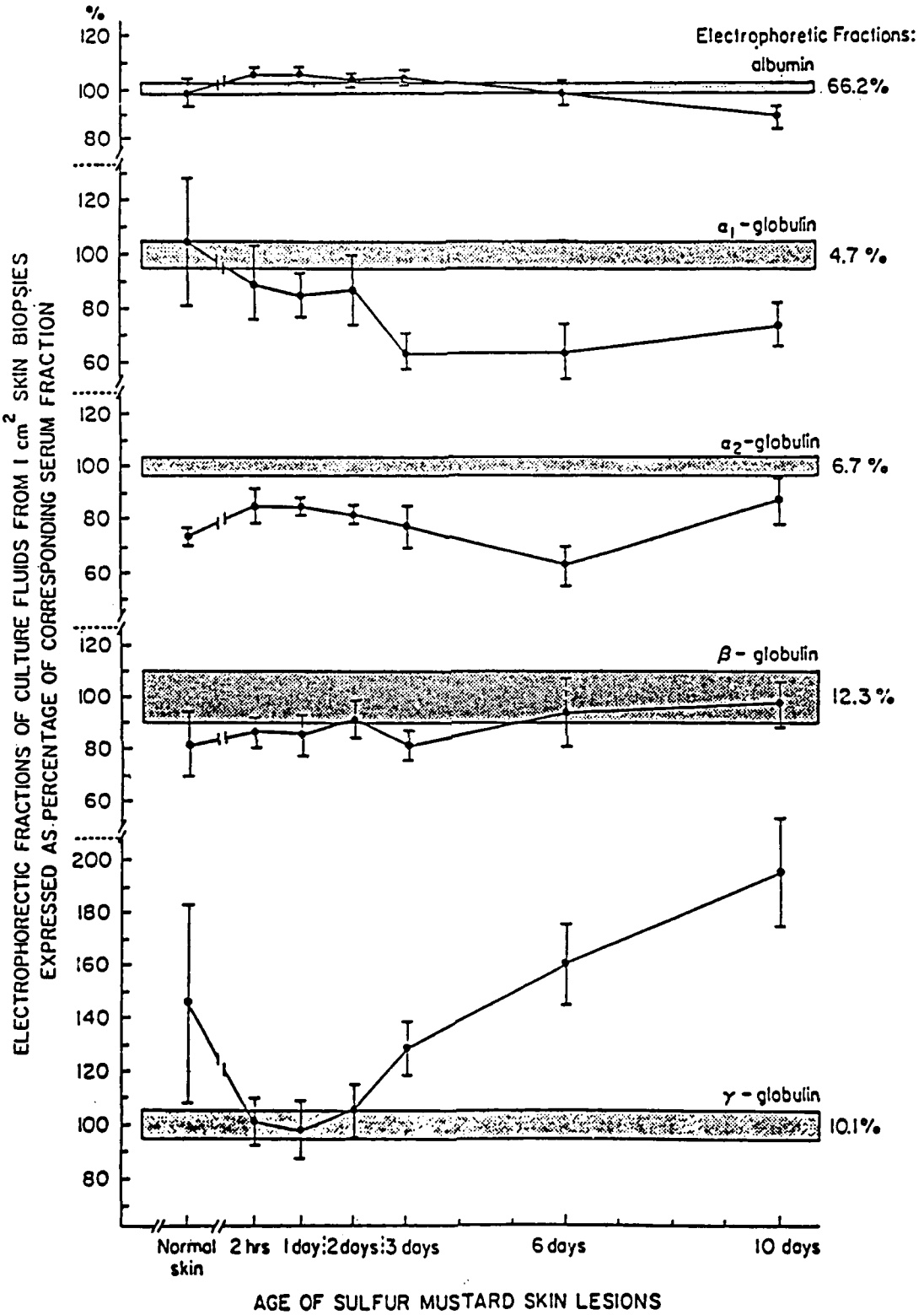

%
Electrophoretic Fractions:
albumin
66.2%
α1-globulin
4.7 %
α2-globulin
6.7 %
β-globulin
12.3 %
γ-globulin
10.1%
ELECTROPHORECTIC FRACTIONS OF CULTURE FLUIDS FROM 1 cm² SKIN BIOPSIES EXPRESSED AS PERCENTAGE OF CORRESPONDING SERUM FRACTION
Normal skin
2 hrs
1 day
2 days
3 days
6 days
10 days
AGE OF SULFUR MUSTARD SKIN LESIONS

Figure 2.

a. Polyacrylamide gel slab containing the culture fluid from a 1-day SM lesion, after two-dimensional electrophoresis and silver staining.

b. Schematic of this polyacrylamide gel slab in which the location of a given spot on the gel is represented by an ellipse and the amount of protein in the spot (i.e., staining intensity) is represented by the size of the ellipse. A schematic similar to this was used to identify the spots on a color video screen, where the spots from each gel were portrayed in different colors and the overlapping spots were portrayed in a third color (see text).

The gels have microlocal distortions. Therefore, to identify the same spots in two separate gels, we matched as "tie points" 10 to 15 spots in different areas. A glyceraldehyde-3-phosphate dehydrogenase charge train (between the horizontal arrows) provided some of these "tie points."

The diagonal arrow points to the most prominent group of spots stained by the α_1M-α_2M antibody and the immunoperoxidase technique. (Duplicate gels were not available for staining with antibody to α_1PI.)

The family of spots representing most of the polydispersed albumin was not included because the protein concentration was too high. In the stained gel (above), the albumin appears as a horizontal dark line. In the schematic (below), a few of the albumin spots are represented by the horizontal group of circles below the αM group.

Figure 2

a.

b.

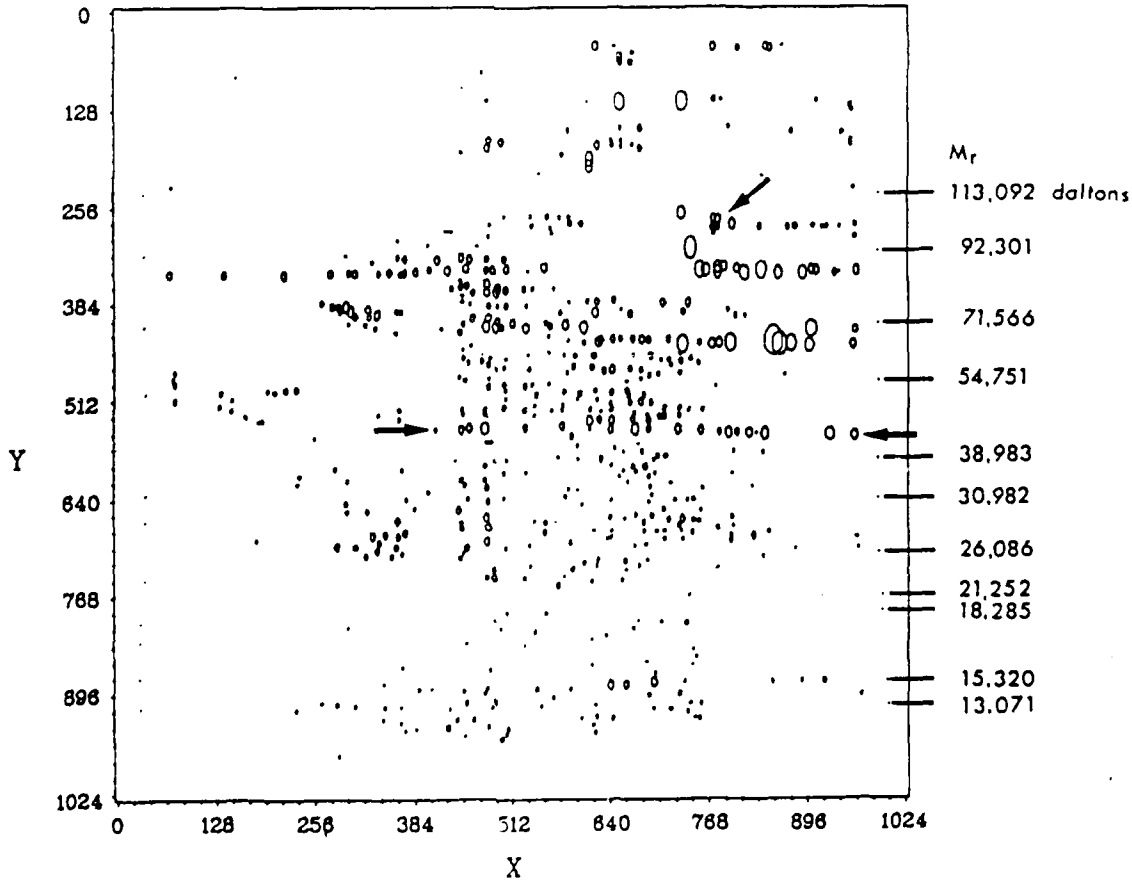

0
128
256
384
512
640
768
896
1024
Y
0
128
256
384
512
640
768
896
1024
X
M_r
113,092 daltons
92,301
71,566
54,751
38,983
30,982
26,086
21,252
18,285
15,320
13,071

Figure 3. A polyacrylamide gel slab, containing (a) Prestained High Molecular Weight (M_r) Markers (Catalog No. 6041SA, Bethesda Research Laboratory, Gaithersburg MD 20877), (b and c) undiluted culture fluids from 1- and 6-day SM lesions (8, 4 and 2 ul) and from normal skin (8 and 4 ul), and (d) serum (1 ul, diluted 1:40), after one-dimensional electrophoresis and staining with Coomassie blue. The 65 K band is serum albumin (ALB). SDS-PAGE with mercaptoethanol).

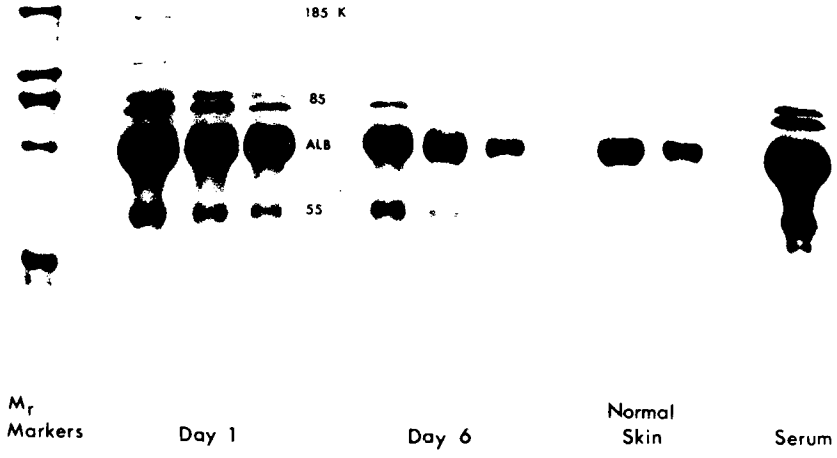


Figure 4. A Western blot of an acrylamide gel, containing first-day SM lesion culture fluids and serum in the amounts described in Figure 3, stained by the immunoperoxidase technique utilizing an antiserum specific for rabbit α_1PI. The 55 K bands probably represent free α_1PI. The 71 K and 88 K bands probably represent α_1PI complexed with proteinases, since treatment with SDS and mercaptoethanol does not dissociate such complexes (47). In other Western blots, the two 55 K bands were fused into one band. The 8-, 4- and 2-ul samples on the gel (see Figure 3) enabled us to estimate the relative concentrations of α_1PI in culture fluids from (a) 1-day and (b) 6-day lesions and from (c) normal skin. They were in a ratio of about 9:3:1 for each of the three major bands (55K, 71K and 88K). Similar results were found with lesion culture fluids of SM lesions and normal skin from two additional rabbits. SDS-PAGE with mercaptoethanol.

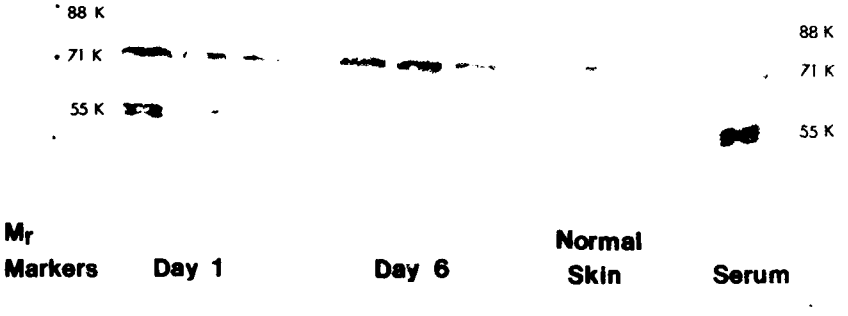


Figure 5. A Western blot of an acrylamide gel, containing SM lesion culture fluids and serum in the amounts described in Figure 3, stained by the immunoperoxidase technique utilizing an antibody specific for rabbit α_1M-α_2M. The 85 K band is thought to be a subunit of αM that had been split in the 'bait' region by a proteinase. Such a split causes molecular rearrangement and trapping (and inhibition) of the proteinase. The αM-proteinase complex is dissociated during the fractionation of αM SDS under reducing conditions. Thus the 85K band represents αM that had been complexed with a proteinase (see Discussion). The portion of the proteinase that is covalently bound should not migrate within the 85 K band. The 185 K band represents αM that had not been complexed with a proteinase. Bands of 68 K and below probably represent fragments derived from spontaneous autolytic cleavage of αM and αM-proteinase complexes.

The 8-, 4- and 2-ul samples on the gel (see Figure 3) enabled us to estimate the relative concentrations of αM in culture fluids from 1-day lesions, 6-day lesions, and normal skin. The 85K bands showed a ratio of about 9:3:1. Other bands showed somewhat different ratios. Similar results were found with lesion culture fluids from 3 additional rabbits. SDS-PAGE with mercaptoethanol.

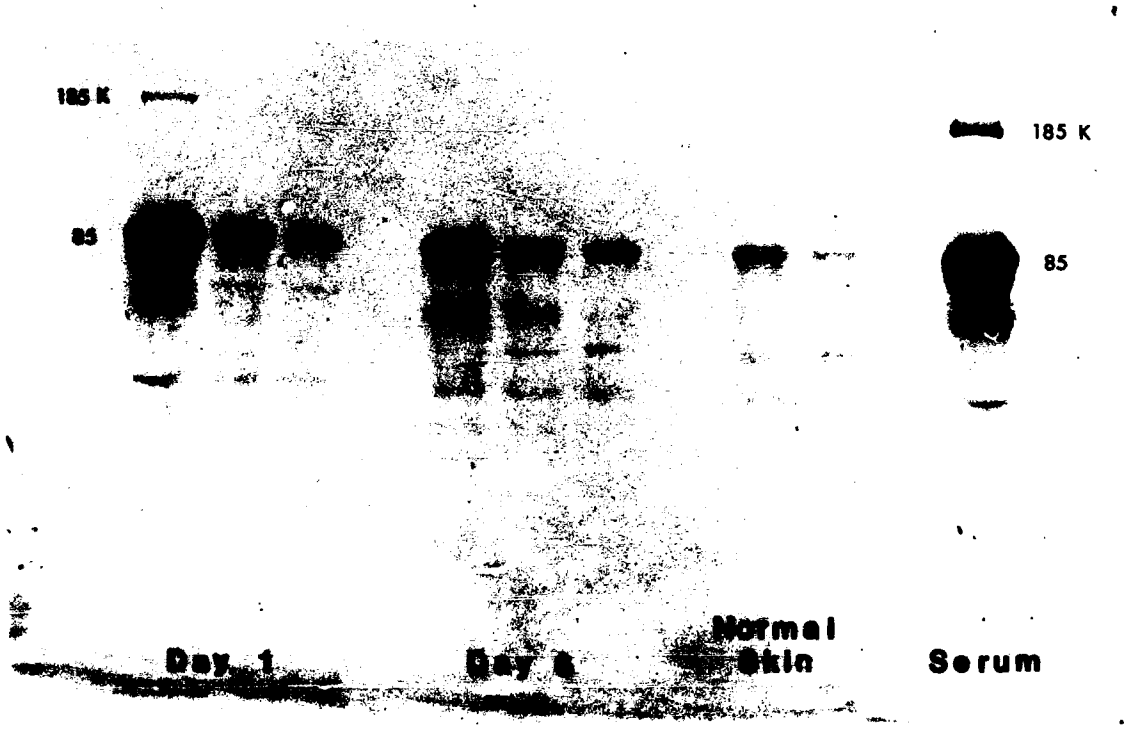


Figure 6. Trypsin-inhibitory capacity in first-, second- and third-day organ culture fluids of 1.0-cm^2 dermal SM lesions of various ages, from 6 rabbits. The means and their standard errors are depicted. The trypsin-inhibitory capacities of organ culture fluids from 1-, 2-, 3-, 6-, and 10-day SM lesions were significantly higher than those of normal skin on all days of culture ($P < 0.001$ for the first-day culture fluids, and < 0.03 for second- and third-day culture fluids). The inhibitory capacities of first- and second-day culture fluids from 1-day lesions were also significantly different from those of the 6- and 10-day lesions ($P < 0.003$). Each 1.0-cm^2 skin biopsy was cultured in 2.5 ml of RPMI 1640.

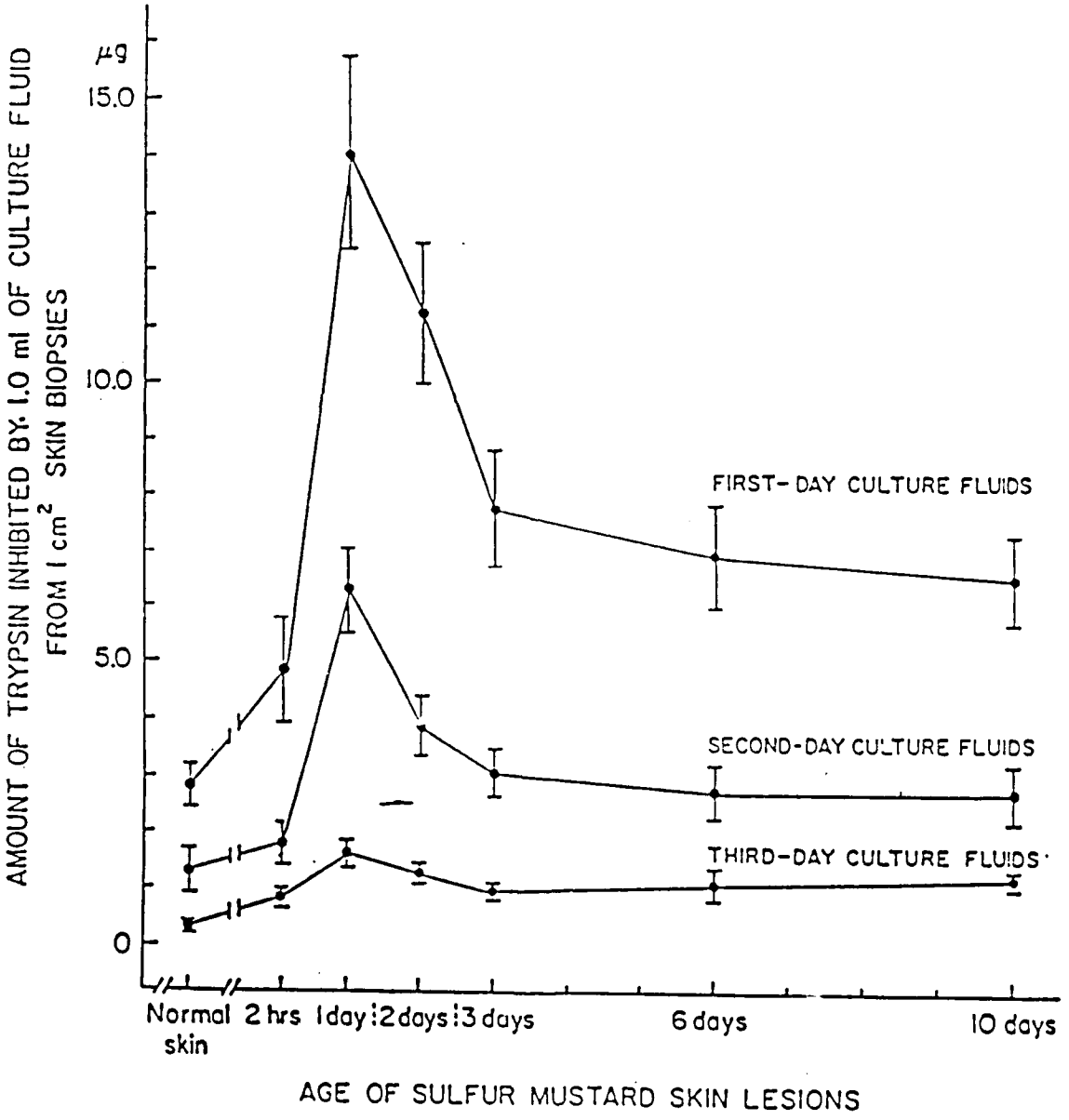
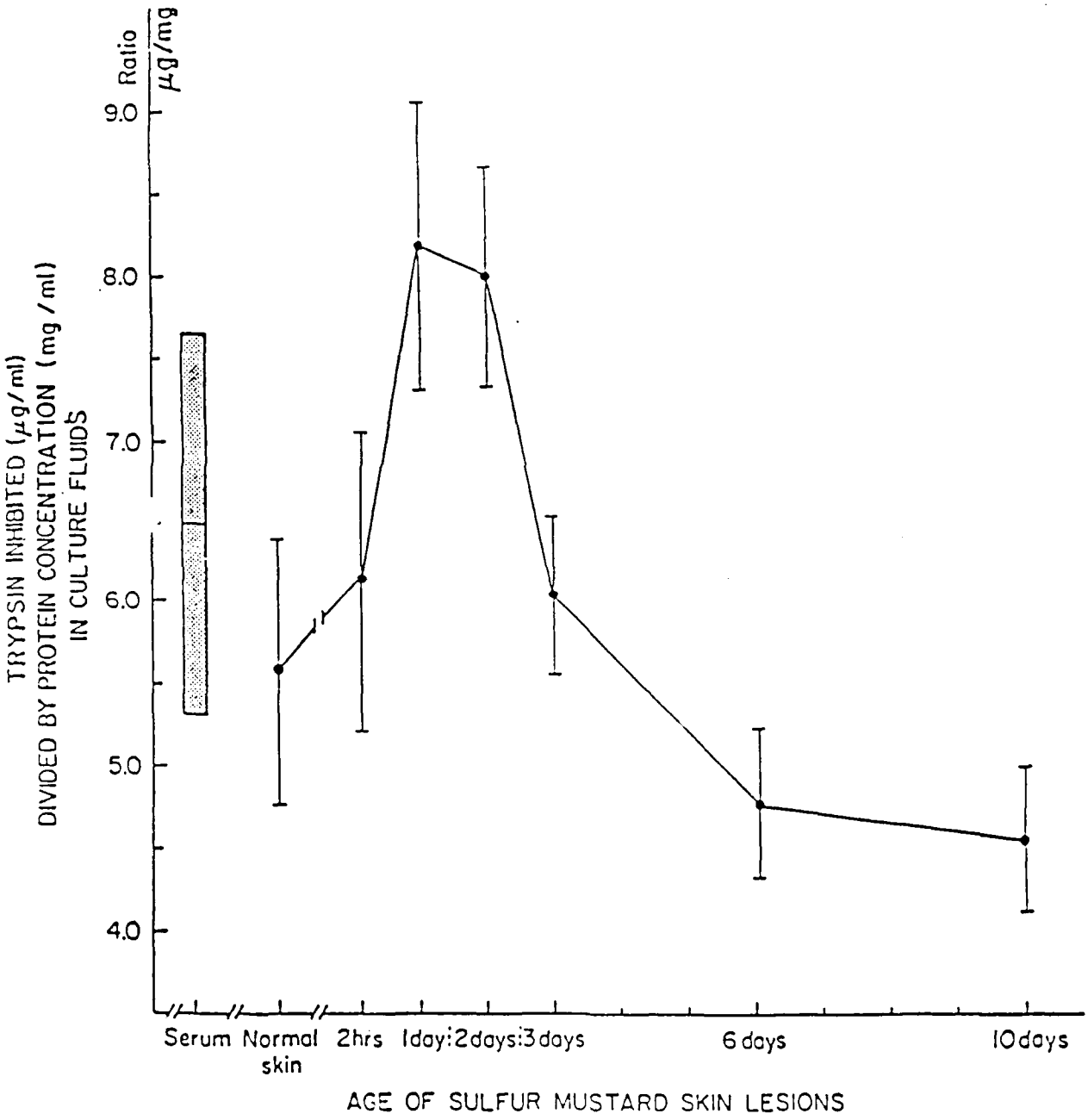


Figure 7. Micrograms of trypsin inhibited per milligram of protein in sera and in first-day culture fluids of 1.0-cm^2 dermal sulfur mustard lesions of various ages from six rabbits. The means and their standard errors are depicted. Culture fluids from 1- and 2-day (peak) lesions showed greater trypsin-inhibitory capacity per milligram of protein than did culture fluids from 6- and 10-day (healing) lesions ($P < 0.003$). The graphs for TIC per milligram of protein in the second- and third-day culture fluids were similar.



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CHAPTER 4

Chemotaxis of Granulocytes and Macrophages by the Culture Fluids from Developing and Healing Dermal Sulfur Mustard Lesions

ABSTRACT

Major sources of the inflammatory mediators in dermal SM lesions are the leukocytes that have infiltrated these lesions from the blood stream. Not only do they release phlogistic substances, e.g., histamine, platelet activating factor and eicosanoids, but they release potentially damaging substances, e.g., reactive oxygen intermediates and proteinases. One of their main functions is the prevention and control of local microbial infection. Thus for the treatment of SM injury (especially ruptured blisters), some leukocytes should be allowed into the site, but an excess of leukocytes, which may cause tissue damage, should be prevented.

Leukocytes are attracted into the site by chemotactic factors. In this chapter, experiments are described which prove that chemotactic factors for both granulocytes and macrophages were present in SM lesion culture fluids. Chemotactic activity was measured in Boyden chambers.

Chemotactic activity in the lesions was detected as early as 2 hours after the application of SM to skin. This activity reached a peak in 1 day, and its level was maintained at least 10 days, i.e., during the period of healing. The levels of chemotactic activity for granulocytes and for macrophages showed the same pattern.

Pilot-type experiments suggest that the chemotactic factors leukotriene B_4 (LTB_4), the C5a component of complement, and Interleukin 1 are present in SM lesions. The production of leukotriene B_4 can be controlled by lipoxygenase inhibitors. It is only a matter of time until similar agents will be available to control Interleukin 1 production because of the numerous industrial and academic institutions now investigating this autocoid.

CHAPTER 4

TABLE OF CONTENTS

INTRODUCTION

This report describes the chemotactic activity in organ-culture fluids from early, peak and healing SM lesions. That such activity was present at each stage of lesion development and healing was conclusively demonstrated, and our findings are presented herein.

Experiments to identify the specific chemotactic factors responsible for chemotactic activity in SM lesions require sophisticated technology and expertise often beyond that possessed by our own laboratory. We therefore enlisted the help of other laboratories, and the results of their pilot-type experiments are also presented herein.

MATERIALS AND METHODS

Assay of Chemotactic Activity for Polymorphonuclear Granulocytes (PMN) and for Mononuclear Phagocytes (MN), i.e., Monocytes and Macrophages

Dr. Donald L. Kreutzer (1,2), of the University of Connecticut School of Medicine and Dr. Ralph Snyderman (3), of Duke University School of Medicine, advised us on setting up these methods.

Lesion culture fluid (110 ul for MN and 115 ul for PMN) was added in the lower compartment of a blind well chemotaxis chamber (Neuro Probe, Inc., Bethesda, MD 20034, Cat. No. 100-187). (The sizes of the upper and lower compartments were 200 ul and 100 ul, respectively.) Upon the convex miniscus of the filled lower compartment, a Millipore or Nuclepore filter was carefully placed, avoiding bubbles. For PMN, 3 um pore-size Millipore filters, (Millipore Corp., New Bedford, MA 01730, Cat. No. SO9Q018A3), cut to about 12 mm in diameter with a cork borer, were used. For MN, 5 um pore-size, polyvinyl pyrrolidone-coated Nuclepore filters, 13 mm in diameter (Nuclepore Corp., Pleasanton, CA 94566, Cat. No. NMF-5) were used. The Nuclepore filter was placed on the miniscus with the dull side up, but the two sides of the Millipore filter are not appreciably different.

After the top half of the Boyden chamber was screwed down tightly, 0.20 ml of the PMN or MN cell suspension (see below) was pipetted into the upper compartment. At 37 C, in humidified air containing 5% CO_2, the chambers were incubated 45 min for PMN and 90 min for MN.

For PMN, the cell migration was stopped by partly submerging the chambers in a cracked ice bath. Then, the cell suspension in the upper compartment was removed by one rapid shake, and the chamber disassembled. The PMN-containing filters were placed for 1 to 5 min in 100% ethanol without prior rinsing. After fixation, the filters were rinsed in tap water for about 5 min and stained for 5 to 15 min with alum hematoxylin (Accra-Lab, Inc, Bridgeport, NJ 08014, Cat. No. 541). They were then rinsed in tap water and dried overnight, between two layers of filter paper (Whatman #3, qualitative, 24 cm in diameter), pressed down with heavy textbooks.

A thin plastic rack, containing 60 two-ml wells to hold the filters, was cut from a 96-well sheet. Four holes (3-4 mm in diameter) were cut in the

sides of each well, and one hole in the base (6 mm in diameter). This holding rack, placed in a shallow stainless steel tray, was used for the ethanol fixation, rinsing and staining procedures.

The air-dried, stained filters were cut with a 6 mm Baker skin biopsy punch (Baker-Cummins Division of Key Pharmaceuticals, Inc., Miami, FL 33169). The central areas, containing the cells, were only 4.7 mm in diameter. Then the filters were mounted on a standard, 1 x 3 inch, microscope slide in cedarwood oil (Fisher Scientific Co., Silver Spring, MD 20910, Cat. No. O-40), in 3 rows, each containing up to 6 filters. For this purpose, two 3 mm strips of paper masking tape were placed near the edge of the slide. The oil was dropped on top of each filter. Flo-Texx Mounting Medium (Lerner Laboratories, Stamford, CT 06902, Cat. No. M770-1) was placed outside the masking tape strips and a 50 x 24 mm coverslip placed over everything. The Flo-Texx sealed the coverslip in place, but was not allowed to mix with the oil because such a mixture becomes cloudy. Immersion oil may be used instead of cedarwood oil, but with immersion oil, cloudiness sometimes occurs in a few days.

For MN, no chilling in the ice bath was used. Instead, we quickly placed the MN-containing filters, dull side down, on a microscope slide and air-dried them at 23 C. Then we fixed the cells to the filters by applying one drop of 100% ethanol and allowing it to evaporate at 23 C. The filters were stained for 20 min with a dilute, buffered Giemsa solution: 2.0 ml of Giemsa Stain (Harleco, Gibbstown, NJ 08027, Cat. No. 6201) mixed with 15 ml of 0.02 M sodium phosphate buffer (pH 6.6). They were rinsed by gently dipping into a large beaker of distilled water. Then they were dipped in a 0.35% $NaHCO_3$-2% $MgSO_4$ solution for about 30 sec, and air-dried. The Nuclepore filters were then mounted in Flo-Texx, a permanent mounting medium.

With MN, all the cells in 5 high power fields (HPFs) (40 X objective lens, 500 X magnification) on the shiny surface of each filter were counted, using the ocular grid described below in the area described below. Our chemotaxis index was the total number of MN in 5 HPFs.

With PMN, counting was more complex, because the method measured the degree of leukocyte penetration into the Millipore filter (4). First, the top of the filter was surveyed for uniformity, but clumping was rare. Then 5 representative grid areas were chosen and counted from the center third of the filter (usually the top, bottom, right, left and center of this central third). A 40 X objective lens and 12.5 X ocular with a 10 X 10 mm ocular grid were used. By turning the fine focus on the microscope 10 um at a time, we counted all leukocytes in the grid in the 10 um, 20 um, 30 um, 40 um (up to 100 um) planes from the top of the Millipore filter. (The filter is about 110 um thick.) Only PMN were present, as the rare macrophage in the preparation did not migrate into the 3-um pore filter.

The chemotaxis index was calculated by multiplying the number of cells at each plane by 1/10 of the depth of the plane in the filter and then adding together the resulting units. For example, 20 cells at 10 um depth gave 20 units, 15 cells at 20 um gave 30 units, 10 cells at 30 um gave 30 units, 5 cells at 40 um gave 20 units, and 1 cell at 50 um gave 5 units. The sum of these units produced a chemotaxis index of 105. The indices from the 5 grid areas were counted and then averaged.

Each culture fluid had six chemotaxis assays performed on it: undiluted, 1:5 dilution, and 1:25 dilution; each in duplicate. When the chemotactic factors were in high concentration, a prozone phenomenon occurred, i.e., the undiluted culture fluids showed less chemotactic activity than did the 1:5 dilution. With some culture fluids, the 1:25 dilution had the highest activity.

N-formyl-L-methanyl-L-leucyl-L-phenylalanine (FMLP) (10^{-8} to 10^{-11} M) and zymosan-activated rabbit serum (1:10 to 1:1000 dilutions) served as standard chemotactic factor controls. FMLP was purchased from Sigma Chemical Co. (Cat. No. F-3506). Zymosan (also from Sigma, Cat. No. Z-4250), was added to fresh rabbit serum, incubated for 30 min at 37 C, and centrifuged. The zymosan-activated rabbit serum was then aliquoted in 0.1 ml amounts and kept frozen at -70 C until used.

PMN and MN preparations for the Boyden chambers

Glycogen-induced PMN and MN exudate cells were obtained from the peritoneal cavities of rabbits and quantitated as described in Chapter 5. With both cell types, 200,000 cells were added to the top half of the Boyden chamber.

Cleaning Chemotaxis Chambers

The chambers were placed in 1% Linbro 7X-O-Matic Cleaning Solution (Flow Laboratories, Inc., 7655 Old Springhouse Rd., McLean, VA 22102, Cat. No. 76-674-94), in hot tap water (about 50 C) for 1 or 2 hr; rinsed several times in hot tap water, left 1 to 3 days in deionized water (changing it 2 or 3 times a day), rinsed individually in warm tap water with a jet stream from a large pipette, rinsed several times in deionized water, and dried in air.

RESULTS

Biopsies of sulfur mustard lesions, 1 through 10 days of age, released, in organ culture, chemotaxins for granulocytes (PMN) (Figure 1) and for mononuclear phagocytes (MN) (Figure 2). With PMN, these levels were about four times those in cultures of normal skin and, on the average, were maintained into the healing period. With MN, culture fluids from normal skin showed as much chemotactic activity as those from the SM lesions (Figure 2). Evidently, the trauma associated with the organ-culture procedure was sufficient to initiate the production of chemotaxins for monocytes.

During the healing phases, at 6 and 10 days, the culture fluids of some lesions showed a marked decrease in chemotaxin levels, while culture fluids of other lesions showed a marked increase in chemotaxin levels. The reason for this variability during healing is not known.* Relatively few PMN were actually within the tissues of 6- and 10-day lesions; most PMN (if still discernible) were in the crust.

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* One factor may be variations in the crusts: Some crusts were thick, containing numerous dead PMN (see Chapters 1 and 5). Others were thin and contained fewer PMN, and still other crusts were shed early, due to rapid healing.

Two of the 8 rabbits evaluated showed very thin (almost absent) crusts at 6 and 10 days. (Each rabbit had 1-, 2-, 3-, 6-, and 10-day lesions at the time of sacrifice.) The culture fluids from their lesions showed peak chemotaxin levels at 2 and 3 days, and decreased levels at 6 and 10 days. Thus healing seems to be associated with a decrease in chemotaxins within the lesions.

Chemotactic Activity Released from PMN and MN Exudate Cells and from SM Lesion Crusts in Culture

PMN and MN peritoneal exudate cells (10.0×10^6 cells in 2.0 ml of serum-free supplemented RPMI 1640) were cultured for 24 hr, and the culture fluids frozen at -70 C until they were assayed for chemotactic activity. Both MN and PMN exudate cells from other rabbits were used as the responding cells. In the few samples that were evaluated, PMN, MN and fibroblasts contained and released (in culture) chemotaxins for both PMN and MN. Crusts removed from healing (6-day) SM lesions (see Chapter 5) also released in culture chemotaxins for PMN. (These crusts were full of dead PMN.)

Pilot-type Experiments Identifying Specific Chemotactic Factors

Leukotriene B_4 (LTB_4) is a potent chemotaxin (see 5). Its level in organ culture fluids from developing and healing SM lesions was analyzed under the guidance of Dr. David S. Newcombe of our department (6-7). High performance liquid chromatography (HPLC) showed a large LTB_4 fraction in SM lesion culture fluids and only a small LTB_4 fraction in culture fluids from normal skin. This result remains to be confirmed by radioimmunoassay (e.g., with the kit available from Seragen, Inc., Boston, MA).

SM lesions and control skin were incubated with the pure C3 and C5 components of complement. The resulting culture fluids were frozen and shipped in Dry Ice to Drs. Tony E. Hugli and Richard G. DiScipio of Scripps Institute and Research Foundation in La Jolla, CA (8). The SM lesions hydrolyzed both C3 and C5, producing the phlogistic fragment C3a and the phlogistic and chemotactic fragment C5a (8).

Finally, culture fluids from developing and healing SM lesions were shipped to Dr. Gail S. Habicht (and Gregory Beck) (9,10), of the School of Medicine, State University of New York, at Stony Brook. They have found that the Interleukin 1 (IL-1) levels of culture fluids from SM lesions were significantly higher than IL-1 levels from culture fluids from normal skin. IL-1 has recently been shown (in their laboratory) to be a potent chemotactic factor for PMN (10).

Conclusions

Many more studies remain to be performed before we shall be able to identify which of the many chemotactic factors are important at each stage of SM lesion development and healing. Such information should enable us to select appropriate pharmacologic agents and select the time during which they are employed in order to control the influx of phagocytes into these lesions. In this way, the beneficial effects of the phagocytes might be retained and their harmful effects might be eliminated.

Figure 1. PMN chemotactic activities in first-day culture fluids from 1.0-cm^2 dermal sulfur mustard lesions of various ages. These activities are plotted as percents of those found in first-day culture fluids from 2-day lesions. The means and their standard errors are depicted. Culture fluids from 1-, 2-, 3-, 6-, and 10-day lesions showed more chemotactic activity for PMN than did fluids from normal skin ($P < 0.01$). The one-tailed Student's t test was used.

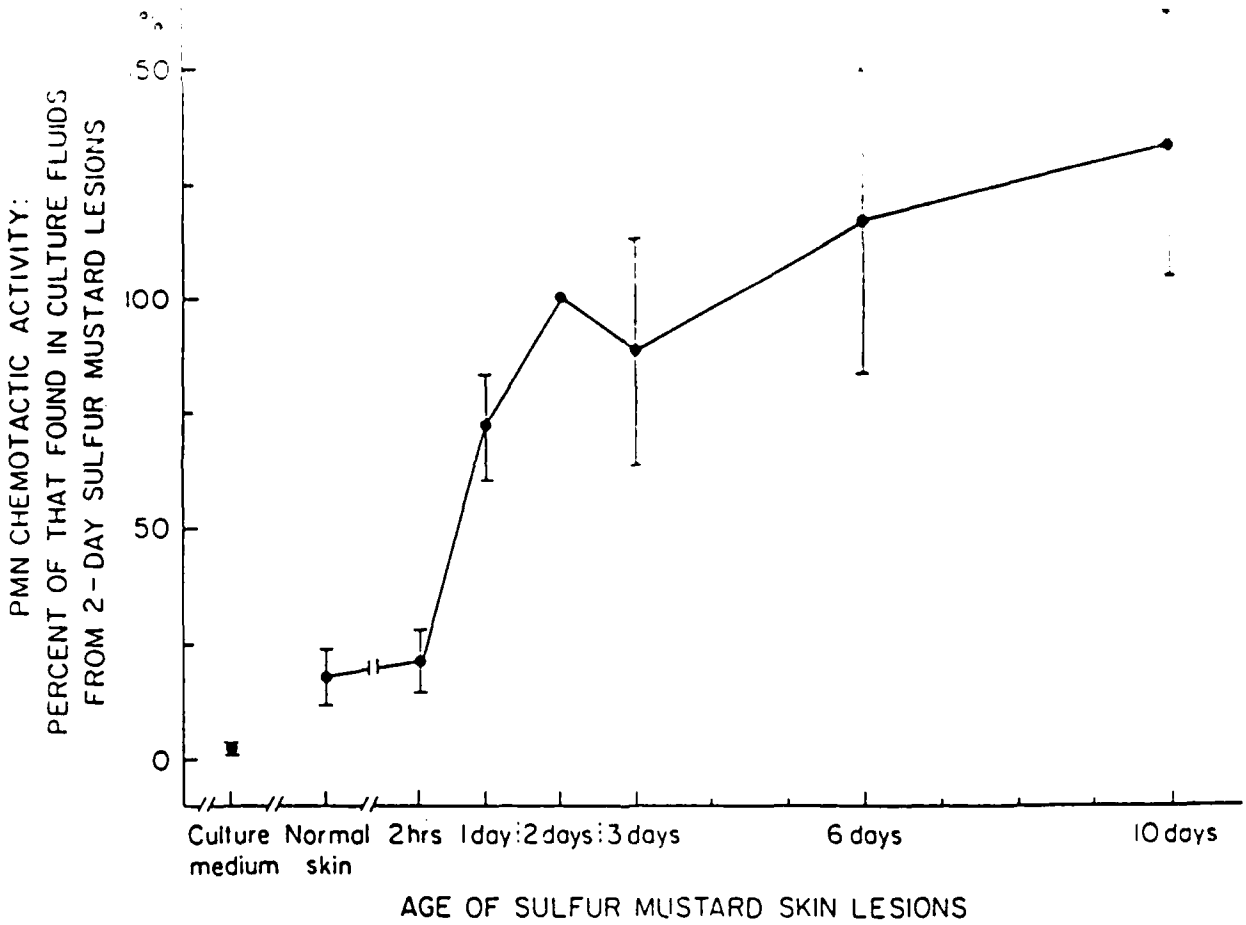
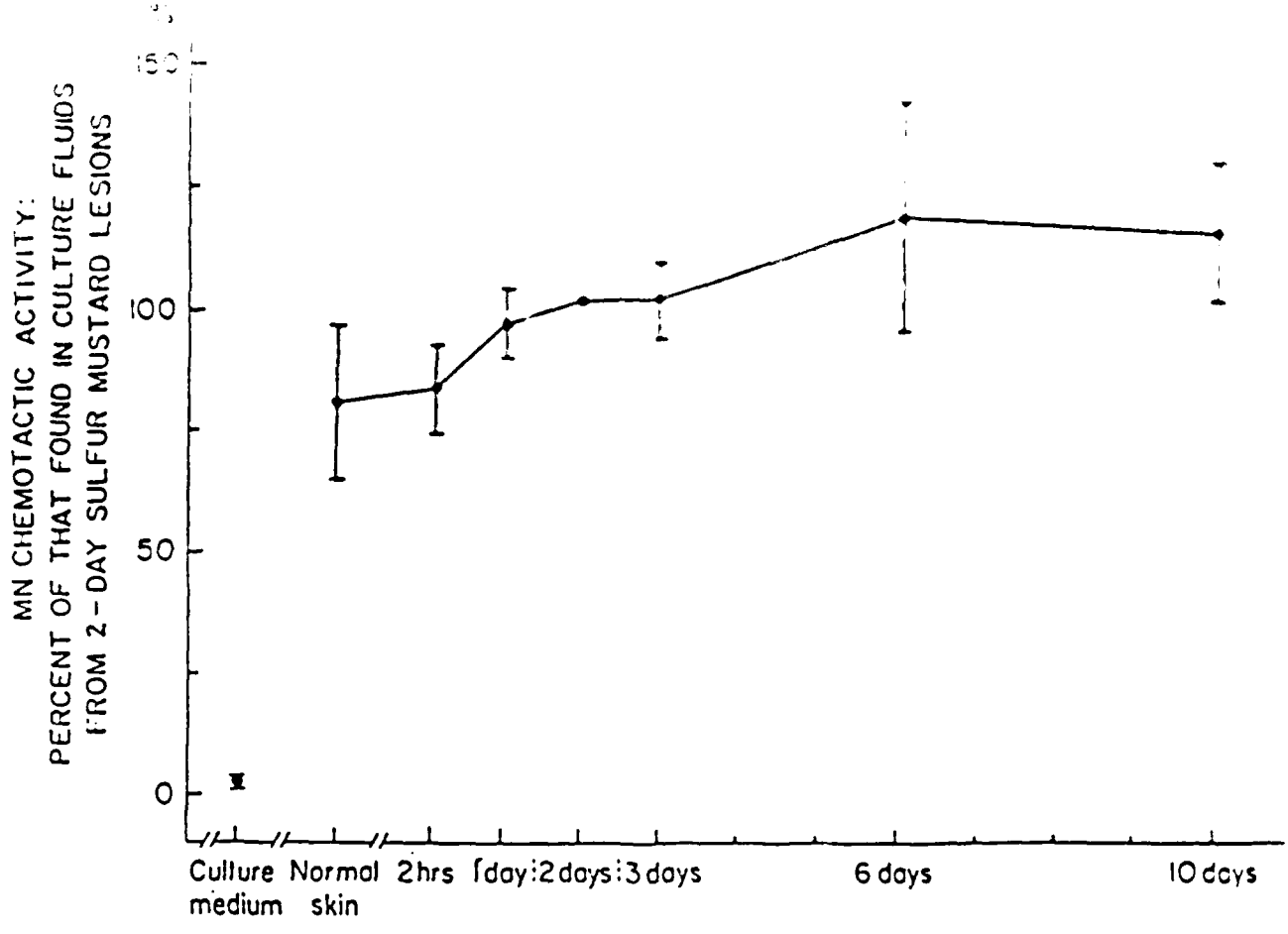


Figure 2. MN chemotactic activities in first-day culture fluids from 1.0-cm^2 dermal sulfur mustard lesions of various ages. These activities are plotted as percents of those found in first-day culture fluids from 2-day lesions. The means and their standard errors are depicted. The culture fluids from both SM lesion and normal skin showed comparable chemotactic activities for macrophages.



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CHAPTER 5

Estimates of the Cellular and Serum Sources of Extracellular Lysosomal Enzymes in Dermal Sulfur Mustard (SM) Lesions:

(a) Acid Phosphatase, β-Glucuronidase, β-Galactosidase, Lysozyme (and Lactic Dehydrogenase) Released In Vitro by Organ-cultured Developing and Healing SM Lesionsμ (b) Histochemical-Biochemical Correlations; and (c) Lysosomal Enzymes Released by Cultured Granulocytes, Macrophages and Fibroblasts

ABSTRACT

SM lesions contain extravasated serum, granulocytes, macrophages, fibroblasts and other cells, each of which may provide mediators (and modulators) that control lesion development and healing. In this chapter, we describe studies that utilize quantitative biochemical, histological and histochemical techniques to estimate which of these five sources provide the extracellular lysosomal enzymes[a] found in SM lesion culture fluids.

Lysosomal enzymes, as a group, represent the major digestive enzymes that cells contain. The release of such enzymes into the tissues from live or autolyzing cells is a potential cause of endogenous tissue injury and blister formation.

Histochemical tests for acid phosphatase and β-galactosidase are among the very best existing. These two lysosomal enzymes could therefore serve as markers for the other lysosomal enzymes and provided a unique opportunity for us to determine the sources of extracellular lysosomal enzymes in SM lesions at various stages of development and healing.

The extracellular lysosomal enzymes (acid phosphatase, β-glucuronidase, β-galactosidase, and lysozyme) extracted from the SM lesions by the organ-culture fluids were measured biochemically along with lactic dehydrogenase (LDH), an enzyme marker for cell death. We found that lysosomal enzymes were indeed secreted or released by the live cells within the inflammatory lesion.

In order to determine the likely sources of these enzymes, the following evaluations were made: (a) Polymorphonuclear leukocytes (PMN), macrophages (MN) and fibroblasts were counted in tissue sections of the lesions. (b) Acid phosphatase and β-galactosidase in these cells were measured histochemically. (c) Lysosomal enzymes in serum and in culture fluids from peritoneal exudate PMN and MN, and from two rabbit fibroblast cell lines, were measured biochemically, and cell smears were measured histochemically. (d) The crusts of healing SM lesions, and the bases of these lesions after the crusts had been removed, were also assayed for these enzymes.

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[a] Lysosomal enzymes is used herein as a general term to designate the classical lysosomal marker enzymes, acid phosphatase, β-galactosidase, and β-glucuronidase, and also lysozyme.

The culture fluids from peak lesions contained much lower levels of all five enzymes than did culture fluids from healing lesions. When histological-histochemical-biochemical correlations were made, serum, MN and activated fibroblasts (but not tissue PMN) appeared to be major sources of extracellular lysosomal enzymes in peak lesions; and the dead PMN in the crusts and activated tissue fibroblasts appeared to be major sources in healing lesions. The high lysosomal enzyme content of crusts suggests that this passive barrier also plays an active role in promoting healing and in protecting against invasion by microorganisms.

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INTRODUCTION

Lysosomal enzymes released from live and dead phagocytes are thought to play a significant role in the inflammatory process (15,19,27,36). The main function of lysosomal enzymes is the intracellular digestion of ingested microorganisms (8,9,29), cellular debris or effete intracellular organelles (autophagy) (14). Primary lysosomes, originating from the Golgi apparatus, fuse with the membranes of the phagosome or autophagic vacuole and discharge their enzymes into these intracellular digestive organelles (8,9,14,20). During autolysis, such lysosomal enzymes are released into the cytoplasm.

Lysosomal enzymes also play an extracellular role in sites of inflammation (15,19,27,36). During the life of the phagocyte, these enzymes may be released by exocytosis or by incomplete closure of phagosomes, e.g., when the particle being ingested is too large to be surrounded (15,19). These enzymes are also released extracellularly when an autolyzing cell ruptures its plasma membrane.

In biological research, the lysosomal enzymes have mainly been used as markers for the digestive type of macrophage activation (10,12). In such activated macrophages, the levels of many acid-acting digestive enzymes (7), and possibly some of the neutral-acting ones (see 6), are increased.

Acid phosphatase, β-glucuronidase and β-galactosidase (as well as many other lysosomal enzymes) apparently exist in their active forms and are not inhibited or inactivated by any known serum component. The extracellular release of these three 'marker' enzymes could be a reflection of the release of other lysosomal enzymes, including those inhibited by extravasated serum components in inflammatory lesions.

The histochemical tests for acid phosphatase and β-galactosidase are among the best existing. They can even be used with paraffin-embedded tissue sections (33) because these enzymes are relatively resistant to various fixatives and solvents. In addition, biochemical methods to measure the activity of these enzymes are readily available and easy to perform.

In the current study, we organ-cultured developing and healing inflammatory lesions produced by SM, measured the extracellular lysosomal enzymes extracted by the culture fluids, and identified the major sources of these lysosomal enzymes. This chapter is presented in four parts: (I) the cellular composition of these lesions and the lysosomal enzymes histochemically demonstrable in the cells; (II) the extracellular lysosomal enzymes extracted from the lesions into the organ-culture fluids; (III) the lysosomal enzymes released in culture from peritoneal PMN and MN and from fibroblast cell lines, and released by freezing and thawing these cells; and (IV) the sources of extracellular lysosomal enzymes estimated from the studies described in I, II and III. We used histochemical techniques on free cells and on cells in inflammatory tissues to ascertain that the function (i.e., the states of activation) of both cell populations was of the same order of magnitude.

This type of biochemical-histological correlation is greatly needed to increase our understanding of the inflammatory response. The literature is replete with studies on the function of free cells in culture, which may or may not represent the function of these same cells in tissues. Because of the mixed cell population within inflammatory lesions, the cellular source of lysosomal enzymes cannot be determined with precision. Yet such semi-quantitative studies can provide information on the in vivo inflammatory response, which cannot be obtained in any other way.

From these studies, we concluded (a) that tissue PMN make only a small contribution to the lysosomal enzymes in the extracellular fluids of sulfur mustard lesions, (b) that MN make their major enzyme contribution to peak (not healing) lesions, (c) that activated fibroblasts make their major enzyme contribution to healing lesions, (d) that extravasated serum makes a substantial contribution to extracellular lysosomal enzymes in every stage of the inflammatory process, and (e) that the crust (containing numerous dead PMN) is a major source of the lysosomal enzymes found in culture fluids from healing lesions. In addition, we provided evidence that lysosomal enzymes are secreted or released from live cells within areas of inflammation.

MATERIALS AND METHODS

Production and organ culture of sulfur mustard (SM) lesions.

Details of these methods have been published in a previous report from our laboratory (13). In brief, 8 ul of sulfur mustard (1% in methylene chloride) was applied topically on various days to the skin of rabbits (after the hair had been removed with electric clippers). The animals were sacrificed with intravenous pentobarbital (65 mg/ml, 1.5 to 2.0 ml), and the lesions (2 hr and 1, 2, 3, 6, and 10 days of age) were excised as 1.0 cm^2 central biopsies. Each explant was then cultured in an atmosphere of 95% O_2-5% CO_2 for 1, 2 and 3 days by gentle rocking in a small Petri dish, containing 2.5 ml of RPMI 1640 (GIBCO Laboratories, Grand Island, NY 14971, Cat. No. 320-1875), penicillin (100 U/ml), streptomycin (100 ug/ml), and additional glutamine (2.0 mM). The culture fluids were centrifuged, and then the supernates were frozen at -70 C until assayed for the enzymes listed below. The enzyme activities seemed stable at -70 C for several months.

Histological and histochemical preparations.

For histological evaluation of epidermal injury and cell infiltration, representative lesions were fixed for 24 hr at 4 C in a modified Karnovsky's formaldehyde-glutaraldehyde fixative (21,35), cold-embedded (25) in glycol methacrylate (GMA), sectioned at 1 to 2 um, and stained with Giemsa (35).

For histochemical evaluation, cold-embedded GMA tissue specimens were sectioned at 6 to 8 um, stained for acid phosphatase by the Burstone method (12,25) and for β-galactosidase by the Pearson method (25,38). The sections were counterstained with hematoxylin (12,25). Staining for acid phosphatase was performed at 4 C for 6 days, whereas staining for β-galactosidase was performed at 37 C for 24 hours. With acid phosphatase, the long incubation period at the cold temperature retains the staining of activated macrophages and activated fibroblasts, but reduces the staining of PMN and connective tissues, which may be nonspecific.

Collection and quantitation of exudate granulocytes (PMN) and macrophages (MN)

Glycogen (Oyster type II glycogen, Cat. No. G8751, Sigma Chemical Co., St. Louis, MO 63178) (200 ml of a 0.1% solution in pyrogen-free, sterile saline (0.9% NaCl)) was injected intraperitoneally into rabbits with a 50 ml syringe with a blunt 18-gauge needle (12). A mixture of ketamine (10 mg/kg) and acepromazine (1 mg/kg) was used to tranquilize each rabbit. PMN were obtained 4 hrs later after injecting 150 ml of heparinized-saline (10 units of heparin per ml of 0.9% saline) intraperitoneally and then draining the fluid from the peritoneal cavity (of the live tranquilized animal) with a blunt 18-gauge needle attached to intravenous tubing.

MN were obtained 4 days after the intraperitoneal injection of 200 ml of 0.5% glycogen in saline (31) into other rabbits. The animals were sacrificed by the intravenous injection of pentobarbital, followed by exsanguination from a femoral artery. Then the peritoneal cavity was opened, and the cells were collected by flushing the cavity with the heparinized saline solution.

The suspensions of PMN or MN exudate cells were centrifuged (for 5 min, at 2000 RPM [900 g]). The cells in the sediment were washed twice with the supplemented RPMI 1640, counted in a hemacytometer, smeared and stained with a combination Wright-Giemsa stain for differential cell counting (37). Their viability was assessed with Trypan blue (see below). Smears were also made and stained histochemically for acid phosphatase (12) and β-galactosidase (38).

The PMN exudates were 95 to 100% PMN (with 0 to 5% MN). The MN exudates were 98 to 100% mononuclear cells, i.e., macrophages containing variable numbers of lymphocytes (see Table 4 footnote). The cells were suspended in supplemented RPMI 1640 at concentrations of 5.0 million per ml. A given exudate contained 100 to 500 million PMN or 30 to 100 million MN.

Cultures of intact PMN and MN

Freshly collected, washed and resuspended PMN and MN (10.0 million in 2.0 ml) were each cultured in small Petri dishes, as described above for the skin explants. After 24 hr, the cells were removed from the culture fluids by centrifugation at 2000 RPM [900g] for 5 min, and the supernates were frozen at -70 C until used for enzyme assays.

With the Trypan blue dye exclusion method (11,32), the PMN were 94±2% viable before culture and 71±5% viable after culture; the MN were 87±3% viable before culture and 79±1% viable afterwards. Following 24 hr in culture, about 50% of the PMN and MN was still suspended in the culture fluids with variable amounts of clumping. The other half was not available for evaluation, probably because of adherence to the culture dishes and cell lysis.

Fibroblast cultures

The two fibroblast cell lines, ATCC CCL-193 (R9AL, F-1517) from rabbit lung and ATCC CRL-1414 (RAB9, F-3769) from rabbit skin, were obtained from the American Type Culture Collection (12301 Parklawn Dr., Rockville, MD 20852), and maintained in RPMI 1640 culture medium containing 10% heat-inactivated calf serum (GIBCO, Cat. No. 230-6170), penicillin (10 u/ml), streptomycin (10 ug/ml), and additional glutamine (2.0 mM).

The fibroblasts were harvested with a trypsin-EDTA (1 X) preparation (GIBCO, Cat. No. 610-5300), washed three times by centrifugation and resuspension in fresh, supplemented serum-free RPMI 1640 medium. Then 3.6 ±0.2 X 10^6 cells were incubated in 1.5 ml of this medium for 24 hr, as described above for the skin explants. Each Petri dish had a sterile coverslip on its bottom. After incubation, the supernatant culture fluid was cleared by centrifugation and stored at -70 C until it was assayed for lysosomal enzymes and LDH. The coverslip was air-dried and stained for acid phosphatase and β-galactosidase.

Before culture the cell suspensions were 75% to 95% viable with the Trypan blue dye-exclusion method (11,32). After culture, the viability of the cells (adherent plus nonadherent) was unchanged, but the total cell count had decreased to about 72% of the original count.

Extracts of PMN, MN and fibroblasts

Freshly harvested, washed PMN, MN and fibroblasts in serum-free, supplemented RPMI 1640 culture medium (5.0 million in 1.0 ml for PMN and MN, 2.4 ±0.1 million in 1.0 ml for fibroblasts) were stored in a frozen state, at -70 C for 1 or more weeks. Then the cells were thawed at 37 C and refrozen in a Dry Ice-acetone bath 4 times, with vigorous pipetting of the thawed suspensions. Following centrifugation, at 2000 RPM for 5 min, the supernates were stored at -70 C until they were assayed for lysosomal enzyme content.

Histochemistry of the PMN, MN and fibroblasts

Representative PMN and MN exudate cells and fibroblast suspensions were centrifuged. The cell pellets were suspended in about 4 to 6 volumes of 15% gelatin (dissolved in water and cooled to 37 C). After solidification of the gelatin, the pellets were fixed in a modified Karnovsky's solution (21,35) and cold-embedded in glycol methacrylate (25). Thick sections (6 to 8 um) were prepared and stained for acid phosphatase and β-galactosidase, as described above for the sections of the SM lesions. The coverslips from the bottom of Petri dishes containing cultured fibroblasts were stained by the same method used to stain the PMN and MN exudate smears (12,38).

Acid phosphatase assays, adapted from Bergmeyer et al. (33)

To each 1.2 X 10.0 cm standardized colorimeter tube (placed in cracked ice), were added 0.50 ml of 0.09 M sodium citrate buffer (pH 4.8), 0.50 ml of 15.2 mM disodium p-nitrophenyl phosphate (phosphatase substrate, Sigma, Cat. No. 104-0) in the same buffer, and 0.20 ml of a given culture fluid. The tubes were incubated for 30 min in a water bath at 37 C, and the reaction was stopped by placing the tubes in water containing cracked ice. Immediately before the optical density (OD) of each tube was read at 410 nm, the tubes were removed from the ice, and 2 ml of 0.10 N NaOH at 23 C was added. The OD's were read in a Bausch and Lomb Spectronic 20 spectrophotometer (Rochester, NY) against a reagent blank containing incubated substrate and culture medium RPMI 1640. The acid phosphatase of both the culture fluids and the PMN- and MN-exudate cells showed substantial activity over a pH range of 3.5 to 6.0.

β-Glucuronidase assay, adapted from Bergmeyer et al. (33)

To each 1.5 X 15.0 cm screw-capped Pyrex glass test tube (placed in cracked ice) were added 0.80 ml of 0.05 M sodium acetate buffer (pH 5.0), 0.10 ml of 45 mM p-nitrophenyl-β-D-glucuronide (Sigma, Cat. No. N-1627) in the same buffer, and 0.10 ml of a given culture fluid. The tubes were incubated for 5 hours in a water bath at 37 C, and the reaction was stopped by placing the tubes in water containing cracked ice. Two ml of 0.10 M NaOH at 23 C was then added to each tube just before its OD was read at 410 nm. These OD's were read in the Beckman DB-G spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) against a reagent blank containing incubated substrate and culture medium, RPMI 1640. β-Glucuronidase of both the culture fluids and the PMN- and MN-exudate cells showed substantial activity over a pH range of 3.5 to 5.5.

β-Galactosidase assay, adapted from Bergmeyer et al. (33)

To each 1.2 X 10.0 cm standardized colorimeter tube (placed in cracked ice) were added 0.8 ml of 0.10 M sodium acetate buffer (pH 4.5), 0.10 ml of 49.8 mM p-nitrophenyl-β-D-galactopyranoside (Sigma, Cat. No. N-1252) in the same buffer, and 0.10 ml of a given culture fluid. The tubes were incubated for 90 min in a water bath at 37 C, and the reaction was stopped by placing the tubes in water containing cracked ice. Two ml of 0.10 N NaOH at 23 C were added to each tube just before its OD was read, at 410 nm, in a Bausch and Lomb Spectronic 20 spectrophotometer. The tubes were read against a reagent blank, containing incubated substrate and culture medium RPMI 1640. The β-galactosidase of both the culture fluids and the PMN- and MN-exudate cells showed substantial activity over a pH range of 3.5 to 4.8.

Standard curves

For the acid phosphatase, β-glucuronidase and β-galactosidase assays, a stock solution of p-nitrophenol (10 umoles per ml) (Sigma, Cat. No. 104-1) was diluted in 0.02 M NaOH just before use, in concentrations ranging from 4.54 to 45.45 nmoles per ml. An almost straight-line relationship with the OD occurred in this concentration range.

SM lesion culture fluids, diluted 1 to 1 with RPMI 1640, usually showed about 50% of the acid phosphatase, β-glucuronidase and β-galactosidase activities of corresponding, undiluted culture fluids.

Note

Although minimal hydrolysis occurred when nitrophenyl substrates for these 3 lysosomal enzymes were incubated at acid pH and 37 C, appreciable hydrolysis occurred when these substrates were placed in 0.10 N NaOH at room temperature. To prevent further hydrolysis from occurring, the reactions were terminated by placing the tubes in a water bath containing cracked ice. Then the NaOH (at 23 C) was added to each tube immediately before the tube was read in a spectrophotometer.

Lysozyme assay, adapted from Sugimoto et al., Vogt et al., and Weissmann et al. (34-36)

Preparation of the agar plates. One gram of agar noble (Difco Laboratories, Detroit, MI 48232, Cat. No. 0142-02) was suspended in 80 ml of 1/15 M sodium-potassium phosphate buffer (pH 7.0) and boiled in a water bath for 20 min. In another flask, 30 mg of Micrococcus lysodeikticus (Micrococcus luteus, spray-dried, Miles Laboratories, Inc., Elkhart, IN 46515, Cat. No. 21-751-1) was suspended in 20 ml of the same buffer and vortexed. The hot agar was cooled to 50 C and mixed with the M. lysodeikticus suspension. This lysozyme substrate (6.0 ml) was poured into plastic (60 X 15 mm) Petri dishes. The dishes were left at room temperature for 15 min to allow their contents to solidify, then placed in the cold room at 4 C, upside down in a stainless steel tray (with 2-inch sides) containing a wet paper towel. Before use (usually within 3 days), they were left covered at room temperature for 5 hrs to dry the surface of the agar. Then 3 to 6 holes were punched in each plate with a 3 mm gel puncher (Bio-Rad, Richmond, CA 94804, Cat No. 170-4028), using a plastic template so that the walls of the punched holes were perpendicular to the bottom of the dish.

Assay procedure. Ten ul of a given culture fluid (or lysozyme standard solution) was placed into each well with a Finnpipette (Labsystems OY 00810, Helsinki 81), and the agar plates were incubated 18 to 24 hr at 37 C. The diameter of the area of lysis was measured with a ruler resting on the bottom of the inverted Petri dish.

Standard solutions of lysozyme from egg white (Sigma, Grade 1, Cat. No. L-6876) in RPMI 1640 culture medium were made in concentrations from 0.5 to 1000 ug/ml. Stock solutions of 1.0 mg/ml in RPMI were kept frozen at -70 C, and used to construct a standard curve each time lesion or cell culture fluids were assayed for lysozyme.

A full analysis of factors influencing this agar plate method has been published (37).

Lactic dehydrogenase (LDH) assay, adapted from Yarborough et al. (38) (see 34)

Reagents. β-NADH (reduced β-nicotinamide adenine dinucleotide) (Sigma Chemical Co., St. Louis, MO 63178, Cat. No. N-8129) (9.34 mg) was dissolved in 1.0 ml of a 1.0% aqueous $NaHCO_3$ solution. Pyruvic acid (Sigma, Cat No. P-2256)(0.63 mM) was made up in 0.05 M potassium phosphate buffer (pH 7.5).

LDH units. The Sigma unit is equal to 1.0 umole of pyruvate reduced to lactic acid per min at pH 7.5 and 37 C. It is equivalent to 0.077 Bergmeyer units (see below).

Standards. *Standard suspensions of* LDH (Sigma, Cat. No. L-1254) dissolved in culture medium (RPMI 1640) were made up in concentrations ranging from 2 to 10 units per ml. An almost straight-line relationship between units and change in optical density per min at 365 nm was observed. Since the standard LDH suspensions seemed to vary from day to day, and since our experiments were internally controlled, we did not run LDH standards with most of our assays.

Procedure. Into a 1.0 cm quartz cuvette at 23 C were added 3.0 ml of the buffered pyruvic acid solution, 50 ul of the β-NADH solution, and then 0.10 ml of culture fluid (or standard LDH suspension). One, 2, 3, 4 and 5 min after mixing, the optical density at 365 nm was read (in a Beckman DB-G grating spectrophotometer) against a distilled water blank. The LDH activity was expressed as the average change in OD per min. This change in OD per min is multiplied by 9265 to convert it to the Bergmeyer units per liter (U/L) (38).

Protein Determination (16)

We used Bradford's Coomassie Blue procedure (39), as modified by Bio-Rad Laboratories (Richmond, CA 94804). This test is based on a shift in absorbance from 465 to 595 nm when Coomassie Brilliant Blue G-250 binds with protein (39). It is as sensitive as the Lowry procedure (40), and easier to use. The Bio-Rad Dye Reagent comes with an instruction manual containing complete directions and background information.

Stability of these enzymes

One 24-hr culture fluid from a 6-day SM lesion, one centrifuged PMN cell lysate and one centrifuged MN cell lysate were each incubated for 24 and 48 hr at 37 C. Then each was assayed for these five enzymes. An average of 50% of the acid phosphatase activity, 45% of the β-glucuronidase activity, and 35% of the β-galactosidase activity remained after 24 hr; and 35%, 25%, and 10%, respectively, remained after 48 hr. Almost all of the lysozyme and LDH activities remained after 48 hr. Thus all of these enzymes seem stable.

RESULTS

PART I

HISTOLOGICAL AND HISTOCHEMICAL STUDIES

General description and histopathology of the sulfur mustard (SM) lesions

The SM lesions in this experiment followed the same course as those in previous experiments (13,17,18). Briefly, by day 1, the epidermal cells had died, and an influx of leukocytes (mainly PMN and basophils) had occurred (Table 1). By day 3, an ulcer covered by a firmly adherent crust, was well established. The crust consisted of dead leukocytes (which were almost all PMN) and dried serum protein, held together with fibrin strands. By day 6, this crust was thicker and beneath it re-epithelialization had begun. By day 10, a layer of thin epidermal cells usually covered the ulcer bed, but the crust usually remained adherent.

Histological identification of macrophages and fibroblasts in SM lesions

Macrophages and fibroblasts cannot always be differentiated in tissue sections, but the shape of the nucleus and the cell itself enabled us to assign most of the cells to each group. A typical macrophage had a large ovoid or kidney-shaped nucleus, a round or ovoid contour, and a distinct border. A typical fibroblast had an elongated nucleus, a spindle-shaped or stellate contour, and an indistinct border. These morphologic criteria enabled us to count the total number of macrophages and fibroblasts in 1.0-cm-long histologic sections of normal skin and 1- and 6-day SM lesions (Table 2).

Histochemical identification of activated macrophages and fibroblasts in SM lesions

In cold-embedded glycol methacrylate tissue sections of the lesions, both macrophages and fibroblasts (Figure 1) were often activated; i.e., they stained + to ++++ for acid phosphatase and β-galactosidase (Table 2). In general, activated macrophages stained darker for β-galactosidase than did activated fibroblasts, but both groups stained equally well for acid phosphatase.

Normal skin contained very few β-galactosidase-positive and acid phosphatase-positive macrophages; in fact, normal skin contained few identifiable macrophages (Table 2). In contrast, normal skin contained many fibroblasts, some of which were activated (Table 2).

Peak inflammatory (1-day) lesions contained large numbers of activated macrophages, i.e., acid phosphatase- and/or β-galactosidase-positive MN (Table 2); and the number of activated fibroblasts was somewhat increased, compared to that in normal skin (Table 2).

Six-day (healing) SM lesions showed a substantial increase in the number of activated fibroblasts (Table 2), but by this time, the number of macrophages had decreased considerably.

About half of the activated macrophages and fibroblasts were distributed throughout the upper third of sections of 1-day and 6-day SM lesions, and about half were distributed throughout the lower two thirds. The few activated cells found in normal skin were in the upper third. The number of darkly stained (+++ to ++++) macrophages and fibroblasts increased progressively from normal skin to 1-day SM lesions to 6-day lesions.

In summary, both activated and nonactivated macrophages were most numerous in peak (1-day) SM lesions (Table 2), whereas the activated fibroblasts were most numerous in healing (6-day) lesions (Table 2).

Histochemical staining of other cells in SM lesions

PMN often stained + to ++ (and sometimes +++) for β-galactosidase, but were usually negative for acid phosphatase. Mast cells stained negatively or palely (+ to ++) for β-galactosidase and did not stain at all for acid phosphatase. Epidermal cells in normal skin and in SM lesions did not stain for either enzyme.

Although the crust sometimes contained scattered areas of acid phosphatase or β-galactosidase activity, it usually did not stain for these enzymes. Both enzymes, however, could be extracted from crusts by the culture fluids (see Part II).

PART II

BIOCHEMICAL STUDIES

Levels of lysosomal enzymes and lactic dehydrogenase (LDH) in first-day culture fluids of developing and healing SM lesions

The levels of acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme, and LDH in first-day culture fluids from SM lesions of various ages became elevated as the lesions healed (Figures 2 to 4). The healing lesions had crusts containing numerous dead phagocytes. Such crusts were a major source of lysosomal enzymes and LDH (see below).

The lysozyme levels in the various culture fluids did not parallel the other lysosomal enzymes, but increased almost exponentially (Figure 3). First-day culture fluids from 1-day lesion explants showed an 18-fold increase in lysozyme when compared to culture fluids from normal skin. Those from 6-day lesions showed a 36-fold increase when compared to culture fluids from 1-day lesions, and a 660-fold increase when compared to culture fluids from normal skin. Both PMN and MN contain substantial amounts of lysozyme (5,22,24). It is actively manufactured and secreted by MN (16, see 26).

Levels of lysosomal enzymes and LDH in second- and third-day culture fluids of SM lesions

The fluids in which the lesions were cultured were changed each day. For normal skin, 1-day SM lesions and 6-day SM lesions, the first-day fluids contained the greatest amount of extravasated serum protein (Table 3). (About 85% of the protein in first-day culture fluids was of serum origin

(17,18).) Second-day culture fluids contained about one third as much protein as first-day fluids, and third-day culture fluids contained about half as much protein as second-day fluids (Table 3). This decrease in protein concentration seemed to be due to the removal of extravasated serum protein when each culture fluid was replaced with fresh culture medium.

Extravasated serum protein was decreased in second- and third-day lesion culture fluids, and serum was low in lysosomal enzymes per mg of protein (Table 3). Therefore, a comparison of the first-, second- and third-day culture fluids should indicate whether lysosomal enzymes were released during culture from the cells in the lesions. In other words, if lysosomal enzymes continue to be released during the second and third days of culture, their levels per mg of protein should increase.

For 1-day SM lesions, this prediction was confirmed (Table 3): The second- and third-day culture fluids showed more lysosomal enzymes per mg of protein than did the corresponding first-day culture fluids. The increase in extracellular lysosomal enzymes (per mg of protein) with time in culture seemed to be partly due to cell death and partly due to the release of these enzymes from still viable cells. The latter possibility is supported by the fact that the lysosomal enzyme levels per unit of LDH (see below) were highest in the third-day culture fluids from these 1-day lesions (Table 3).

This pattern, however, was not always duplicated with culture fluids from 6-day SM lesions. In fact, the enzyme activity (per mg of protein) of first-day culture fluids from 6-day lesions was sometimes higher, sometimes lower, and sometimes the same as that of second- and third-day culture fluids (Table 3). A portion of the lysosomal enzymes in 6-day lesions was released from the dead cells in the crust (see below). One-day lesions had no crust.

Lysosomal enzyme released by cell death

Since LDH is released from cells only when they die, it can be used to differentiate the secretion or leakage of lysosomal enzymes from live cells from the general release of all cell constituents due to cell death and autolysis. In order to obtain the lysosomal enzyme levels per unit of LDH, we divided the levels of lysosomal enzymes in first-, second- and third-day lesion culture fluids by the levels of LDH in the same fluids.

Our results, in general, resembled those in Table 3, which lists "enzyme levels per mg of protein": (a) In 1-day SM lesion explants, the live cells apparently secreted or released lysosomal enzymes. (b) In 6-day lesion explants, the release of such enzymes from live cells was undetectable, probably because their crusts, which contained many dead cells, released such large quantities of lysosomal enzymes and LDH.

Contribution of the enzymes in the crust to the enzymes in the culture fluids of healing SM lesions.

Crusts, present in healing 6- and 10-day SM lesions, are composed of inspissated plasma and serum components (including fibrin) and dead (and some live) PMN and MN (see Table 1 and its footnote). Peak 1-day lesions have no crusts. The question arose whether the increased extracellular lysosomal enzymes and LDH found in healing lesions could be due to enzymes

released from the crusts into the culture fluids. In other words, the tissues of healing lesions might contain the same levels of lysosomal enzymes as the tissues of peak lesions, and the enzyme elevations shown in Figures 2, 3, and 4 might be due to crust enzymes and not tissue enzymes. To address this question, we removed the surface layers of 1.0-cm^2 1-day and 6-day SM lesions with a scalpel, cultured the bases in supplemented RPMI 1640 for 24 hr, and cleared the culture fluids by centrifugation.

The culture fluids from 6-day lesion bases contained higher enzyme levels than the culture fluids from 1-day lesion bases (Table 4). Thus the extracellular fluids within 6-day (healing) lesions indeed contained more lysosomal enzymes than the extracellular fluids within 1-day (peak) lesions. Examination of tissue sections prepared from the 'surfaceless' lesions showed that we had removed about 80 to 90% of epidermis and usually all of the crust, but had retained the hair follicles and most of the corium.

When free crusts from 6-day lesions were cultured for 24 hr in supplemented RPMI 1640, the culture fluids (after centrifugation) contained high lysosomal enzyme levels. These levels were equivalent to those found in culture fluids from intact lesions (Table 4). Thus crusts could be a major source of lysosomal enzymes in culture fluids of healing lesions, especially if the crusts had been made thoroughly wet by these fluids (see Discussion).

In contrast to the other lysosomal enzymes, the level of acid phosphatase was lower in culture fluids from free crusts than in culture fluids from 'crustless' 6-day lesion bases (Table 4). Crusts are composed of many dead PMN (and relatively few MN) (see Table 1), and PMN contain relatively low amounts of acid phosphatase when compared with macrophages and fibroblasts (Table 4).

PART III

LYSOSOMAL ENZYMES OF SERUM, PMN, MN AND FIBROBLASTS

Lysosomal enzymes in serum

Per milligram of protein, serum contains only low levels of acid phosphatase, β-glucuronidase and β-galactosidase; very low levels of lactic dehydrogenase; and almost no lysozyme (Table 3). But serum contains large amounts of protein (Table 3). Thus serum is a major source of acid phosphatase, β-glucuronidase and β-galactosidase in 1-day SM lesion culture fluids in which over 85% of the protein comes from the serum (17,18). (See Discussion and Table 5.)

Lysosomal enzymes released from rabbit PMN, macrophages (MN) and fibroblasts in culture, and released from these cells after lysis by freezing and thawing

PMN and MN peritoneal exudate cells and fibroblast cell lines from the American Type Culture Collection were incubated for 24 hr in serum-free supplemented RPMI 1640. The culture fluids were then cleared by centrifugation and assayed for lysosomal enzymes and LDH. Details of these procedures are presented in Materials and Methods. Suspensions of each cell type were also lysed by freezing and thawing, and cleared by centrifugation.

The cultured cells released only part of their lysosomal enzymes into the culture fluids. Extracts of frozen and thawed PMN and MN contained about twice the total protein, and usually twice the amount of each enzyme, that PMN and MN culture fluids contained (Table 4). Similarly, extracts of frozen and thawed fibroblasts contained five times the total protein, and often five times the amount of each enzyme, that fibroblast culture fluids contained (Table 4).

Certain differences among the four cell types were apparent (Table 4). MN contained and released more acid phosphatase and β-glucuronidase, but less lysozyme, than did PMN. MN also contained and released more β-glucuronidase and lysozyme, but less LDH, than did fibroblasts.

Extracts of the lysed cells of each type and supernatant fluids from cultured viable cells of each type showed roughly the same levels of lysosomal enzymes per mg of protein (with the possible exception of MN lysozyme). These data can be calculated from Table 4. Thus in contrast to the cells in 1-day SM lesions, viable PMN, MN and fibroblasts, cultured in serum-free medium, did not seem to preferentially release lysosomal enzymes over constitutive enzymes, such as LDH.

Histochemical studies on PMN, MN and fibroblasts

An attempt was made to determine whether the activation of the PMN exudate cells, MN exudate cells, and fibroblast cell lines was similar to the activation of these cell types within the SM lesions. Our histochemical stains for acid phosphatase and β-galactosidase were used (12,38). The PMN and MN peritoneal exudate cells, as well as cells from the two rabbit fibroblast lines, were stained both as smears and as 6 um glycol methacrylate-embedded sections of centrifuged cell pellets (resuspended in gelatin) (see Materials and Methods).

Cell smears of the MN exudates and fibroblast cell lines often stained comparably (+, ++ and +++) to each other for acid phosphatase and β-galactosidase. Many PMN stained + and ++ for β-galactosidase, but PMN did not usually stain for acid phosphatase.

Quantitative histochemical comparisons, however, could not be made between these free cells and comparable cells in SM tissue sections: Cell smears and glycol methacrylate sections of the cell pellets required different staining times. In cell smears, more cells stained (and with more intensity) in thick areas than in thin areas. In the glycol methacrylate pellet sections, cells in some areas also stained more darkly than cells in other areas.

Nevertheless, these studies clearly demonstrated (a) that numerous cells, both in the cell suspensions and in the sections of the pellets, showed the lysosomal type of cell activation, and (b) that the PMN, MN and fibroblast cell suspensions were reasonable models for the same cells within the SM lesions.

PART IV

SOURCES OF LYSOSOMAL ENZYMES AND LDH IN THE EXTRACELLULAR FLUIDS OF SM LESIONS

From the cell counts on the histological sections (see Table 1 for PMN and Table 2 for macrophages and fibroblasts), we calculated the total number of PMN, MN and activated fibroblasts in 1.0-cm^2 explants of normal skin, 1-day (peak) lesions and 6-day (healing) lesions. Then we calculated the amount of each enzyme derived from these sources, assuming that the enzyme levels produced by exudate PMN and MN and fibroblast cell lines (listed in Table 4) were representative of the PMN, MN and activated fibroblasts in the lesions. Similarly, we calculated the amount of each lysosomal enzyme that came from the serum in the lesions, assuming that the protein in the culture fluids was 85% serum protein with the same enzyme composition as whole serum (see 18). Our rough estimates are presented in Table 5.

These estimates are based on a number of assumptions, some of which are more certain than others: (a) PMN in peritoneal exudates and PMN in SM lesions contain and release similar amounts of lysosomal enzymes. This assumption seems reasonable, since the lysosomal enzymes of PMN are packaged into granules before the cells leave the bone marrow (see Discussion). (b) MN in peritoneal exudates and MN in SM lesions also contain and release similar amounts of these enzymes. This assumption is less certain. MN are highly adaptive cells that manufacture such enzymes locally in the inflammatory area (10). However, our histochemical studies (see above) suggest that peritoneal MN and lesion MN are activated for lysosomal enzymes to roughly the same degree. (c) The fibroblast cell lines and the activated fibroblasts in SM lesions contain and release similar amounts of these enzymes. Again, our histochemical studies suggest some correlation (see above and see Discussion). (d) Serum (obtained from blood) and the extravasated serum within the lesions contained comparable amounts of these enzymes per milligram of protein. This assumption is quite reasonable, especially because most lysosomal enzymes have molecular weights resembling those of serum albumin and globulin, and because plasma lysosomal enzymes are in the same concentration as serum lysosomal enzymes (see Discussion).

If we accept these assumptions concerning the sources of lysosomal enzymes in the extracellular fluids within these dermal inflammatory lesions, we can conclude that MN make their main contribution to peak lesions, that fibroblasts are the main source of such enzymes in healing lesions, and that PMN are a minor source, and serum a major source, of these enzymes in lesions at all stages of development and healing.

The actual contribution of the crust to the enzyme content of the extracellular fluids of 6-day SM lesions is unknown. Certainly, some enzyme leakage occurs into the bed of the ulcer, but most of the enzymes in the crust remain desiccated and would not participate in healing, unless the crust becomes moist (see Discussion).

DISCUSSION

Developing and healing inflammatory lesions were produced in vivo in the skin of rabbits by the military vesicant sulfur mustard (SM), and 1.0-cm^2 full-thickness explants of these lesions were organ-cultured. The culture fluids extracted lysosomal enzymes from the extracellular fluids of these lesion explants and, when present from their crusts. Low levels of such lysosomal enzymes were found in developing and peak lesions, but high levels were found in healing lesions.

Certain aspects of these studies warrant further discussion:

(a) Extraction of extracellular lysosomal enzymes. The culture fluids should extract the unbound extracellular lysosomal enzymes within the lesions, but the bound extracellular lysosomal enzymes were probably unbound before they became fixed (discussed in reference 17). Therefore, after the 24 hours of culture, representative samples of lysosomal enzymes in the extracellular fluids of the lesions should have been obtained, at least, samples that could be used to compare the levels of these enzymes in developing, peak and healing lesions.

(b) Serum vs. plasma. The clotting of blood to obtain serum may release lysosomal enzymes from platelets and leukocytes. We therefore measured serum and plasma from two blood specimens for acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme, and lactic dehydrogenase. The lysosomal enzyme levels were almost identical in serum and in plasma, except for serum LDH, which was 50 to 100% higher than plasma LDH. Because plasma rapidly clots when it leaks into the connective tissues, serum, not plasma, should be present in the extracellular fluids of the SM lesions.

(c) Granulocytes. PMN contain substantial amounts of lysosomal enzymes (Table 4), but compared to the number of macrophages and fibroblasts, the number of PMN in the inflammatory tissues was small. Thus tissue PMN probably contributed only low amounts of lysosomal enzymes to the culture fluids of SM lesions. The number of PMN in the crusts of healing lesions was much larger. Although most of these crust PMN were dead, their lysosomal enzymes were still active when the crusts became hydrated by the culture fluids (see below).

PMN are relatively short-lived cells (13,29). They can release lysosomal enzymes by regurgitation (15,19) and by cell autolysis. They are thought to have a full complement of lysosomal enzymes in azurophil and specific granules when they leave the bone marrow (see 1) and probably do not synthesize more of these enzymes during their sojourn in the blood and tissues. Thus in contrast to mononuclear phagocytes, the PMN in peritoneal exudates should have the same content of lysosomal enzymes as PMN in the inflammatory lesions.

We did not measure the lysosomal enzymes in basophils, mast cells, or eosinophils because pure cell preparations of these granulocytes are not readily available.

(d) The macrophage-fibroblast group. The lysosomal enzyme contribution from macrophages and fibroblasts in the SM lesions is difficult to

estimate. Both cell types can increase their lysosomal enzyme content tremendously when they become activated (see 10). We used MN exudate cells to represent the macrophages in the tissues, and two rabbit fibroblast cell lines to represent the activated fibroblasts in the tissues. Such a representation is better than none at all, but it is full of uncertainties: Macrophages and fibroblasts in tissues (a natural environment) may or may not produce and release the same amounts of lysosomal enzymes as do free MN exudate cells or fibroblast cell lines in culture.

We stained histochemically the macrophages in both SM lesions and cell suspensions. Since the percentages of MN that stained positively for acid phosphatase and β-galactosidase were similar in both preparations, we considered MN exudate cells to be fairly representative (see Results, Part III).

However, the two rabbit fibroblast cell lines may or may not be representative of the fibroblasts staining positively for acid phosphatase and β-galactosidase in histological sections of the SM lesions. Histochemical preparations of these cell line suspensions sometimes, but not always, stained well for these enzymes. Because of the large numbers of fibroblasts in the SM lesions, we equated the lysosomal enzymes produced by the two cell lines with the enzymes produced by the activated fibroblasts in the lesions, rather than with those produced by the total fibroblasts in the lesions (see Appendix). Since the two cell lines were actively multiplying before they were incubated overnight in serum-free medium, one would expect them to be more comparable to activated than to dormant fibroblasts.

Histochemistry is only roughly quantitative and measures only the enzymes stored in the cells. But as far as lysosomal enzymes are concerned, the enzymes stored in the cells should give some indication of the enzymes released from the cells, especially as the cells die and lyse (see Table 4). (The release of LDH into the organ-culture fluids was rather similar to the lysosomal enzyme release.)

(e) Epidermal cells. Epidermal cells are not an integral part of the inflammatory process within the tissues, although they probably initiate it. The epidermis is killed by sulfur mustard, desquamates, and then regenerates from uninjured epithelial cells, both in the hair follicles and in the peripheral epidermis. In a 1.0-cm^2 SM lesion explant, the number of epithelial cells (which varies with skin thickness and the density of the hair follicles) is roughly the same as the number of activated fibroblasts in the dermis (unpublished observations).

Epidermal cells probably release only very small amounts of lysosomal enzymes into the lesion culture fluids. Normal, dying and regenerating epidermal cells did not stain histochemically for acid phosphatase in tissue sections where positively stained macrophages and fibroblasts were found. In the hair follicles, acid phosphatase-positive keratinizing cells were occasionally present, but they were just as frequent in normal skin as in SM lesions.

Since primary cultures of newborn mouse epidermal cells were available, we decided to confirm biochemically these histochemical results. Frozen and thawed extracts of epidermal cells (5 million/ml) had, respectively, 1, 4, 7, and 2% of the acid phosphatase, β-glucuronidase, β-galactosidase and lactic

dehydrogenase activities of extracts of the fibroblasts (2.4 million/ml) listed in Table 4 (unpublished observations). No lysozyme activity was found in either cell type.

Culture fluids from the same epidermal cells (incubated in serum-free medium for 24 hr) had, respectively, 1, 2, 10, and 2% of the enzyme activities of culture fluids of the fibroblasts listed in Table 4. Again, no lysozyme activity was found. Thus both biochemical and histochemical criteria support the conclusion that epidermal cells make only a small contribution to the high levels of the lysosomal enzymes in the culture fluids of healing (6-day) lesions.

With microanalytical techniques, Im and Hoopes studied healing wounds produced surgically in guinea pigs (reviewed in 20a). They found that acid phosphatase was increased in keratinizing epithelium and that β-glucuronidase was increased in basal and regenerating epithelium. These two enzymes were present in roughly the same concentration (per mg of protein) in the extracts of guinea pig epithelium as they were in our extracts of mouse epidermal cells (see above).

(f) Crusts. The rabbits showed considerable variability in crust formation. Some developed good, distinct crusts, whereas others developed poor, indistinct crusts. This variability was caused by variations in hair follicle density, in skin thickness, and in the amount of injury.

To our surprise, substantial amounts of lysosomal enzymes and LDH could be extracted by culture fluids from crusts removed from healing lesions (Table 4). Peak lesions did not have crusts. The process of removing crusts by cutting with a scalpel blade squeezed and partly macerated both the crust and the underlying tissues. For this reason, and because of the greater surface area exposed, the culture fluids extracted larger amounts of each enzyme from the two parts of the same lesion (crust plus base) than culture fluids extracted from the intact lesion. Thus the actual contribution of the 6-day lesion crust to the enzymes released into the culture fluids cannot be estimated satisfactorily. Nevertheless, assigning a value of 33% to the contribution of the crust (rather than assigning no value at all) enabled us to estimate the contributions from other sources more accurately.

In vivo, the crusts probably make little contribution to the extracellular enzyme content of SM lesions. Crusts are strongly adherent and somewhat waterproof, and most of the crust usually remains dry until it falls off. Therefore, larger quantities of crust enzymes would be released into the culture fluids than would be released into the lesions. Nevertheless, the bases of crusts are wet with tissue fluids, so that crust enzymes could influence the adjacent tissues.

The fact that crusts are such a rich source of enzymes and probably of other mediators of inflammation increases their importance in the healing process. They not only act as a mechanical barrier to protect the wound but also may stimulate the regrowth of epithelium. In addition, the microbicidins in the crust (e.g., lactoferrin and lysosomal enzymes) should help control infection.

APPENDIX

Calculations and assumptions used to estimate the sources of enzymes

Full-thickness 1.0-cm^2 explants from normal skin, a 1-day SM lesion and a 6-day SM lesion contained, on the average, 16,000, 880,000 and 290,000 PMN; 100,000, 3,700,000 and 500,000 macrophages (MN); and 1,000,000, 1,900,000 and 7,400,000 activated fibroblasts, respectively. To obtain these figures, we counted microscopically the number of each of these cell types in the 2.0 um thick tissue section of a given lesion (Tables 1 and 2) and multiplied this number by 1000. [Since each cell is about 10 um in diameter, the 1.0-cm^2 lesion biopsy would be about 1000 cells across. PMN are 10 to 15 um in diameter (23), MN are 12 to 20 um (23), and fibroblasts resemble MN in size. Therefore, the use of 10 um for each cell type is a bit low, and the factor 1000 is a bit high . . but quite satisfactory for a rough estimate.] The amount of each enzyme released by each cell type into a lesion's culture fluid was then calculated from the averages in Table 4, and expressed as a percentage of the total enzyme in the culture fluid (listed in Table 5). First-day culture fluids were used for all of these enzyme assays.

For example, MN provided 33.0% of the total acid phosphatase in first-day culture fluids from 1-day SM lesions (Table 5). These lesions contained 3700 MN in the tissue section (Table 2) X 1000 = 3,700,000 MN per 1.0-cm^2 lesion explant. Five million MN released an average of 100 nmoles of p-nitrophenol (per ml of culture fluid) from the acid phosphatase substrate (Table 4); *therefore, 3,700,000 MN would release 100 X (3.7/5.0) = 74 nmoles.* Culture fluids from 1-day lesions contained 226 nmoles (43 nmoles X 2.10 mg protein/ml X 2.5 ml) (see Table 3). The contribution of MN to the acid phosphatase content of the lesion culture fluids was, therefore, 74 nmoles *divided by 226 nmoles = 33.0% (see Table 5).*

Serum, in appropriate dilutions, was also assayed for these enzymes (Table 3): The amount of serum enzyme in first-day culture fluids would be the amount of enzyme per mg of protein (Table 3), times the protein concentration (mg/ml) in the lesion culture fluids (Table 3) times 2.5 ml times 0.85. (For these calculations, we assumed that 85% of the protein in the culture fluids was serum protein of identical composition (see 18).) The means were used for these calculations.

No allowance was made for the circular nature of the lesions, since their diameters usually exceeded the central 1.0-cm^2 biopsy removed from them. Also, no allowance was made for the differences (if any) between exudates and tissue sections with regard to the proportions of PMN and MN that were activated.

Since the number of fibroblasts in the skin is so high, we used only activated fibroblasts (detected histochemically) in our calculations (see Discussion). Activated fibroblasts (not the dormant fibroblasts) were probably the main source of extracellular lysosomal enzymes in the lesions. The sum of the acid phosphatase-positive and β-galactosidase-positive cells in Table 2 was used to represent the number of activated fibroblasts in Table 5. Even though a given cell may stain for both enzymes, many fibro-

blasts would probably stain for only one or the other enzyme, just as activated MN do (33).

Comparison of PMN and MN lysosomal enzymes.

We attempted, without success, to find characteristics of PMN lysosomal enzymes that were different from MN lysosomal enzymes, in order to use this information to determine the contribution of each cell type to the lysosomal enzyme content of SM lesion culture fluids. Extracts of frozen and thawed PMN exudate cells, extracts of frozen and thawed MN exudate cells, and culture fluids from 6-day SM skin lesions showed the same pH optima, when assayed for acid phosphatase, β-glucuronidase and β-galactosidase (with the possible exception of acid phosphatase in PMN exudate cells). The inhibitor spectra of the three preparations were also similar when sodium molybdate (5.0 mM) was used to inhibit acid phosphatase, and 1.0% d(+)galactose to inhibit β-galactosidase (see 38). Finally, the stability of the enzymes in these three preparations was rather similar when the preparations were incubated at 37 C for 24 and 48 hr (see Materials and Methods). These comparisons were of a pilot nature. We include them here because they are pertinent to this report.

Table 1

Number and types of cells in tissue sections (1.0 cm long), cut from a central plane of SM lesions of various ages

Age of SM lesions	PMN	Basophil-mast cell group	Mononuclear-fibroblast group	PMN in crust	Dead epithelial cells
Normal skin	16 ± 4	110 ± 30	10,700 ±300	0	0
2 hr	76 ± 31	120 ± 10	10,700 ±500	0 (0-200)	0
1 day	880 ±140	550 ±260	12,100 ±400	0 (0-500)	+++
2 days	310 ±170	570 ±220	15,200 ±2200	1900 (0-20,000)	+++ (++ - ++++)
3 days	270 ± 90	590 ±180	13,600 ±1200	6300 (1100-13,000)	+++ (++ - ++++)
6 days	290 ±150	370 ±100	13,700 ±1400	8400 (1000-16,000)	+++
10 days	160 ± 40	620 ±110	14,700 ±1700	6000 (3500-28,000)	+ (0 - ++)

Footnotes for Table 1:

The cell counts presented here are from a different set of six rabbits (Experiment II) than the cell counts presented in the first chapter of this report (Experiment I) (13). The counts were also made by different investigators. A thorough evaluation of the methods used by each investigator indicated (a) that the ocular grid method used for counting the cells reported in this table was more precise than the high power field method used in Chapter 1, and (b) that the SM lesions of the rabbits used in both series of experiments were not markedly different in PMN, basophil, and mononuclear-fibroblast content. The lesions were embedded in glycol methacrylate, cut at 2 um. and stained with Giemsa (13,35). The means and their standard errors are presented.

For this table, the following criteria were used:

(a) The PMN were identified by their granules in the superficial tissues and in the deep tissues. This group included a small percentage of eosinophils. (Both cell types are eosinophilic in the rabbit.)

(b) The basophils and mast cells were also counted by the ocular grid method. These cells were grouped together in this table because they were identified by their red-purple granules (whether or not their nuclei were visible). Without seeing the nucleus, one cannot always tell to which group the red-purple granules belong.

(c) In counting the mononuclear-fibroblast group, we included in this table all connective tissue cells, macrophages, fibroblasts, endothelial cells, pericytes, lymphocytes and many of the round cells surrounding the hair follicles. Our previous counts (13) included only macrophages and the few fibroblasts and large lymphocytes that could not be distinguished from them. Both counting methods provide useful information on the cellular content of the lesions. The mononuclear-fibroblast group is very difficult to quantitate, and each method provides only rough estimates. (Also see Table 2.)

(d) The number of PMN in the crust was quite variable in both this and our previous experiment (13). No meaningful difference between them could be detected. We estimate that about 85% of the cells in the crusts were PMN and 15% were MN. These cells were in various stages of disintegration, and most of the PMN had lost their granules. Therefore, no satisfactory differential cell count could be made. At the base of the crust, small numbers of apparently live PMN and MN could often be found.

Table 2

Number of activated macrophages and fibroblasts in peak (1-day) and healing (6-day) SM lesions, recognized histochemically by staining for acid phosphatase and β-lactosidase

	Number of macrophages			Number of fibroblasts		
Source	Total	Acid P'ase positive (% of total)	β-gal. positive (% of total)	Total	Acid P'ase positive (% of total)	β-gal. positive (% of total)
Normal skin	100 ± 50	30 ± 10 (30%)	30 ± 10 (30%)	10,000 ±1000	800 ±100 (8%)	200 ± 50 (2%)
1-day SM lesion	3700 ±500	1400 ±200 (38%)	2400 ±300 (65%)	10,000 ±1000	1200 ±200 (12%)	700 ±100 (7%)
6-day SM lesion	500 ±100	150 ± 60 (30%)	400 ±100 (80%)	16,000 ±1000	4300 ±600 (27%)	3100 ±400 (19%)

Footnote for Table 2:

For this table, cells of the mononuclear-fibroblast group were assigned to the macrophage or the fibroblast group according to the criteria described in Part I of "Results," and the cells staining + to ++++ for acid phosphatase and β-galactosidase were counted. The figures listed represent the number of each cell type in the entire full-thickness central tissue section of the skin lesions (1.0 cm long and 6 um thick). We estimated that the use of 6 um (instead of 2 um) sections should increase the cell counts only about 3 to 5%: The diameter of activated macrophages and fibroblasts was several times the thickness of the tissue sections, and the spaces between these cells averaged several times the cell diameter. The six rabbits used for this table were different animals from those used for Table 1.

Table 3

Lysosomal enzymes and lactic dehydrogenase in organ-culture fluids of normal skin, 1- and 6-day SM lesions, and serum, expressed as activity per mg of protein

Source		Acid-phosphatase	β-glucuronidase	β-galactosidase	Lysozyme	Lactic dehydrogenase	Protein concentration
		nanomoles of p-nitrophenol per mg protein			ug/mg protein	mU/mg protein	mg/ml
Culture Fluids of 1st day	Normal skin	117 ±23	68 ± 9	73 ± 9	2.1 ±0.6	119 ±22	0.53 ±0.04 (0.52 ±0.04)
	1-day SM lesions	43 ± 9	23 ± 2	17 ± 3	5.5 ±0.9	34 ± 9	2.10 ±0.10 (1.72 ±0.13)
	6-day SM lesions	158 ±24	380 ±150	120 ±26	240 ±120	156 ±24	1.33 ±0.21 (1.44 ±0.09)
Culture Fluids of 2nd day	Normal skin	175 ±36	123 ± 16	166 ±16	6 ± 1	168 ±35	0.19 ±0.03 (0.16 ±0.02)
	1-day SM lesions	70 ±22	53 ± 7	41 ± 4	10 ± 2	68 ±16	0.69 ±0.03 (0.57 ±0.03)
	6-day SM lesions	260 ±40	174 ± 39	109 ±15	78 ± 33	98 ±29	0.43 ±0.06 (0.56 ±0.03)
Culture Fluids of 3rd day	Normal skin	370 ±50	350 ± 60	270 ±50	33 ± 8	200 ±55	0.09 ±0.01 (0.07 ±0.01)
	1-day SM lesions	161 ±28	166 ± 13	95 ±11	31 ± 5	80 ±34	0.33 ±0.04 (0.25 ±0.01)
	6-day SM lesions	440 ±50	220 ± 10	104 ±13	72 ± 16	119 ±38	0.25 ±0.04 (0.27 ±0.03)
Serum		47 ± 8	27 ± 5	9 ± 1	0.15 ±0.02	5 ± 2	51.0 ±0.80

Footnotes for Table 3:

The 1.0 cm^2 skin explants were cultured in 2.5 ml of supplemented RPMI 1640. The data presented are pooled from two experiments of six rabbits each: The data for lysozyme and lactic dehydrogenase (LDH) came from Experiment I, described in the first report of this series (13); the data for acid phosphatase, β-glucuronidase, β-galactosidase, and protein concentration came from Experiment II, described in this report. The protein concentrations in the culture fluids of Experiment I are listed in parentheses. The two experiments were identical: The culture fluids from each contained almost the same mean protein concentrations, and the histological sections from each contained about the same mean number and types of cells (when representative histological sections were counted by the same method). An additional experiment, containing three rabbits, confirmed the trends presented in this table.

The means and their standard errors are listed. When the one-tailed, paired-sample Student's t test was used, the 6-day enzyme levels per mg of protein were significantly higher than the 1-day enzyme levels per mg of protein: P values were 0.004 to 0.05 on the first day of culture, and often on the other 2 days of culture. Similar results were obtained for lysosomal enzyme levels per unit of LDH, but are not presented. Their P values were similar.

Two-hr, 2-day, 3-day, and 10-day SM lesions were also evaluated in this manner, but not included here to save space. The enzyme levels relative to the 1-day and 6-day enzyme levels (shown here) can be approximated from the data in Figures 1, 2 and 3. For 1-day SM lesions, with 5 enzymes listed, first-day culture fluids had lower levels than second-day fluids: P values were NS, 0.004, 0.003, 0.05 and NS. (NS = not significant.) Second-day fluids had lower levels than third-day fluids: P values were 0.03, <0.001, 0.003, 0.005 and NS. First-day fluids vs. third-day fluids had P values of <0.01, <0.001, <0.001, 0.005 and NS.

Sera: The sera came from the six rabbits in Experiment II. Their pelts contained SM lesions of various ages at the time of sacrifice, when the sera were obtained.

Table 4

Lysosomal enzymes and lactic dehydrogenase from rabbit SM lesion crusts, and from PMN, macrophages (MN), fibroblasts and serum, expressed as activity per ml

Source	Acid-phosphatase	β-glucuronidase	β-galactosidase	Lysozyme	Lactic dehydrogenase	Protein concentration
	nanomoles of p-nitrophenol per ml			ug/ml	mU/ml	mg/ml
CFs from intact 6-day SM lesions	83 ± 3	500 ±120	210 ± 90	1400 ±670	30 ±12	0.86 ±0.03
CFs from crusts of 6-day SM lesions	65 ±10	690 ± 70	310 ± 50	1800 ±540	116 ±44	0.53 ±0.06
CFs from 6-day SM lesions with crusts removed	122 ±14	141 ± 15	53 ± 8	480 ±100	32 ± 7	0.78 ±0.04
CFs from 1-day SM lesions with epidermis removed	71 ±12	46 ± 7	18 ± 5	195 ± 52	37 ± 8	1.20 ±0.12
PMN extracts	25 ± 5	290 ± 30	126 ± 10	33 ± 13	17 ± 2	0.14 ±0.01
PMN CFs	14 ± 3	79 ± 5	59 ± 2	10 ± 3	13 ± 2	0.06 ±0.01
MN extracts	119 ±20	1820 ±140	320 ± 50	8 ± 1	65 ± 5	0.35 ±0.08
MN CFs	100 ± 8	770 ±130	118 ± 15	13 ± 3	47 ± 7	0.19 ±0.06
Fibroblast extracts	160 ±30	200 ± 30	150 ± 10	0	220 ±30	0.30 ±0.02
Fibroblast CFs	26 ± 4	37 ± 5	38 ± 5	0	100 ±50	0.06 ±0.01
Serum (1:29)	83 ±14	48 ± 9	16 ± 2	0.26±0.04	9 ± 4	1.76 ±0.28
(1:45)	53 ± 9	31 ± 6	10 ± 1	0.17±0.02	6 ± 2	1.13 ±0.18

Footnotes for Table 4:

Culture fluids (CFs) from crusts and from 'surfaceless' SM lesions: Crusts from three or four 6-day SM lesions on each of 4 rabbits were removed, cultured separately for 24 hr in 2.5 ml of RPMI 1640, and centrifuged. Then the supernates were assayed for these enzymes. For comparison, intact 6-day lesions, the 'crustless' bases of these lesions, and the 'surfaceless' 1-day lesions were also cultured. With the five enzymes listed, the P values for 6-day lesion crusts vs. 6-day bases were, 0.003, <0.001, <0.001, 0.008, and 0.03. For 6-day bases vs 1-day bases, they were 0.013, <0.001, 0.003, 0.024, and 'not significant.' The one-tailed Student's t test was used. See Part II and Discussion for details on this crust experiment.

PMN and MN extracts: Ten million PMN or MN peritoneal exudate cells (in 2.0 ml) were frozen and thawed 4 times and centrifuged. The supernates were assayed for these enzymes. See Materials and Methods.

PMN and MN culture fluids (CFs): Ten million PMN or MN exudate cells were cultured in 2.0 ml of RPMI 1640 for 24 hr 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% PMN in the MN exudates. The MN group contained variable numbers of small mononuclears that resembled small lymphocytes. The lysates and culture fluids from MN exudates containing about 30% "lymphocytes" and those from MN exudates containing only about 2% "lymphocytes" showed no differences in acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme, LDH, or protein contents. Nor were any differences found (before and after culture) in cell counts and viabilities with the Trypan blue dye-exclusion tests. Nonadherent mononuclear cells, resembling small lymphocytes, were still present after 1 day in culture.

Fibroblasts: The two fibroblast cell lines were freed from the culture flasks with trypsin (see Materials and Methods), washed 3 times by centrifugation and cultured 24 hr 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 viabilities of these cells before and after culture are presented in Materials and Methods.

The means and standard errors are listed. Four rabbits were used for MN; 4 other rabbits were used for PMN. Three different cell suspensions were obtained from each of the fibroblast cell lines.

Sera: Serum dilutions of 1:29 and 1:45 approximate the amount of serum protein in first-day culture fluids from 1-day and 6-day SM lesions, respectively. We calculated these dilutions from the data in Table 3 by assuming that 85% of the protein in the culture fluids was serum protein (see 18). The sera from six rabbits of Experiment II were assayed.

Table 5

Rough estimates of the percentage of lysosomal enzymes (and LDH) from serum, PMN, macrophages (MN), fibroblasts and crusts in culture fluids (CF) of 1.0-cm^2 SM lesions of various ages

Source	Acid phosphatase	β-glucuron-idase	β-galacto-sidase	Lysozyme (ug)	Lactic dehydrogenase (mU)
	(nanomoles p-nitrophenol)				
Normal skin: enzyme activity in 2.5 ml CF (100%)	155	90	97	1.3	74
Serum	34.0 %	34.0%	10.5%	12.8%	7.4%
PMN	0.03	0.3	0.2	2.5	0.1
MN	1.3	17.0	2.4	20.0	1.3
Fibroblasts	7.0	17.0	16.0	0	56.0
Remainder	58.0 %	32.0%	71.0%	65.0%	35.0%
1-day lesions: enzyme activity in 2.5 ml CF (100%)	226	121	89	24	146
Serum	93.0%	100.0%	45.0%	2.3%	12.5%
PMN	1.0	11.0	12.0	7.3	2.0
MN	33.0	470.0	98.0	40.0	24 0
Fibroblasts	0.1	24.0	34.0	0	54.0
Remainder	0 %	0 %	0 %	50.0%	8.0%
6-day lesions: enzyme activity in 2.5 ml CF (100%)	530	1260	400	860	560
Serum	25.0%	6.1%	6.4%	0.1%	2.7%
PMN	0.2	0.4	0.9	0.1	0.1
MN	1.9	6.1	3.0	0.2	0.8
Fibroblasts	15.0	9.1	29.0	0	55.0
Crusts	33.0	33.0	33.0	33.0	33.0
Remainder	25.0%	45.0%	28.0%	67.0%	8.0%

Footnotes to Table 5

This table presents a very rough estimate of the sources of the lysosomal enzymes and LDH in first-day culture fluids of the 1.0 cm^2 normal skin, 1-day SM lesions and 6-day SM lesions. Listed as 100% is the enzyme activity in the 2.5 ml of culture fluid that bathed each lesion: specifically, the enzyme activity per mg protein times the protein concentration (mg/ml) times 2.5 ml (see Table 3). Also listed are the percentages of a given enzyme in the lesion culture fluids attributable to the serum PMN, MN and fibroblasts within the lesion.

To prepare this table, we assumed that the amount of these enzymes released into the organ-culture fluids by the PMN, MN and activated fibroblasts within the SM lesion was the same as the amount released by these cell types when they were free in cell culture. The serum protein in the lesion culture fluids was considered to be 85% of the protein concentration present (see 18). The total amount of enzymes released into the culture fluids from the crusts of 6-day lesions was arbitrarily assumed to be one third of the total amount (see Discussion).

That the sum of the component percentages was sometimes over 100% is not surprising, considering the rough nature of our estimates and the assumptions made.

Our methods of preparing this table are presented in the Appendix.

Figure 1. Activated fibroblasts (+++ to ++++) (located between collagen fibers in the corium) from a darkly stained area in a healing (6-day) dermal SM lesion. The majority of these cells stained bright red, indicating acid phosphatase activity. (In this black and white photograph, red is depicted as a dark intensity.) Edema is no longer present. A small blood vessel containing erythrocytes appears in the lower part of the photograph. Normal skin had fewer acid phosphatase-positive fibroblasts (see Table 2), which, on the average, stained more lightly than those in SM lesions. (A 6 um glycol methacrylate-embedded tissue section, stained with naphthol AS-BI phosphate and fast red-violet LB, counterstained with hematoxylin. X 650.)

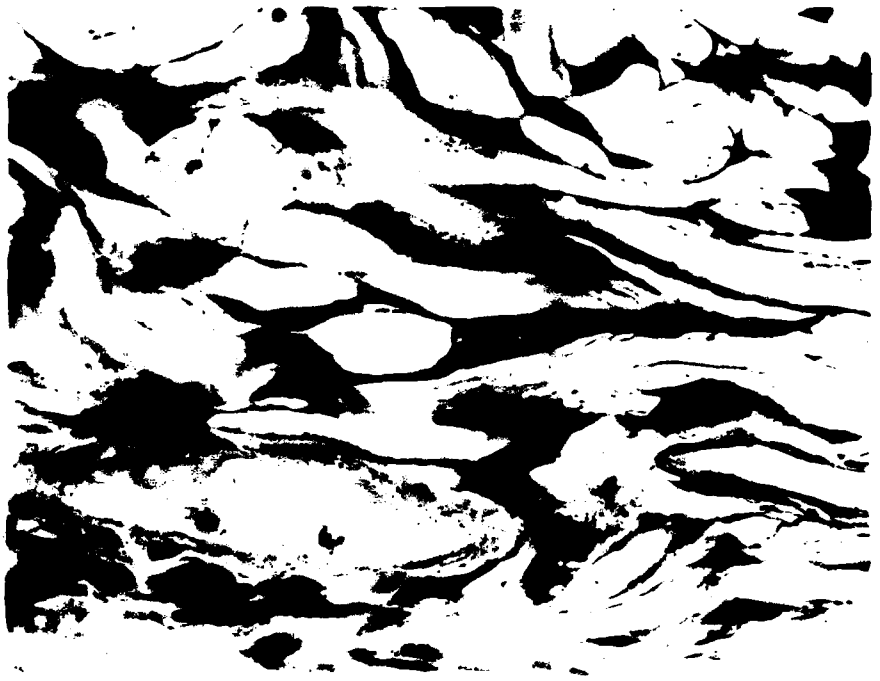


Figure 2. β-glucuronidase, β-galactosidase and acid phosphatase activities in first-day organ-culture fluids from 1.0 cm^2 dermal sulfur mustard lesions of various ages, cultured in 2.5 ml of supplemented RPMI 1640. The means and their standard errors are depicted. (See Materials and Methods for details on these assay procedures.)

The β-glucuronidase activities in the culture fluids of 10-day lesions were significantly different from those of normal skin, 2-hr, 1-day and 2-day lesions. (P values were 0.04, 0.03, 0.04, and 0.04, respectively.)

The β-galactosidase activities in culture fluids of 6-day lesions were significantly different from those of normal skin, 2-hr, 1-day, and 2-day lesions. (P values were 0.03, 0.03, 0.04, and 0.05 respectively.) Those from 10-day lesions were significantly different from those of 2-hr, 1-day, and 2-day lesions. (P values were 0.02, 0.02, and 0.03, respectively.)

The acid phosphatase activities in culture fluids of 6- and 10-day SM lesions were significantly different from those of normal skin, 2-hr, 1-day, and 2-day lesions. (P values were ≤ 0.02, ≤ 0.01, ≤ 0.02, and ≤ 0.03, respectively.) The one-tailed paired-sample Student's t test was used for all.

The data from second- and third-day culture fluids were not included in this figure, for the sake of simplicity. At 6 and 10 days, the acid phosphatase levels in these fluids were about 50% of those found in first-day culture fluids, the β-glucuronidase levels were about 10%, and β-galactosidase levels were about 20%. The data for 1- and 6-day lesions (which can be calculated from Table 3) resemble, in general, those shown for LDH in Figure 4.

Figure 2

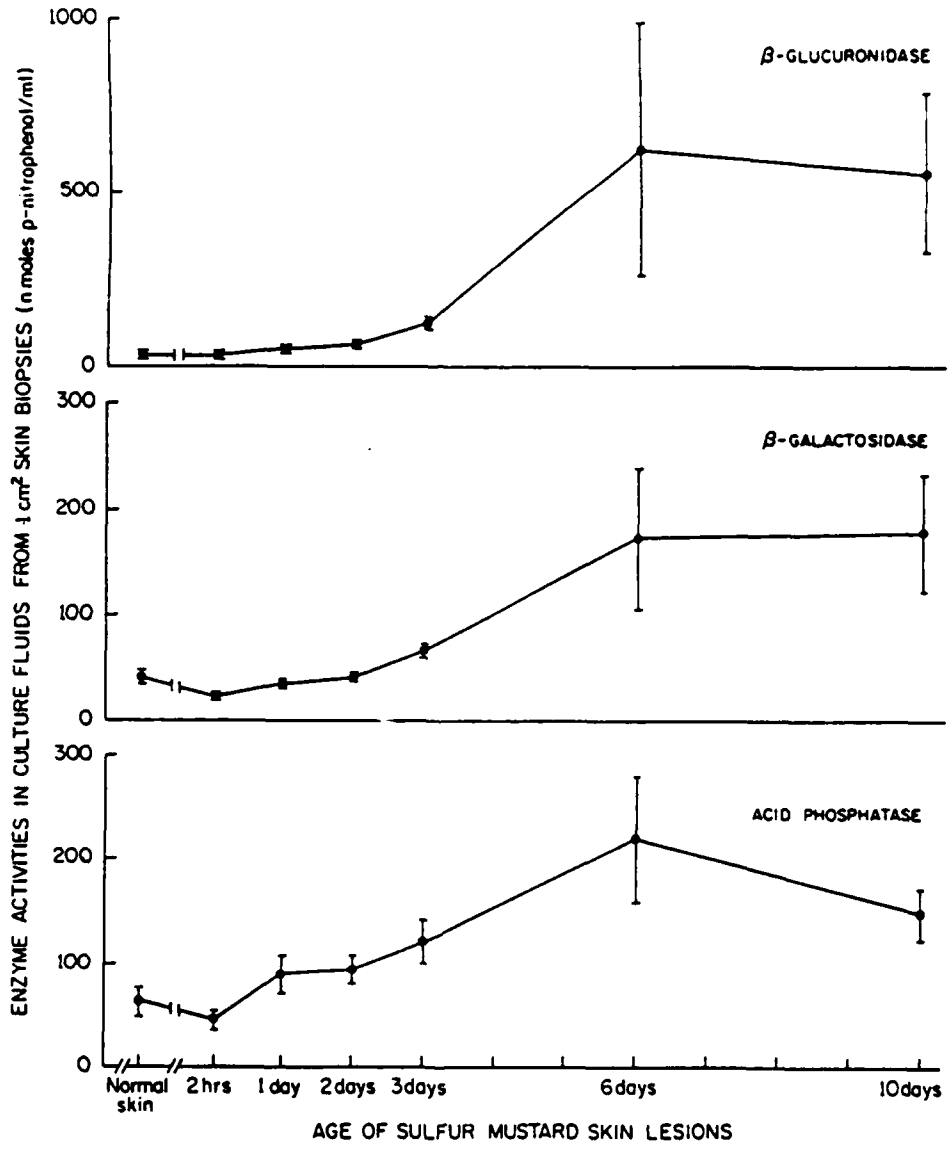

ENZYME ACTIVITIES IN CULTURE FLUIDS FROM 1 cm² SKIN BIOPSIES (n moles p-nitrophenol/ml)
β-GLUCURONIDASE
1000
500
0
β-GALACTOSIDASE
300
200
100
0
ACID PHOSPHATASE
300
200
100
0
Normal skin
2 hrs
1 day
2 days
3 days
6 days
10 days
AGE OF SULFUR MUSTARD SKIN LESIONS

Figure 3. Lysozyme activity in culture fluids from 1.0-cm^2 dermal sulfur mustard lesions of various ages (egg-white lysozyme equivalents). The means and their standard errors are depicted. The diameters of cleared areas of lysis produced by the culture fluids in agar plates containing *M. lysodeikticus* were compared to those of similar areas produced by egg white lysozyme. Note that the lysozyme activity in this graph is expressed on a logarithmic scale so that the lysozyme levels in the second- and third-day culture fluids appear deceptively high.

The lysozyme activity of whole (undiluted) serum from these rabbits is also depicted herein. Since first-day culture fluids from 1-day SM lesions contain about 3% whole serum, the lysozyme in these fluids must be of cellular, not serum, origin (see Table 4).

The lysozyme activities in first-day culture fluids from 10-day lesions were significantly higher than those of normal skin and from 2-hour, 1-day, 2-day, and 3-day SM lesions ($P \leq 0.01$). Because of the large standard errors, similar comparisons for 6-day lesions had *P* values of 0.06 to 0.07. The one-tailed, paired-sample Student's *t* test was used.

Figure 3

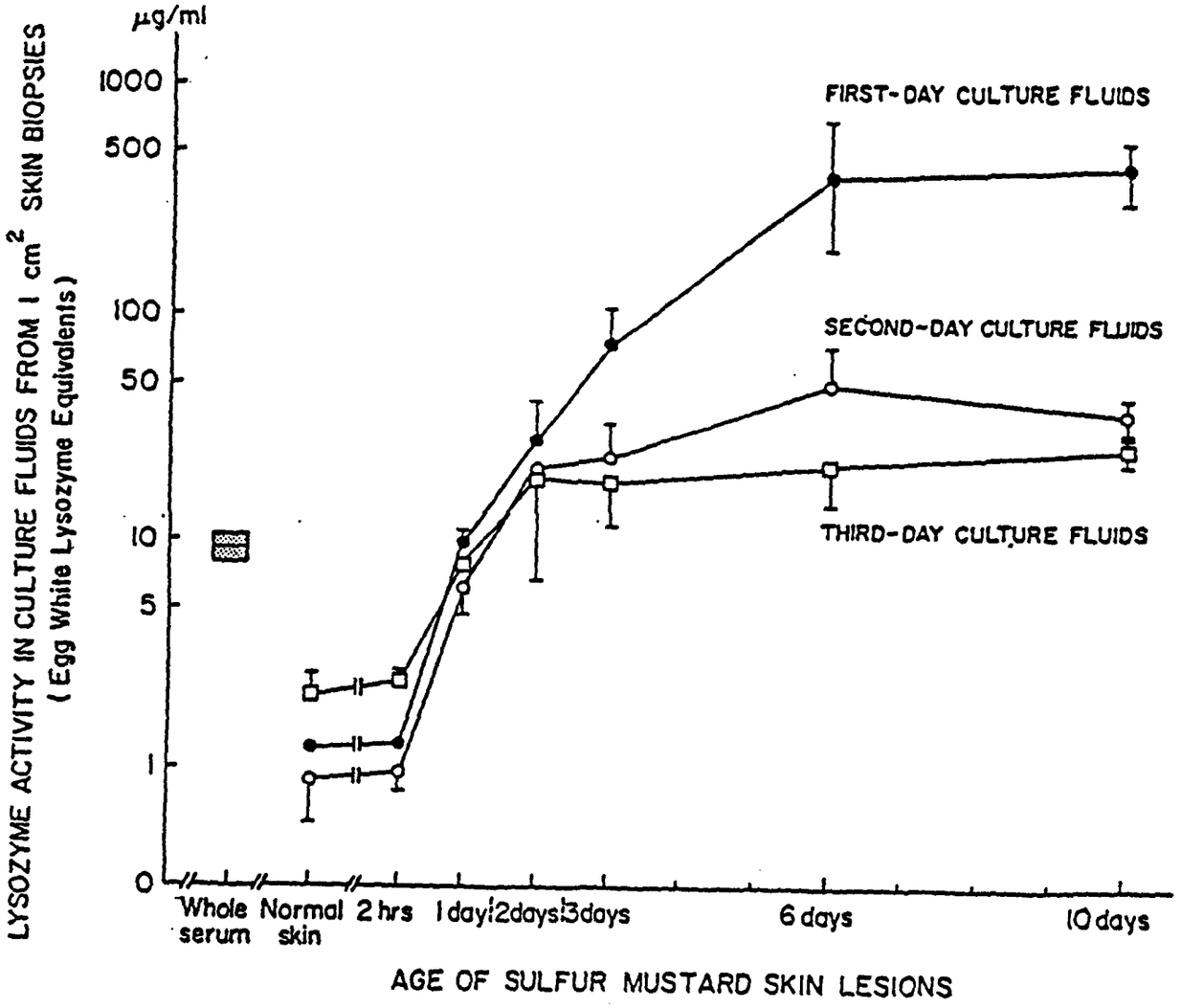
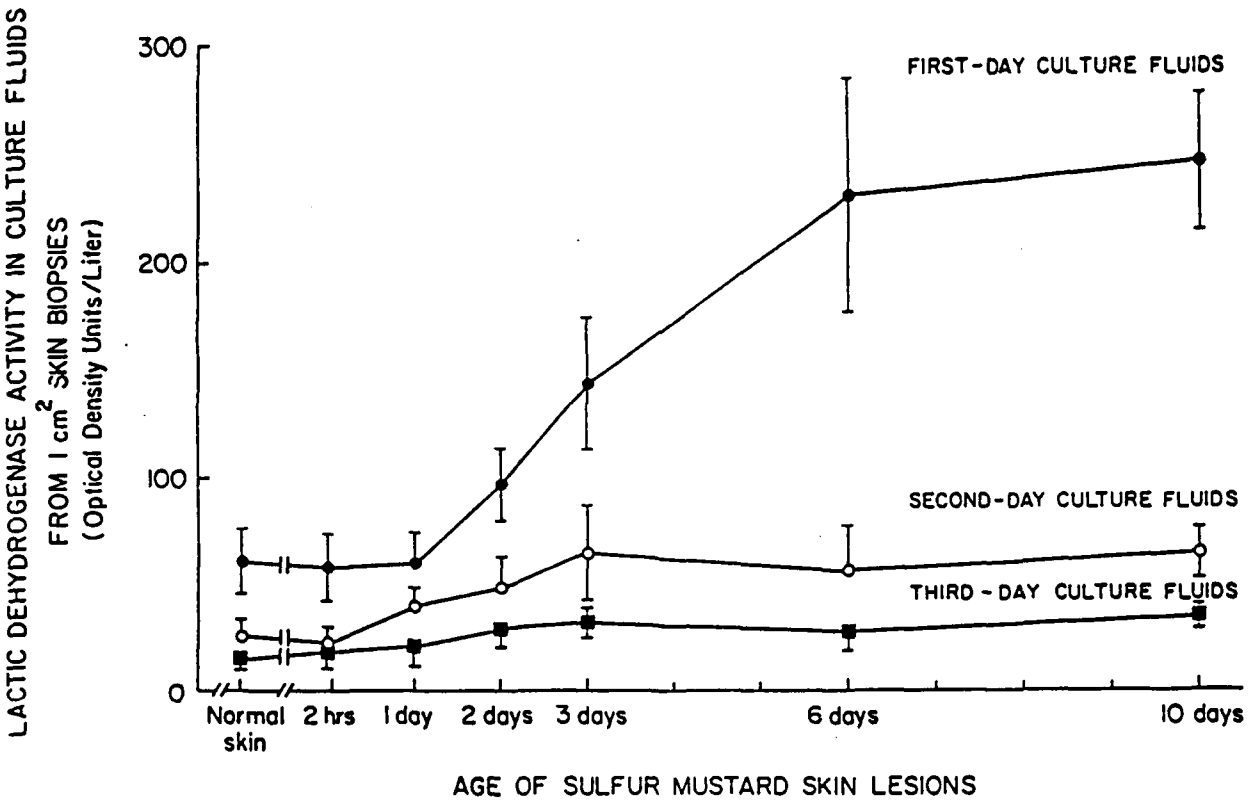

µg/ml
1000
500
100
50
10
5
1
0
LYSOZYME ACTIVITY IN CULTURE FLUIDS FROM 1 cm² SKIN BIOPSIES
(Egg White Lysozyme Equivalents)
FIRST-DAY CULTURE FLUIDS
SECOND-DAY CULTURE FLUIDS
THIRD-DAY CULTURE FLUIDS
Whole serum
Normal skin
2 hrs
1 day
2days
3days
6 days
10 days
AGE OF SULFUR MUSTARD SKIN LESIONS

Figure 4. Lactic dehydrogenase (LDH) activities in first-, second- and third-day organ culture fluids from 1.0-cm^2 dermal sulfur mustard lesions of various ages. The means and their standard errors are depicted. The LDH activities in first-day culture fluids from 6- and 10-day lesions were significantly higher than those from normal skin and those from 2-hr, 1-day and 2-day lesions: $P \leq 0.025$ for 6-day lesions and $\leq .005$ for 10-day lesions. The one-tailed, paired-sample Student's t test was used.



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CHAPTER 6

Trypsin-like and Chymotrypsin-like Enzymes, Hydrolyzing Synthetic Peptide Substrates, Released in Organ Culture by Dermal Sulfur Mustard Lesions

ABSTRACT

The purpose of these studies was to identify the proteases associated with the development and healing of SM lesions. Proteases are known to cause blisters and to hydrolyze the structural proteins of tissues. Their identification in SM lesions could lead to development of specific inhibitors of use in therapy. In this chapter, we describe the hydrolysis of two synthetic protease substrates by culture fluids from developing and healing SM lesions.

When compared to culture fluids from normal skin, the culture fluids from both developing and healing SM lesions had 3 to 6 times the levels of proteases hydrolyzing two synthetic peptide substrates: (a) t-butyloxycarbonyl-Leu-Gly-Arg-4-trifluoromethylcoumarin-7-amide (Boc-Leu-Gly-Arg-AFC, herein abbreviated LGA-AFC), and (b) N-benzoyl-DL-phenylalanine-β-naphthyl ester (BPN). LGA-AFC is a substrate for trypsin, plasmin, plasminogen activator, thrombin, kallikrein, and the C3- and C5-convertasesμ 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 (PMN), macrophages (MN) and activated fibroblasts in histological sections of developing and healing SM lesions, and we measured the levels of these enzymes in serum and 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 PMN and MN seemed to be only minor sources. The crusts of healing lesions, which were full of dead PMN, 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 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 (a) stained for αM with specific antibody with the immunoperoxidase technique and (b) 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.

CHAPTER 6

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INTRODUCTION

In this chapter and in Chapters 7 and 8, we have identified some of the proteases released into SM lesion culture fluids during their development and healing.

The lesion 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 Chapter 3).

Two small, synthetic peptide substrates, however, were hydrolyzed: (a) Boc-Leu-Gly-Arg-AFC (LGA-AFC), a substrate for plasmin, thrombin and other trypsin-like enzymes, and (b) N-benzoyl-DL-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-hr, 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 1, 2 and 3 days. 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 of the organ culture and histologic techniques are given in Chapters 1 and 5.

Trypsin-like proteinase (adapted from references 1-3)

Boc-Leu-Gly-Arg-AFC (LGA-AFC) is a trypsin, plasmin and plasminogen activator and kallikrein substrate (see Table 1). Boc designates t-butyloxycarbonyl, 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, CA 94550, Cat. No. 58-AFC). It was dissolved in dimethylformamide (DMF) to a concentration of 20 mM. Fifty ul of the AFC substrate, 900 ul of 0.05 M TES buffer (pH 8.2), and 50 ul of the lesion culture fluid was incubated for 24 hr at 37 C. TES designates N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (Sigma Chemical Co., St. Louis, Mo., Cat. No. T-1375). The fluorescence of the incubated solutions was read at 0 hr and 24 hr in an AMINCO spectrofluorometer with an excitation wave length of 400 nm, and an emission wave length of 505 nm. As a control, the substrate solution without enzyme (50 ul) was incubated for

24 hr in 950 ul 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, Cat. No. A-8401), used to set the 'sensitivity vernier' at 10, consisted of 50 ul of an AFC solution (20 uM in DMF) in 950 ul of TES buffer. Thus, 1.0 uM 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 of 5.0 to 9.0. Also, the hydrolysis of LGA-AFC by lesion culture fluids and by serum was the same in TES and TRIS (and phosphate) buffers at the same pH.

Chymotrypsin-like esterase (4,5)

N-benzoyl-DL-phenylalanine-β-naphthyl ester (BPN), a substrate for chymotrypsin-like enzymes, was obtained from Sigma Chemical Co. (Cat. No. B-2379). Culture fluids (1.0 ml) were mixed with 2.5 ml of the dilute BPN substrate and incubated 1 hr 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 reaction 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) (o-dianisidine tetrazotized, Cat. No. D 3502, Sigma Chemical Co.) 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 reaction.

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 and 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 subtracted from that produced by the lesion culture fluids. Positive controls of 1.0 ug/ml of crystallized α-chymotrypsin in water (EC 3.4.31.1) (Sigma Chemical Co., Cat. No. C-4129) were assayed with each group of culture fluids. In this assay system, 1 ug of the α-chymotrypsin produced an increase in OD of 0.300 OD units/ml, and 1.0 ug of β-naphthyl 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 uCi/mg) was obtained from New England Nuclear Corp. (Boston, MA 02118, Cat. No. NEC-735) and used as previously described in Chapter 3.

^{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. (Cat. 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, Cat. No. NEF-941) were incubated 5 hr 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 (6).

Granulocyte (PMN) and macrophage (MN) rabbit peritoneal exudate cells and rabbit fibroblast cell lines

PMN exudate cells were obtained 4 hr after the intraperitoneal injection of 0.1% glycogen in physiological saline. MN exudate cells were obtained 4 days after a similar injection of 0.5% glycogen. Details of these procedures are described in Chapter 5. The PMN exudate cells contained 95 to 100% neutrophils. The MN exudate cells contained 98 to 100% mononuclear cells (macrophages with variable numbers of lymphocytes. With the Trypan blue dye-exclusion method (see Chapter 5), we found that PMN were 94 ±2% viable before culture and 71 ±5% viable after culture for 24 hr. Similarly, MN 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. Following culture or extraction, the cells and debris were removed from the RPMI 1640 by a brief centrifugation (2000 rpm [900 g] X 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 Chapter 5. 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 (see Chapter 1).

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 (1-day) and healing (6- and 10-day) SM lesions had 4 to 6 times the LGA-AFCase activity per ml (Figure 1). Culture fluids from healing lesions, but not peak lesions, showedd 2.5 times the LGA-AFCase activity (per mg of protein) of such fluids from normal skin (Figure 2).

Proteases (in SM lesion culture fluids) hydrolyzing N-benzoyl-DL-phenyl-alanine β-naphthyl ester (BPN), a substrate for chymotrypsin-like enzymes

When compared to culture fluids from normal skin, culture fluids from both peak (1-day) and healing (6-and 10-day) SM lesions had 4 to 5 times the BPNase activity per ml (Figure 3). Culture fluids from healing lesions, but not peak lesions, showed 2 times the BPNase activity (per mg of protein) of such fluids from normal skin (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 (see Chapters 1 and 2), the concentration of protein in second- and third-day culture fluids was decreased. If the cells in the SM lesions released appreciable amounts of LGA-AFCase and BPNase, the concentration of these enzymes per mg of protein in the culture fluids should have increased during the second and third day of culture. No such increase occurred (see Figures 2 and 4). The LGA-AFCase abd BPNase activities per mg of protein in the second- and third-day culture fluids 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. In other words, during organ culture, these proteases did not seem to be preferentially released over other protein constituents by the cells in the lesions.

Plasmin and plasminogen activator: probable sources of the LGA-AFCase activity in the lesion culture fluids

Aprotinin is known to inhibit plasmin, but not plasminogen activator (7). 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. We also tested the effect of aprotinin on several commercially available proteinases as controls.

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 non-inhibitable types of proteases, plasmin and plasminogen activator, respectively, because the culture fluids contained extravasated serum (see Chapters 1, 2 and 3), 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 (8,9) or by the sensitive ^{125}I-fibrin method (10). The latter method was performed by Dr. Pamela Jensen, who is with Dr. Gerald S. Lazarus's group at the University of Pennsylvania (10). 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 α-macroglobulin inhibitors (see below).

LGA-AFCase and BPNase activities in serum

LGA-AFCase activity per mg of protein was 2.5 to 5 times higher in serum than in SM lesion culture fluids (Figure 2), whereas BPNase activity per mg of protein in serum was similar to that in the culture fluids (Figure 4).

The studies presented in Table 1 (and those of others [11,12]) 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 mg 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 the proteases hydrolyzing LGA-AFC and BPN

Cells. One-cm^2 explants of normal skin, 1-day SM lesions and 6-day SM lesions contained an average of 16,000, 880,000 and 290,000 PMN; 100,000, 3,700,000 and 500,000 macrophages (MN); and 1,000,000, 1,900,000 and 7,400,00 activated fibroblasts, respectively (see Chapter 5). Rabbit peritoneal exudate PMN and MN and rabbit fibroblast cell lines (obtained and cultured as described in Chapter 5) 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 1 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 Chapter 5. The many assumptions made to produce these rough estimates are discussed therein. For comparative purposes, the amounts of these enzymes extracted from frozen and thawed PMN, MN 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 Chapter 5), and that intravascular and extravascular serum had the same composition.

Crusts. Healing SM lesions have crusts containing fibrin; inspissated serum; large numbers of PMN (mostly dead); and variable, but smaller, numbers of MN (again, mostly dead) (see Chapter 5). We removed most of the crusts of 6-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 (see Chapter 5), probably because of the cell injury and the alteration of the physical properties of the ground substance.] The crusts and the 1- and 6-day lesion bases (lesions without epidermis or crust) were cultured for 24 hr, and the culture fluids were then assayed for LGA-AFCase and BPNase.

The crusts of the 6-day lesions released 2 to 3 times the LGA-AFCase and BPNase into the culture fluids that the bases of 6-day lesions released ($P < 0.01$) (Table 2). Together, the crust plus the base released 3 to 4 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 6-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 1-day lesions released into the culture fluids over 3 times the amount of LGA-AFCase that the bases of 6-day lesions released ($P < 0.001$) (Table 2). However, the bases of 1-day lesions released about the same amount of BPNase into the culture fluids as the bases of 6-day lesions released (Table 2).

Serum had 2.5 to 5 times the level of LGA-AFCase per mg of protein (Figure 2) and about the same level of BPNase per mg of protein (Figure 4) that 1- and 6-day lesion culture fluids had. These facts may explain why higher LGA-AFCase levels were found in culture fluids from 1-day lesion bases than in culture fluids from 6-day lesion bases: One-day lesions were edematous and rich in extravasated serum (Table 2).

Our studies clearly suggest (see Table 3) (a) that 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, (b) that fibroblasts may also be a major source of LGA-AFCase, c) that PMN and MN (because of their low numbers) released only small amounts of these two enzymes into these extracellular fluids, and (d) that the dead (and live) cells (mostly PMN) in the crusts 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 the LGA-AFCase and BPNase in the lesion culture fluids have at least some endopeptidase activity.

Endopeptidases are able to split the internal peptide bond in the bait region of α-macroglobulin proteinase inhibitors (αM) (13). Such splitting causes the αM to undergo a conformational change that traps the endopeptidase and inhibits its proteolytic activity (13). This property of αM can be used to distinguish endopeptidases from exopeptidases: By definition, exopeptidases cannot hydrolyze an internal peptide bond and therefore can not cause the conformational change in αM, or be inhibited by αM (see Discussion).

First-day culture fluid from 6-day SM lesions was applied uniformly along the top of a 7.5% polyacrylamide gel containing 2% sodium dodecyl sulfate and was electrophoresed, in both the presence and absence of 5% mercaptoethanol. From single gel, multiple identical strips were cut. Details of our procedures are presented in Chapter 3.

Some of the gel strips were incubated at 30 C for 20 hr 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, CA) because of their fluorescence.

Some of the strips were incubated at 30 C for 20 hr at pH 6.5 in the benzoyl-DL-phenylalanine-β-naphthyl ester substrate solution (described in Materials and Methods). We found that postcoupling (of the released naphthol) with Naphthylanil Diazo Blue B was not as effective as simultaneous coupling. Therefore, NDBB (dissolved in a drop of dimethylformamide) was mixed with the substrate solution, at a final concentration of 1.0 mg per 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 (see Chapter 3) (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. 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).

Figure 5 depicts a gel that was representative of the five culture fluids that we evaluated. Each was from a 6-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 α 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_r that stained in this manner.

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 Chapter 3).

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 (see Chapter 3) 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 that were 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 hr with several first-day culture fluids from 1-day and 6-day SM lesions. The proteins, ^{14}C-casein (see Chapter 3) and solubilized ^{14}C-elastin, were incubated at 37 C, at pH 8.0 and 8.5, respectively, for 18 hr (see Materials and Methods). Then trichloroacetic acid or ammonium sulfate was added to precipitate the unhydrolyzed proteins and, after centrifugation and adding ECONOFLUOR (see Chapter 3), the supernatant fluids were read in a scintillation counter.

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

The hydrolysis (by the culture fluids) of (c) the synthetic substrate for cathepsinG, L-glutaryl-L-alanyl-L-alanyl-L-phenylalanyl-4-methoxy-2-naphthylamine (Enzyme Systems Products) and (d) the synthetic substrate for the trypsin-like Cathepsin B, N-benzoyl-DL-arginine-β-naphthylamide (Sigma Chemical Co.) were measured under the same conditions as (b) above, except that cathepsin B was assayed at pH 5.6. Little or no hydrolysis of (c) and (d) was found. With (d), 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 (1- and 2-day) SM lesions. Thus the elastase could be present in lesion culture fluids, but merely inhibited (see Chapter 3).

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 (14). In addition, proteolytic enzymes from epithelial cells, endothelial cells, and fibroblasts, and from the infiltrating leukocytes play major roles in the breakdown of connective tissues during the inflammatory process and in the remodeling of connective tissues during repair (15-20).

We found no active proteinase (endopeptidase) in the organ-culture fluids of inflammatory skin lesions produced by sulfur mustard. 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 (see Chapter 3).

Detection of inhibited proteinases

Previously active proteinases can be detected in several ways: (a) by using antiserum to purified enzymes with immunoperoxidase techniques, (b) by measuring the products of their proteolytic action, (c) by removing or inactivating the inhibitor part of proteinase-inhibitor complexes, and (d) by using small synthetic peptides as substrates. Such peptides can enter the 'cleft' in α-macroglobulin-proteinase complexes and become hydrolyzed. This chapter concerns the approach in (d). Subsequent chapters concern (b) and (c). Purified rabbit proteinases to test (a) are not readily available, but our laboratory has used the method histochemically to demonstrate cathepsin D in macrophages found in chronic inflammatory lesions (21,22).

The α-macroglobulins (αM) are unique proteinase inhibitors (see Chapter 3 and reference 13). They will bind a proteinase 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 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 are then diffused in the surrounding medium, where they can be measured.

These principles can be used to distinguish exopeptidases from endopeptidases (13). 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 (a) to hydrolyze the fluorogenic peptide substrate LGA-AFC or the chromogenic peptide substrate BPN, and (b) to stain with a specific antibody to αM and the immunoperoxidase technique (Figure 5). Other electrophoretic bands stained either 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.

Sources of LGA-AFCase and BPNase activities in SM lesions

Serum 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 11, 12), but specific enzymes in serum that hydrolyze BPN were not found in the literature. When 2 to 4 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).

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-α-macroglobulin 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 mg of protein than did lesion culture fluids (Table 2).

BPN is an excellent substrate for cathepsin G (23), which resembles chymotrypsin. Homogenates of PMN (from blood) and of whole spleens are rich sources of cathepsin G, but other sources of this enzyme have not been thoroughly evaluated (23).

Mast cells (23-25), and probably basophils, hydrolyze BPN. From reference 24 and Table 2, we estimated that their extracts should contain roughly 2 to 4 times the BPNase activity of PMN (per million cells). However, the number of mast cells and basophils within the SM lesions is small, approximately that of PMN (excluding those in the crusts) (see Chapter 5). Since PMN make such a 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 PMN could release. Basophils and mast cells remain close to the venules and do not seem to enter the crusts (see Chapter 1).

Epidermal 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 of healing lesions 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 PMN that the crusts contain.

The plasma protease inhibitors

Plasma (and serum) contain α_1-proteinase inhibitor (formerly α_1-antitrypsin), α-macroglobulins, α_2-plasmin inhibitor, α_1-antichymotrypsin, antithrombin III, C1-inactivator, and inter-α-trypsin inhibitor (inter-α-globulin) (26). 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 irhibitor, but the concentration of α_2M is higher (26). Plasmin is bound to both inhibitors (27) in proportions depending (a) on the amount added to serum and (b) on whether or not it was produced by urokinase or added directly as an active enzyme (27). The fact that some plasmin is bound to α_2M (in both serum and SM lesion culture fluids), however, explains why LGA-AFC is hydrolyzed and why its substrate, fibrin, is not (see Results).

Table 1

The hydrolysis of the protease substrate Boc-Leu-Gly-Arg-AFC by culture fluids from SM lesions and by several purified proteases. Inhibition by the plasmin inhibitor aprotinin

Culture fluids[a] from	Fluorescence units/ml[b] A	Fluorescence units/ml in presence of aprotinin[c] B	Percent of protease activity remaining B/A
normal skin	8.3	3.4	41%
2-hr lesions	7.4	2.3	31%
1-day "	20.3	9.7	48%
2-day "	23.1	9.8	42%
3-day "	16.4	5.8	35%
6-day "	21.1	6.9	33%
10-day "	21.9	7.3	33%
Serum (1:40)[d]	90.3	66.6	74%
Trypsin[e] (2.5 ng/ml)	64	0	0
Kallikrein[e] (0.005 units/ml)	34	16	47%
Plasmin[e] (0.5 ug/ml)	26	0	0
Urokinase[e] (5 units/ml)	16	30	188%

Table 1: The hydrolysis of the protease substrate Boc-Leu-Gly-Arg-AFC by culture fluids from SM lesions and by several purified proteases. Inhibition by the plasmin inhibitor aprotinin (continued)

Footnotes

[a] After 24 hr, the culture fluids (2.5 ml) were collected and centrifuged to remove the suspended cells and debris. Fifty ul 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 ug/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 was listed.

Table 2

Hydrolysis of the protease substrates Boc-Leu-Gly-Arg-AFC and N-benzoyl-DL-phenylalanine-β-naphthyl ester (BPN) by culture fluids from SM lesions and their crusts; by PMN, macrophages (MN) and fibroblasts; and by serum

Culture fluids (CFs), or extracts	Number of Samples	LGA-AFCase (Fluorescence units per ml)	Protein concentration mg/ml	Number of Samples	BPNase (Optical density units per 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
PMN CFs from 5×10^6/ml	4	0.5 ± 0.3	0.04 ± 0.01	7	0.50 ± 0.19	0.03 ± 0.01
PMN extracts: 5×10^6/ml	4	0.6 ± 0.2	0.12 ± 0.02	7	1.39 ± 0.39	0.20 ± 0.05
MN CFs from 5×10^6/ml	5	1.7 ± 0.7	0.08 ± 0.01	7	0.16 ± 0.09	0.11 ± 0.03
MN extracts: 5×10^6/ml	5	2.4 ± 0.7	0.35 ± 0.06	5	1.31 ± 0.90	0.53 ± 0.16
Fibroblast CFs: 2.4×10^6/ml	5	61 ± 13	0.14 ± 0.01	4	0.15 ± 0.06	0.07 ± 0.03
Fibroblast extracts: 2.4×10^6/ml	5	189 ± 18	0.64 ± 0.03	5	1.09 ± 0.17	0.46 ± 0.12
Serum 1:29 1:45	6	83 ± 10 53 ± 6	1.74 ± 0.28 1.13 ± 0.18	6	0.96 ± 0.21 0.62 ± 0.13	2.09 ± 0.04 1.35 ± 0.02

Table 2: Hydrolysis of the protease substrates Boc-Leu-Gly-Arg-AFC and N-benzoyl-DL-phenylalanine-β-naphthyl ester (BPN) by culture fluids from SM lesions and their crusts; by PMN, macrophages (MN) and fibroblasts; and by serum (continued)

Footnotes

General. The results are presented as enzyme activity per ml of culture fluid. The enzyme activity per mg of protein can be derived from this table by dividing the activity per ml 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 3 or 4 rabbits were removed, cultured separately for 24 hr in 2.5 ml of RPMI 1640, and centrifuged. Then the supernates were assayed for these enzymes. For comparison, intact 6-day lesions, the "crustless" bases of these lesions, and the "surfaceless" 1-day lesions were also cultured.

PMN and MN extracts: Ten million PMN or MN peritoneal exudate cells (in 2.0 ml) were frozen and thawed 4 times and centrifuged. The supernates were assayed for these enzymes. (See Materials and Methods.)

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

No corrections were made in the PMN and MN extracts (of CFs) for the 3 ±1% MN in the PMN exudates and the 1 ±1% PMN in the MN exudates. The MN group contained variable numbers of small mononuclears that resembled small lymphocytes (see Chapter 5).

Fibroblasts: The two fibroblast cell lines were freed from the culture flasks with trypsin (see Materials and Methods), washed 3 times by centrifugation, and cultured for 24 hr 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 7 rabbits were used for PMN or MN. Three different cell suspensions were obtained from each of the 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 (see Chapters 3 and 5).

Comment. In contrast to our findings in this table, BPNase was previously found to be absent in rabbit PMN (see reference 4). 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 the percentages of these enzymes derived from serum, PMN, macrophages (MN), fibroblasts and crusts[a]

Source	LGA-AFCase	BPNase
Normal skin: enzyme units per 2.5 ml of 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
Remainder	0 %	0 %
1-day SM lesions: enzyme units per 2.5 ml of 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
Remainder	0 %	0 %
6-day SM lesions: enzyme units per 2.5 ml of culture fluids	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
Remainder	0 %	0 %

Table 3: Sources of extracellular LGA-AFCase and BPNase in 1.0-cm^2 SM lesions of various ages: Rough estimates of the percentages of these enzymes derived from serum, PMN, macrophages (MN), fibroblasts and crusts[a] (continued)

Footnotes

[a] This table presents a very rough estimate of the sources of the LGA-AFCase and BPNase in first-day culture fluids of the 1.0-cm^2 normal skin, 1-day SM lesion, and 6-day SM lesion explants. To prepare it, we assumed that the amount of these enzymes released in cell culture by PMN, MN 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 Chapter 5). LGA-AFCase was measured in culture fluids from Experiment II of Chapter 5, and BPNase was measured in culture fluids from Experiment I of Chapter 5.

Crusts were estimated to contribute 33% of the enzyme activity to culture fluids of 6-day lesions. The 33% is a guess, but seems reasonable in light of the data, presented in Results, and of other experiments, described in Chapter 5.

Figure 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 biopsy is 2.5 times the fluorescence 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 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 Chapter 5.

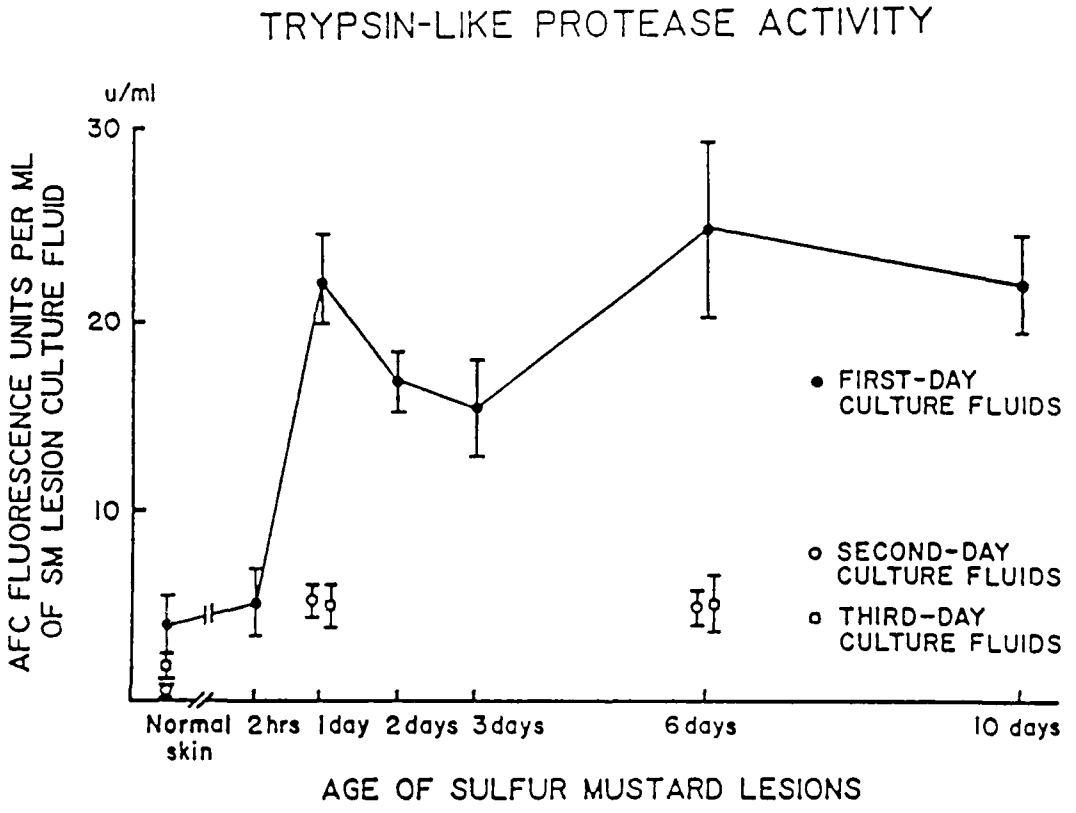


Figure 2. Trypsin-like (LGA-AFCase) activity per mg of protein in first-day culture fluids from 1.0-cm^2 dermal SM lesions of various ages. The culture fluids from 6- and 10-day SM lesions showed significantly higher LGA-AFCase activity per mg of protein than did culture fluids from normal skin and from 1-, 2- and 3-day SM lesions. ($P < 0.025$). The one-tailed Student's t test was used.

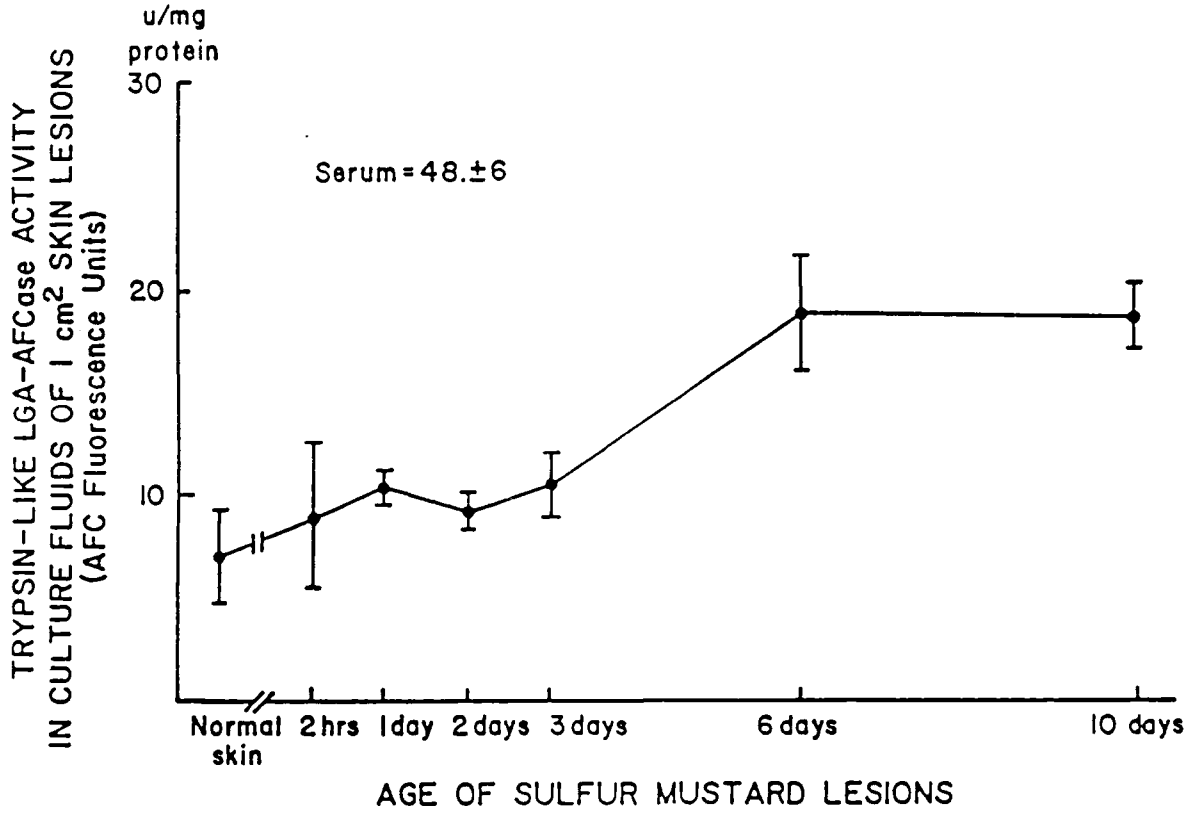


Figure 3. Chymotrypsin-like esterase activity in first-, second- and third-day culture fluids from 1.0 cm^2 dermal sulfur mustard lesions. N-benzoyl-DL-phenylalanine-β-naphthyl ester (BPN) was used as the substrate. The optical density unit is defined in Materials and Methods The BPNase activity for each 1.0-cm^2 biopsy 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 depicted. 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 Chapter 5.

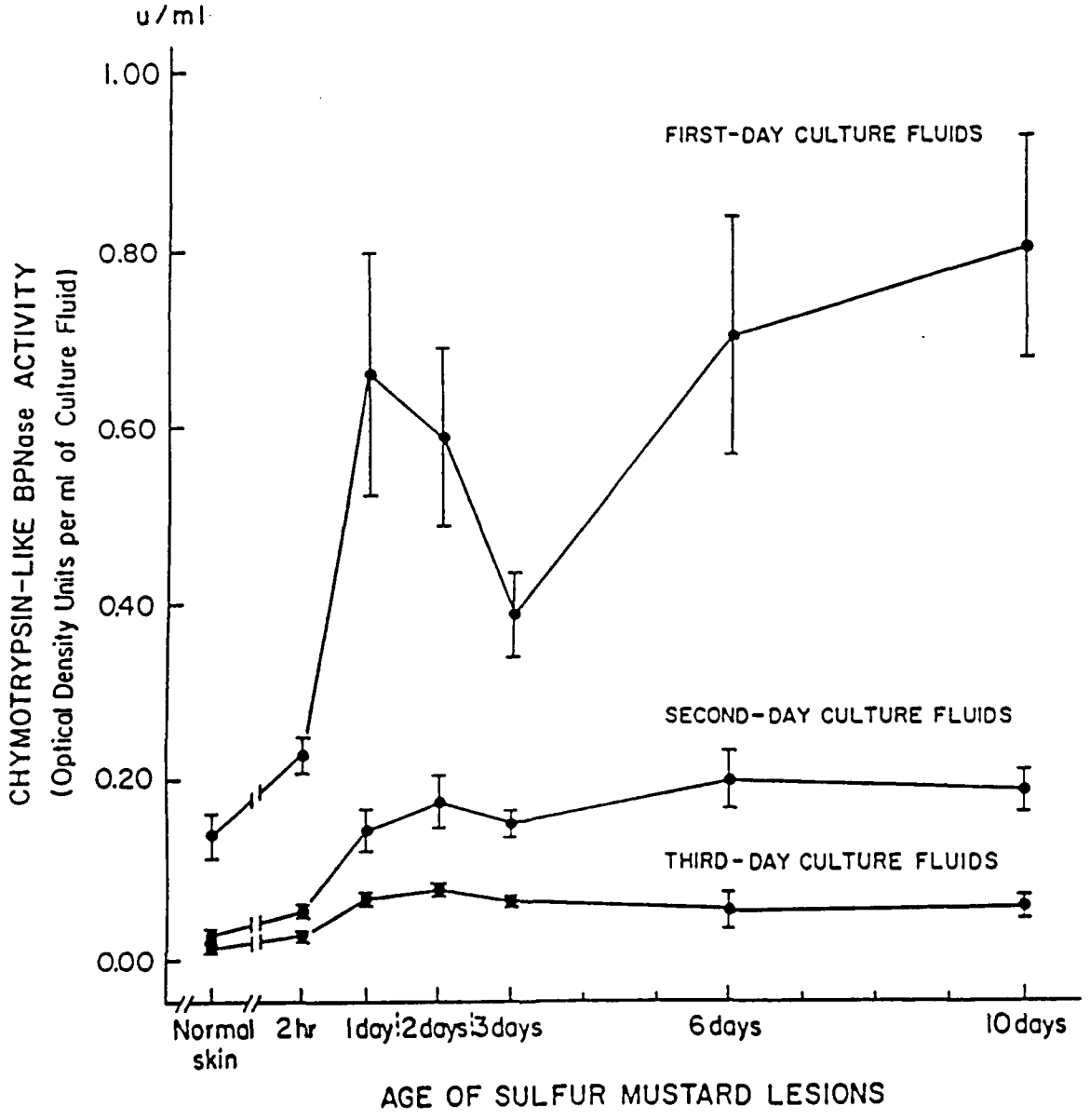


Figure 4. Chymotrypsin-like esterase (BPNase) activity per mg protein in first-day culture fluids from 1.0-cm^2 dermal sulfur mustard (SM) lesions of various ages. The culture fluids from 2-, 6- and 10-day SM lesions showed significantly higher BPNase activity per mg of protein than did culture fluids from normal skin ($P < 0.022$). The one-tailed Student's t test was used. The stippled rectangle represents serum values (the mean and its standard error).

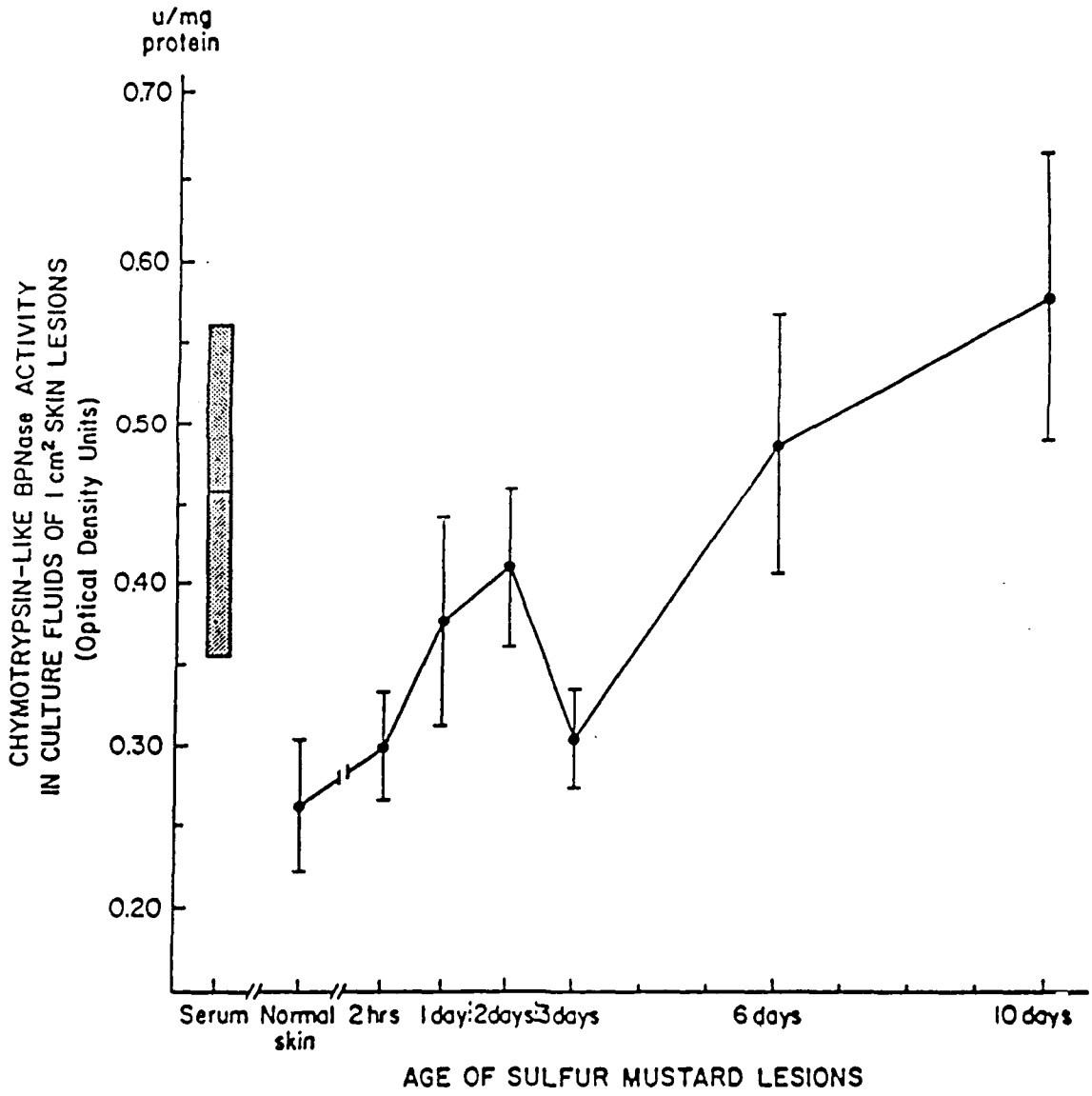
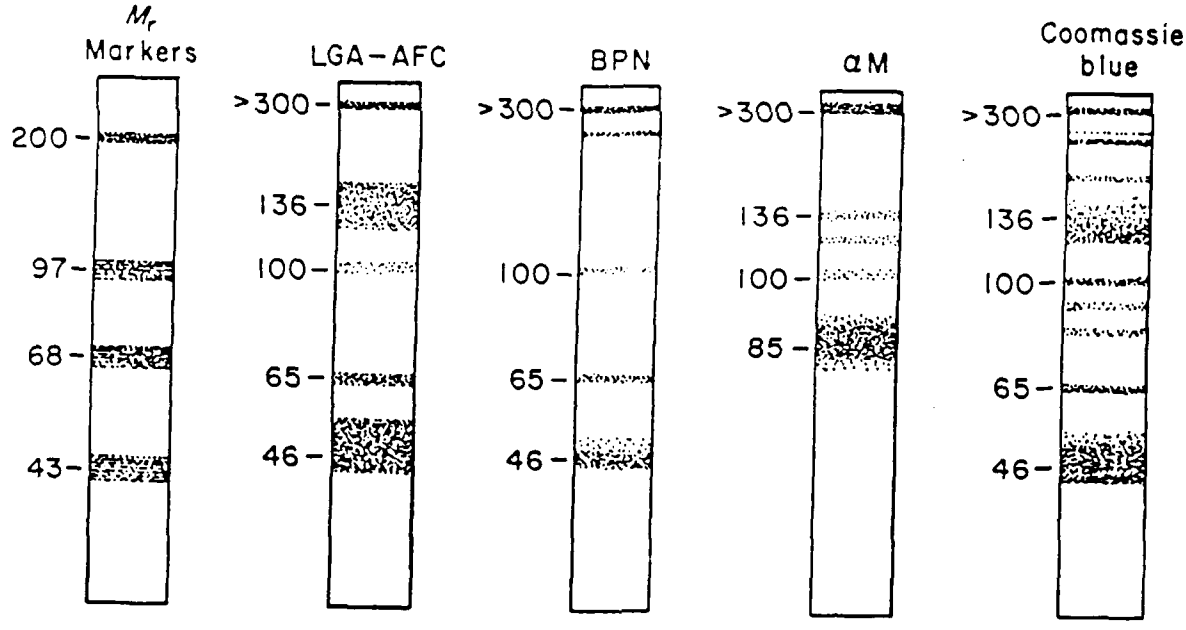


Figure 5. A diagram representing the location of bands produced by the electrophoresis of 6-day SM lesion culture fluids on polyacrylamide gels containing sodium dodecyl sulfate. Details on these procedures were published in Chapter 3.

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 Chapter 3). 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 proteins bands than did the other procedures.



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CHAPTER 7

Collagenase and Proteoglycanase Released in Organ Culture by Dermal Sulfur Mustard Lesions

A collaborative project with Dr. J. Fred Woessner, Jr., Department of Biochemistry, School of Medicine, University of Miami, Miami, Florida

ABSTRACT

By activating latent proteinases and inactivating the inhibitors in proteinase-inhibitor complexes, we could measure collagenase and proteoglycanase activity in SM lesion culture fluids. Healing (6- and 10-day) lesions usually showed higher levels of C'ase and PGase activity than peak (1- and 2-day) lesions. This suggests that the breakdown of the extracellular matrix is mainly due to the remodeling processes of repair.

Second- and third-day lesion culture fluids usually showed more Case and PGase activity than first-day culture fluids. This suggests that dying cells directly either cause the breakdown of the extracellular matrix or stimulate the surviving cells to do so.

These studies support the concept that active proteolytic enzymes are produced by the cells in SM lesions, act on tissues nearby, and then are inactivated by serum and tissue inhibitors in the extracellular spaces.

Collagenase is probably the main enzyme that causes epidermal cells to separate from their basement membrane. In other words, it is probably a major cause of the blisters that are characteristic of sulfur mustard lesions in man. Proteoglycanase may contribute to blister formation, because glycosaminoglycans are a likely component of blister fluid.

CHAPTER 7

TABLE OF CONTENTS

INTRODUCTION

Proteolytic enzymes are known to be released from injured epidermal cells, infiltrating phagocytes and activated fibroblasts. However, we were unable to detect any proteolytic activity in SM lesion organ-culture fluids with ^{14}C-casein as the substrate (Chapter 6). Evidently, these enzymes act locally, and then are inhibited by protease inhibitors in the lesions' extracellular fluids (Chapter 3).

This chapter describes experiments in which active collagenase and proteoglycanase were recovered from SM lesion culture fluids. We inactivated the inhibitor part of the proteinase-inhibitor complexes and released the active proteinases. Chapter 8 provides evidence that these two major proteinases had been previously active on their respective substrates: We found hexosamine-containing and hydroxyproline-containing components of extracellular matrix in organ-culture fluids of SM lesions.

Collagenase can be produced by a variety of cells, particularly skin fibroblasts (1,2), and its synthesis is stimulated by modulators such as interleukin-1 (3,4). Proteoglycanase digests the ground substance of the extracellular matrix. Such proteases have been given various names, including stromelysin (5) and matrix metalloprotease 3 (6).

These proteases are believed to play major roles in the remodeling of the extracellular matrix that accompanies inflammatory and repair processes. They also participate in the blister formation found in human SM lesions (7).

MATERIALS AND METHODS

General

Developing and healing sulfur mustard lesions were produced in the skin of rabbits and were organ-cultured, as described in Chapter 1. The culture fluids were centrifuged, and the supernates were frozen at -70 C until assayed.

Giemsa-stained glycol methacrylate-embedded tissue sections of these lesions were made and evaluated microscopically, as described in Chapters 1 and 5. Granulocytes, macrophages and fibroblasts were obtained and cultured for 24 hr, as described in Chapter 5.

To determine the statistical significance, either the one-tailed Student's t test or the ANOVA and Student-Newman-Keuls tests were used.

Treatment of lesion culture fluids for enzyme assays.

After thawing, about 2 ml of each culture fluid was placed in small dialysis bags and dialyzed overnight, at 4 C, against 4 volumes of 3 M KSCN in assay buffer (50 mM Tris/HCl [pH 7.5] containing 0.15 M NaCl, 0.01 M $CaCl_2$, and 0.02% NaN_3). The samples were again dialyzed overnight against 400 volumes of the same buffer without KSCN. This step is known to destroy the α_1- and α_2-macroglobulin inhibitors (8).

The first-day culture fluids (but not the second- and third-day culture fluids) were subjected to further processing, namely, reduction and alkylation to destroy the tissue inhibitor of metalloproteases (TIMP). First, we added dithiothreitol to a final concentration of 2 mM and incubated the samples for 30 min at 37 C. Next, we added iodoacetamide to a final concentration of 5 mM, and incubated the samples another 30 min at 37 C. Then we dialyzed the samples overnight against assay buffer to remove the reagents.

Collagenase assay

The method of Dean and Woessner (9) was used. Aliquots of lesion culturefluids (5, 2 and 1 ul) were incubated with 10 ul of tritiated collagen (17.6 ug ^{3}H-acetylated telopeptide-free type I collagen, 90,000 cpm) for 18 hr at 30 C, in a total volume of 100 ul assay buffer. To activate latent collagenase, we added aminophenylmercuric acetate (APMA) (0.2 mM, final concentration) to all tubes.

After incubation, the collagen was hydrolyzed further with trypsin and chymotrypsin. Collagenase cleaves the triple helix of the collagen molecule into peptides that are 3/4 and 1/4 its original length. These products denature rapidly at 30 C and become susceptible to hydrolysis by nonspecific proteinases (10). Therefore, after the lesion culture fluids were incubated in tritiated collagen, they were further incubated for 2 hr with trypsin and chymotrypsin, as described in reference 9.

At this point, the original method was modified. We added an equal volume of 20% trichloroacetic acid, and removed the resulting precipitate by centrifugation. An aliquot of the supernate was then placed in a 7 ml polyethylene vial with 5 ml Aquasol scintillant (New England Nuclear, Boston, MA), and counted in a Packard TriCarb Scintillation Spectrometer.

In order to provide blanks with no collagenase activity, we added 1,10-phenanthroline (1 mM, final concentration) to duplicate culture fluid samples (containing APMA). Phenanthroline is the classic inhibitor of metalloproteinases and does not inhibit thiol and serine proteinases. Thus the amount of true collagenase in the samples is calculated by subtracting the cpm (of the released tritiated peptides) in the phenanthroline blanks from the cpm in the samples without phenanthroline.

Digestion was first expressed as percent of the collagen digested due to the phenanthroline-inhibitable enzyme. (The amount of enzyme was always adjusted to give between 5% and 35% digestion.) The percent digestion was then converted to a standard unit of micrograms of collagen digested per min. Finally, the units in 2.5 ml of medium were calculated. This corresponds to the total units of enzyme produced by the 1.0 cm^{2} skin explant during 24 hr in culture.

Proteoglycanase assay.

This method follows closely the procedure published by Nagase and Woessner (11). Culture medium (50 ul) was incubated in a scintillation vial with 2.0 $\pm$0.4 mg dry polyacrylamide beads (containing 180 ug tritiated proteoglycan/mg bead), suspended in 150 ul of assay buffer. Following incubation for 18 hr at 37 C, Aquasol was added directly to the vial, and the contents were counted.

The proteoglycan was prepared from bovine nasal cartilage. It was disaggregated in 4 M guanidine into 2.5 X 10^6 M_r subunits with a single protein chain backbone and about 100 side chains of chondroitin sulfate and keratin sulfate (11). Proteolytic cleavage of the backbone releases large fragments consisting of a peptide portion with varying numbers of covalently attached polysaccharide chains.

The proteoglycan subunits were labeled with ^{3}H-acetic anhydride (9), and dissolved in the TEMED (N,N,N',N'-tetramethylethylenediamine) that was used to polymerize the acrylamide (11). The resulting proteoglycan-containing polyacrylamide was homogenized in cold distilled water with a VirTis homogenizer (Catalog No. H-3500, Baxter Scientific [formerly American Scientific] Columbia, MD 21045). The fines were collected and dried with acetone. The particles were then sized with a 100 mesh screen (0.150 mm opening) and stored in a desiccator. Each milligram of beads showed 11,000 tritium counts per minute.

All tubes contained 1 mM aminophenylmercuric acetate to activate latent enzymes. In addition, the control tubes contained 1 mM 1,10-phenanthroline to inhibit metalloproteinases.

Digestion was calculated as cpm of proteoglycan released per mg of beads, due to phenanthroline-inhibitable protease activity. By assuming that 2.0 mg of beads were used in each assay, we converted these cpm to ug proteoglycan digested/min/2.5 ml culture fluid. Each 1.0-cm^2 skin lesion was organ-cultured in 2.5 ml of RPMI 1640 (see Chapter 1).

RESULTS

Treatment of SM lesion culture fluids.

In most cases, little or no collagenase or proteoglycan-digesting proteases were found in untreated lesion culture fluids, because of the presence of inhibitors (see Chapter 6). Two major inhibitors would be (a) the α-macroglobulins (see Chapter 3) from extravasated serum, and (b) the tissue inhibitor of metalloproteases (TIMP) (12). Treatments of the medium, namely, dialysis against KSCN, followed by reduction and alkylation (see Materials and Methods), were therefore performed to destroy sequentially these two inhibitors.

First-day culture fluids from all SM lesions showed maximal proteoglycan and collagen digestion after such treatment. Without treatment, about 15% of the total activity could be detected, and following KSCN treatment, about 40%. Second- and third-day culture fluids showed maximal collagen and proteoglycan digestion after KSCN treatment, so that the reduction/alkylation step was omitted.

In all cases, aminophenylmercuric acetate was added to activate latent forms of the metalloproteases. Efforts to distinguish active and latent forms of the proteases following the chemical treatments were not satisfactory. Therefore, only the total detectable activity is reported in each case.

Collagenase in first-, second- and third-day culture fluids from developing and healing SM lesions

With both normal and SM-treated full-thickness skin explants, the release of collagenase was lowest after 1 day in culture ($P < 0.05$), increased markedly on the second day of culture ($P < 0.05$), and increased still further on the third day ($P < 0.05$) (Figure 1).

During the second and third days of culture, sulfur mustard lesions showed a significant increase in the production of collagenase when compared to the normal skin controls. This increase first became significant ($P < 0.05$) in second-day culture fluids when the lesions were 3 days of age, and in third-day culture fluids when the lesions were 1 day of age. In general, the collagenase production was still rising in peak (1-day) SM lesions and reached a maximum in healing (6-day) lesions.

Proteoglycanase in first-, second- and third-day culture fluids from developing and healing SM lesions

The proteoglycanase activity was increased in second- and third-day culture fluids (Figure 2), just as in the case of the collagenase. This increase, however, was not as striking because first-day culture fluids already showed considerable proteoglycanase activity. Second-day culture fluids showed significant increases for all explants ($P < 0.02$), but by the third day of culture, further increases were found only in culture fluids from control skin and 1- and 2-day SM lesions.

On each day of culture, the SM lesion explants produced more proteoglycanase than did normal skin explants. This was first significant ($P < 0.05$) in first-day culture fluids from 1-day SM lesions. Maximal proteoglycanase production usually occurred in healing (6-day) lesions.

Sources of collagenase and proteoglycanase in SM lesions

Pilot-type experiments were performed to determine the sources of collagenase and proteoglycanase in the extracellular fluids of SM lesions. These experiments were similar to those presented in Chapters 5 and 6, but were so few in number that only tentative conclusions were possible.

The extravasated serum did not seem to be sufficient to account for the collagenase activity in the lesion culture fluids. In fact, the highest collagenase activities were in second- and third-day culture fluids, in which most of the serum had been removed (see Chapter 1). (Serum was not assayed for proteoglycanase activity.)

Granulocyte (PMN) and mononuclear (MN) peritoneal exudate cells and two fibroblast cell lines (see Chapter 5) were assayed for collagenase and proteoglycanase. From the number of these cells in the lesions (see Chapter 5), we could roughly estimate the contribution of these cells to the collagenase and proteoglycanase found in the lesion culture fluids. Within the lesion proper, only the fibroblasts appeared to make an appreciable contribution. PMN and MN seemed to contribute very little.

The crusts of healing lesions, which are full of PMN, seemed to contain both collagenase and proteoglycanase. But, as in Chapters 5 and 6, we were unable to estimate what proportion of collagenase and proteoglycanase in the culture fluids from intact healing lesions came from the crusts. Again, 33% seemed a reasonable guess.

DISCUSSION

The experiments described herein, and those in Chapter 8, suggest that within developing and regressing SM lesions, considerable hydrolysis of collagen and ground substance occurred at various local sites. Such hydrolysis seemed especially active during the remodeling phase of the healing process.

The relatively low levels of collagenase and proteoglycanase in first-day culture fluids, moderate levels in second-day culture fluids, and relatively high levels in third-day culture fluids suggest that dying cells either directly cause the breakdown of the extracellular matrix or stimulate the surviving cells to do so.

Figure 1. Collagen digestion by organ-culture fluids from developing and healing SM lesions. One-cm^2 full-thickness lesion explants were cultured in 2.5 ml medium. The medium was replaced each day, and the first-, second- and third-day culture fluids (marked 1, 2 and 3) were assayed for collagenase as described in Materials and Methods. The number of micrograms of collagen digested per minute by 2.5 ml of lesion culture fluid (i.e., by each 1.0-cm^2 lesion explant) is shown on the ordinate. Depicted are the means (and their standard errors) of culture fluids from lesions from each of six rabbits. Statistical differences are presented in Results.

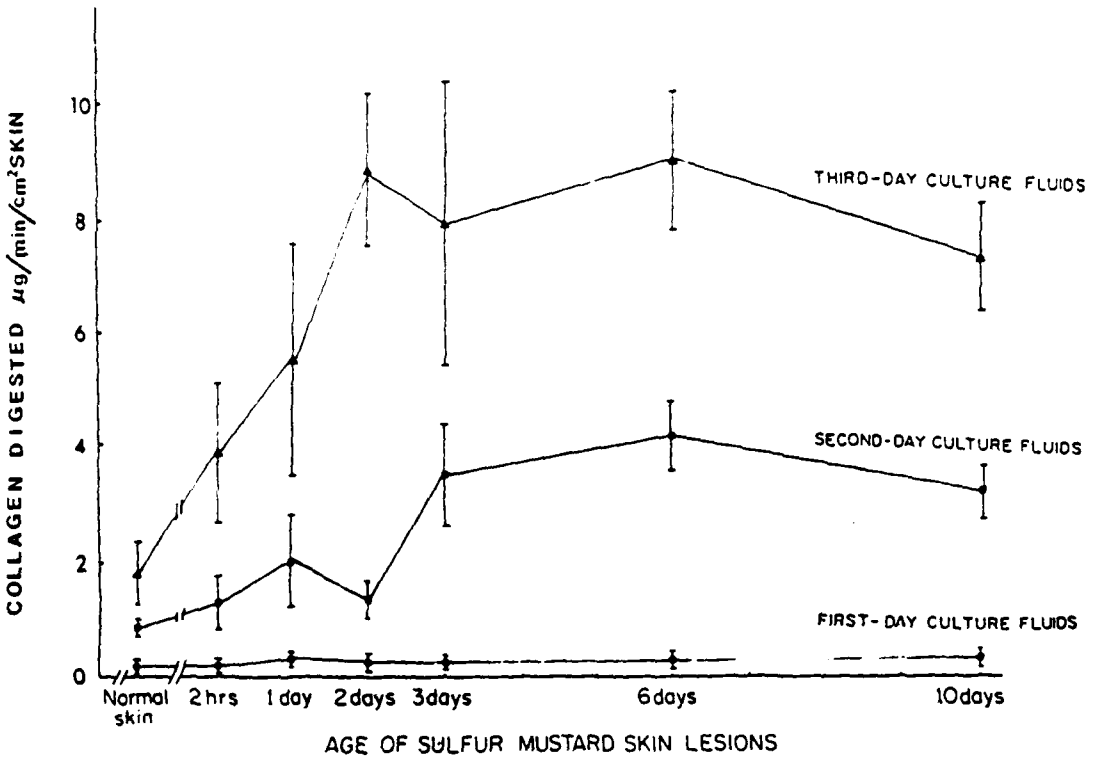
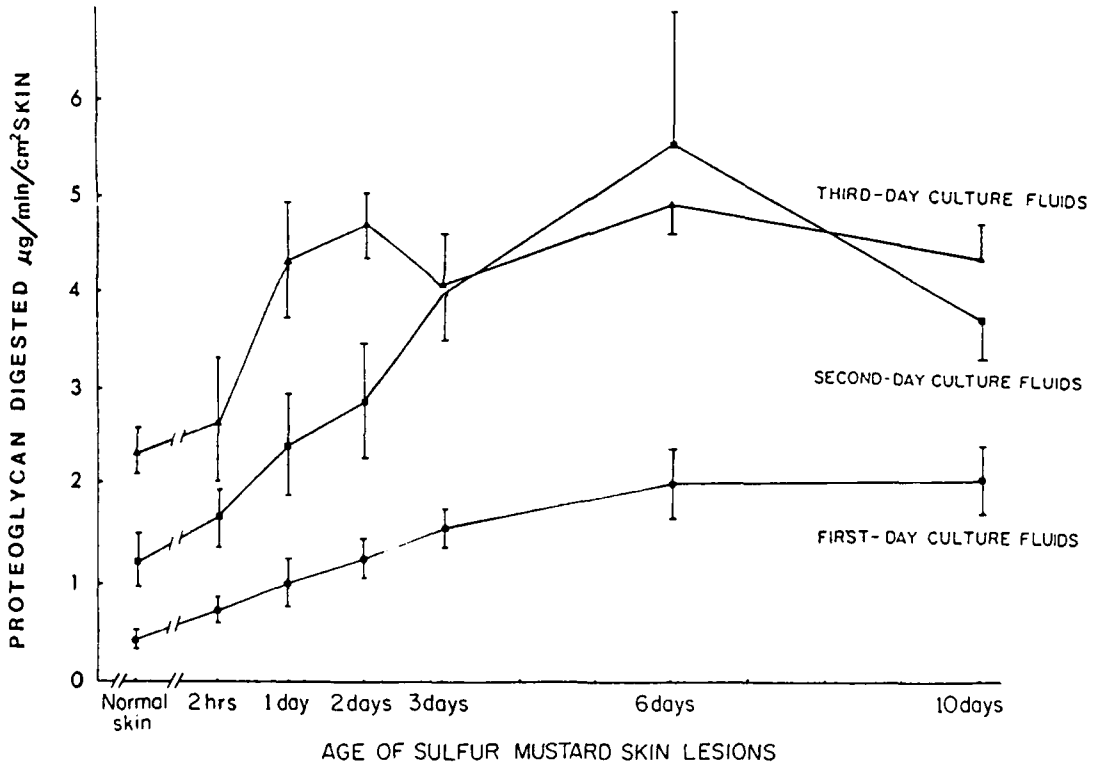


Figure 2. Proteoglycan digestion by organ-culture fluids from developing and healing SM lesions. One-cm^2 full-thickness lesion explants were cultured in 2.5 ml medium. The medium was replaced each day, and the first-, second- and third-day culture fluids (marked 1, 2 and 3) were assayed for proteoglycanase as described in Materials and Methods. The number of micrograms of proteoglycan (PG) released per minute from PG-containing polyacrylamide beads by 2.5 ml of lesion culture fluid (i.e., by each 1.0-cm^2 lesion explant) is shown on the ordinate. Depicted are the means (and their standard errors) of culture fluids from lesions from each of six rabbits. Statistical differences are presented in Results.



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CHAPTER 8

Extracellular Matrix Hydrolysis: Hexosamine-containing and Hydroxyproline-containing Products Released in Organ Culture by Dermal Sulfur Mustard Lesions

A collaborative project with Gerald W. Hart and Nancy M. Dahms of the Johns Hopkins School of Medicine and with Janet S. Kerr and Carol L. Ruppert of the Rutgers School of Medicine of the State of New Jersey in New Brunswick

ABSTRACT

Blister formation, a major occurrence following SM injury to the skin of human beings, is due to the separation of epithelial cells from their basement membrane and/or their separation from the corium beneath their basement membrane. Blistering is thought to be due to the digestion of these collagen-containing connective tissue structures by proteolytic enzymes, released by epidermal cells, local fibroblasts, and/or infiltrating leukocytes. Collagenase is thought to play a major role in blister formation, and enzymes hydrolyzing the ground substance are probably contributory.

Since all major proteolytic enzymes are inhibited in SM lesion culture fluids, we assayed these fluids for some of the products of hydrolysis, namely, (a) hydroxyproline-containing peptides, which are formed when collagen is hydrolyzed, and (b) glycosaminoglycans (GAGs), which are formed when ground substance is hydrolyzed.

The levels of OH-proline-containing peptides were markedly elevated in culture fluids from healing (6-day) lesions, whereas the GAG levels were highest in peak (1-day) lesions. The OH-proline levels in third-day culture fluids were higher than those in second-day culture fluids, which, in turn were higher than those in first-day culture fluids. In other words, the breakdown of collagen increased as the serum inhibitors were extracted from the explants and as granulocytes (and some of the other cells) died (releasing their collagenase).

CHAPTER 8

TABLE OF CONTENTS

INTRODUCTION

In Chapter 7, we described collagenase and proteoglycanase in organ-culture fluids from dermal SM lesions. These proteinases, evidently, acted locally near the cells (that produced them) and then were inactivated by the proteinase inhibitors in the extravasated serum within the lesions. These proteinases were inactive because they were complexed with proteinase inhibitors. To identify the collagenase and proteoglycanase, we had to restore their activities by inactivating these inhibitors.

In the present chapter, we provide direct proof (a) that active collagenase and active proteoglycanase (and/or glycosidases) had existed in the SM lesions, and (b) that before they were inhibited, these enzymes had hydrolyzed (locally) the collagen and ground substance within the lesions. We proved this by finding hydroxyproline-containing and hexosamine-containing breakdown products in SM lesion culture fluids.

MATERIALS AND METHODS

Assay of the hydroxyproline in SM lesion culture fluids (1)

OH-proline is almost unique to collagen, in that few other proteins contain this amino acid (1). The release of OH-proline-containing peptides into the culture fluids can therefore be used as a measure of collagen breakdown within the organ-cultured SM lesions.

The culture fluids (1.0 ml) were mixed with concentrated (12 N) HCl (1.0 ml) in screw-capped tubes with Teflon seals, and heated in an oven at 105 C for 18 hr. They were then evaporated to dryness with a stream of nitrogen under a hood. Distilled water (4.0 ml) was added, and the tubes were vortexed or sonicated to get all of the residue into solution. The samples were then frozen until assayed for OH-proline.

For this assay, chloramine-T reagent (1.0 ml) was added to each sample. (This reagent contained 2.82 gm of chloramine-T [Sigma Chemical Co., St. Louis, MO] in 50.0 ml of 2-methoxyethanol [Aldrich Chemical Company, Inc., Milwaukee, WI, catalog no. 15,620-5.) The reaction was allowed to proceed for exactly 25 min with repeated vortexing. Then 3.6 M sodium thiosulfate (3.0 ml) was added, and the tubes were vortexed again. Toluene (5.0 ml) was then added, and the tubes were vortexed again. After centrifugation, the toluene layer was removed and discarded. The tubes were capped, heated for 30 min in a boiling water bath, and then cooled. Toluene (5.0 ml) was again added and the tubes were shaken for 4 min and recentrifuged.

After centrifugation, 2.5 ml of the toluene layer was collected from each tube and mixed with 1.0 ml of a solution containing Ehrlich's reagent in a colorimeter tube. (This solution contains 120 gm of p-dimethylaminobenzaldehyde (Sigma Chemical Co.), 1200 ml of ethanol and 27.4 ml of concentrated sulfuric acid.) After exactly 25 min at room temperature, the optical densities were read in a spectrophotometer at 560 nm.

Various dilutions of hydroxyproline were always included as positive controls.

Assay of the glycosaminoglycans in SM lesion culture fluids (2-4)

First, the glycosaminoglycans were isolated, then they were hydrolyzed, and then their hexosamine content was determined with Ehrlich's reagent.

Isolation. Two ml of the lesion culture fluid was mixed with 18 ml of absolute alcohol, and stored overnight at -20 C. The sample was centrifuged at 2000 rpm (900 g) for 15 min, and the supernate was discarded.

One ml of 0.2 M Tris HCl buffer (pH 8.0), containing 2 mM $CaCl_2$ and 5% ethanol, was mixed with the sediment. Then the sample was placed in a boiling water bath for 10 min, in order to denature proteins and inactivate glycosidases and antiproteases.

The sample was then incubated in the water bath at 50 C for 24-48 hr, with 1.0 ml of Pronase. This enzymatic treatment digests the proteins bound to chondroitin sulfate. To prepare the Pronase solution, we incubated Pronase (2.0 mg per ml of 0.2 M Tris HCl buffer (pH 8.0) containing 2 mM CaCl and 4% ethanol) at 50 C for 30 min to remove the activity of glucosidase. (The Pronase was obtained from Cal-Biochem, Inc., because Pronase from some suppliers may contain starch.)

After the tube containing the sample was placed in an ice-water bath, trichloroacetic acid was added to make a final concentration of 10%, in order to precipitate the remaining protein. (Glycosaminoglycans (e.g., hyaluronic acid and protein-free chondroitin sulfate) are soluble in 10% trichloroacetic acid.) The sample was kept at 4 C overnight, and then centrifuged at 2000 rpm (900 g) for 15 min.

The supernate was placed into a dialyzing tube (with a cut-off of 2000 M_r) and dialyzed against distilled water at 4 C for 48 hr. The dialyzed sample was placed into a loosely capped glass tube and lyophilized.

The lyophilized sample was resuspended in 1.0 ml of distilled water. To hydrolyze it, we added an equal volume of 12 N HCl, tightened the cap of its tube, and heated it overnight in an oven at 105 C. The HCl solution was then evaporated with a stream of nitrogen in a boiling water bath.

The sample was dissolved in 0.5 ml of distilled water and 1.0 ml of the acetylacetone reagent was added. This reagent contained 1.5 ml of acetylacetone diluted with 1.25 N (6.25 M) Na_2CO_3 to a final volume of 50 ml. The sample was then heated in a water bath at 96 C for 60 min and cooled in tap water.

Absolute ethanol (10 ml) and the solution containing Ehrlich's reagent (1.0 ml) were then added. The tubes were shaken vigorously, left at room temperature for 60 min, centrifuged at 2000 rpm (900g) for 5 min, and read against a distilled water blank at 530 nm in a spectrophotometer.

This solution containing Ehrlich's reagent was similar to (but not the same as) the one used to detect OH-proline. It contained 1.6 gm of p-dimethylaminobenzaldehyde (Sigma Chemical Co.) dissolved in a 1:1 mixture of absolute ethanol and concentrated HCl (30 ml of each).

Glucosamine (Sigma Chemical Co.) served as our standard.

RESULTS

Hydroxyproline content of culture fluids from developing and healing SM lesions

Soluble hydroxyproline-containing peptides (and the free amino acid) can be used as a measure of collagen breakdown, because the OH-proline content of collagen is higher than that of any other body tissue.

By this criterion, we found that more collagen was hydrolyzed in healing SM lesions than in peak SM lesions (Figures 1 and 2).

The healing SM lesions hydrolyzed more collagen during the third day of organ culture than during the second day of culture, and even less during the first day of culture (Figure 1).

Glycosaminoglycans in culture fluids from peak and healing SM lesions

During acute inflammatory processes, the ground substance changes from a gel to a sol state (see Chapter 1). One would therefore expect a large increase in GAGs in organ-culture fluids from peak (1-day) SM and a return toward normal in healing lesions when the gel state was re-established. This trend is shown in Figure 3. Culture fluids from peak lesions contained about twice the GAGs of fluids from normal skin. GAGs in culture fluids from healing lesions had intermediate values.

The GAG concentration per milligram of protein in the culture fluids showed the reverse pattern. Fluids from peak lesions contained about half the levels of GAGs in fluids from normal skin, and those from healing lesions were again intermediate (Figure 4). Peak lesions contained a large amount of extravasated serum. The high protein content of such extravasated serum evidently lowers the GAG levels per mg of protein. In fact, such levels per milligram of protein in culture fluids from peak lesions approximate the level in serum itself.

DISCUSSION

We can explain the results of our hydroxyproline studies as having been caused by a progressive removal (from the lesions) of extravasated serum proteinase inhibitors: the culture fluids were replaced daily with fresh (serum-free) culture media. In addition, it appears that continued production and/or release of collagenase (or activation of latent collagenase) occurs during organ culture (see Chapter 7). Activated fibroblasts are probably the main source of collagenase activity, but epidermal cells, macrophages and granulocytes may be minor sources. Collagenase is probably the main enzyme responsible for blister formation in sulfur mustard lesions.

With respect to GAGs, we were surprised that the peak lesion culture fluids did not contain a 10-fold increase in GAGs, considering the marked difference in the physical characteristics of ground substance after it changed from the gel to the sol state. Evidently, hyaluronic acid and chondroitin sulfate, the large molecules that make up the bulk of the ground

substance, were not extensively hydrolyzed. They changed their physical characteristics because of the extravasated serum, but remained in situ, attached to the matrix of collagen fibers (5,6). In other words, ground substance in the sol state was not a true solution. It was a 'soupy' gel, containing a great deal of fluid and protein, loosely bound to still-adherent proteoglycan molecules.

The loosely bound fluid could help form, and readily enter, the cavities of blisters produced by SM.

Figure 1. Hydroxyproline concentration (ug/ml) in organ-culture fluids from normal skin and from developing and healing SM lesions. The OH-proline in these culture fluids can be considered a measure of collagen breakdown within the lesions. The culture fluids from healing (6-day) lesions contained more OH-proline than did culture fluids from peak (1-day) lesions ($P < 0.05$). With 2-, 3-, 6-, and 10-day lesions, the third-day culture fluids contained more OH-proline than did second-day culture fluids ($P < 0.05$), which in turn often contained more OH-proline than did first-day culture fluids.

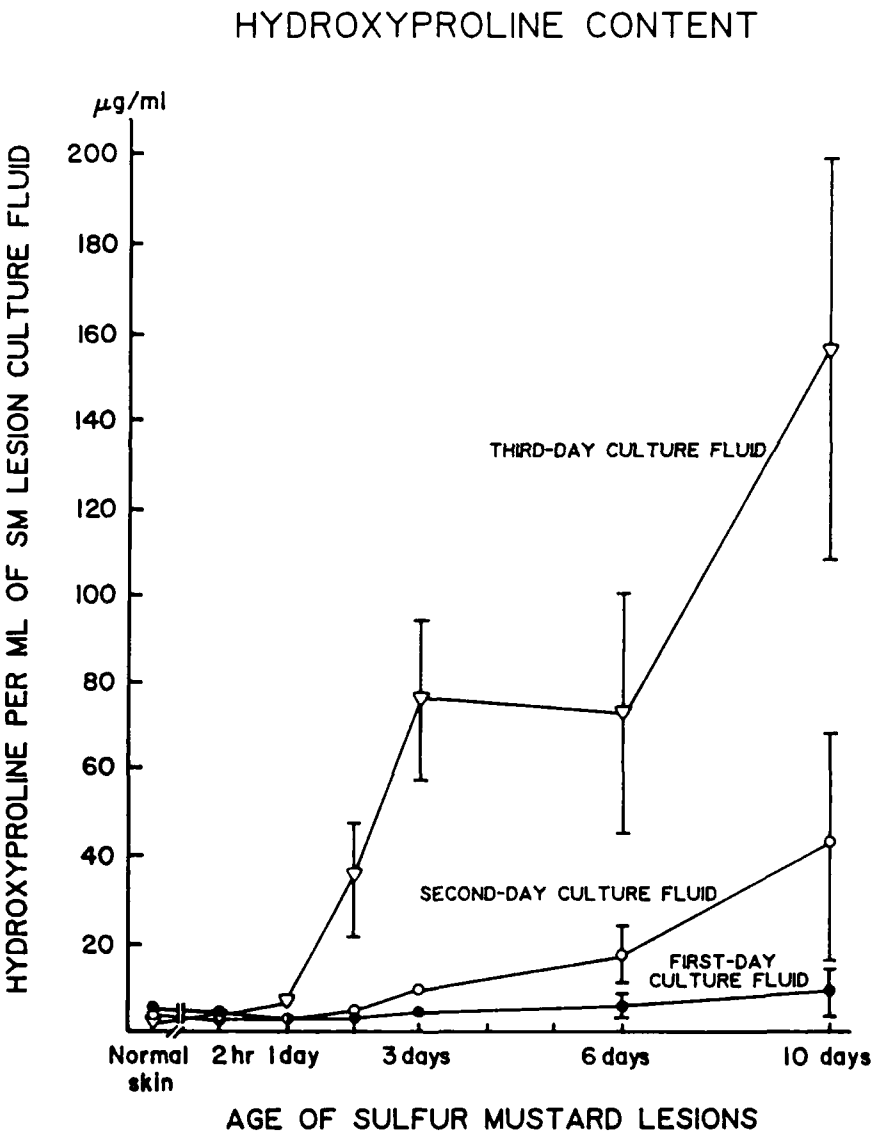


Figure 2. Hydroxyproline concentration (ug/ml) in first-day organ-culture fluids from normal skin and from developing and healing SM lesions. This graph is the same as in the lower graph shown in Figure 1, but it was drawn on an expanded scale. The OH-proline contents of culture fluids from normal skin and from 6-day SM lesions were significantly higher than that from 1-day lesions ($P < 0.002$ and < 0.001, respectively).

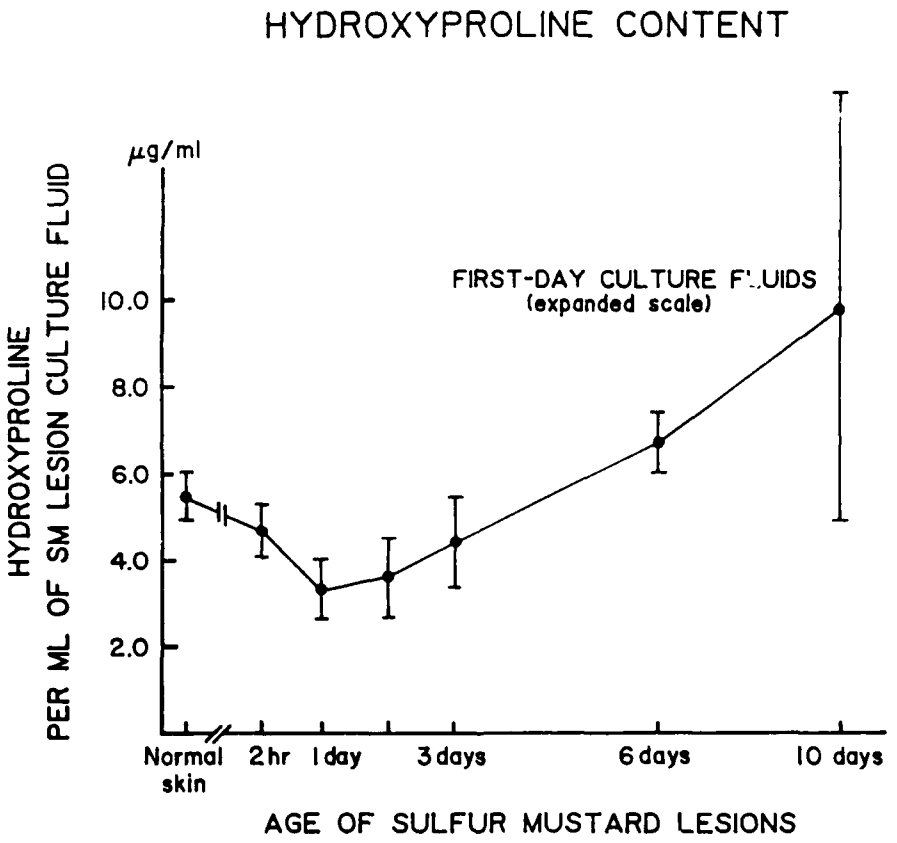


Figure 3. Glycosaminoglycan concentration (ug/ml) in serum (diluted 1:40), and in organ-culture fluids from normal skin, 1-day (peak) SM lesions and 6-day (healing) SM lesions. The GAG concentration in 1-day lesions was significantly higher than that in normal skin ($P < 0.05$). Serum contained appreciable amounts of GAGs, probably because of its numerous glycoproteins. A 1:40 dilution of serum is within the range of the extravasated serum in culture fluids from peak lesions.

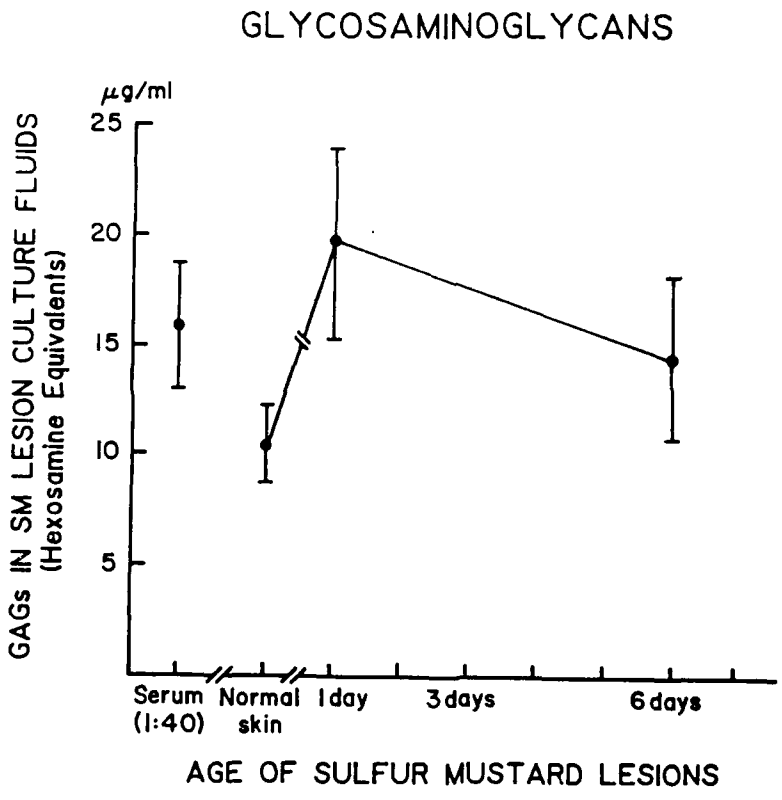
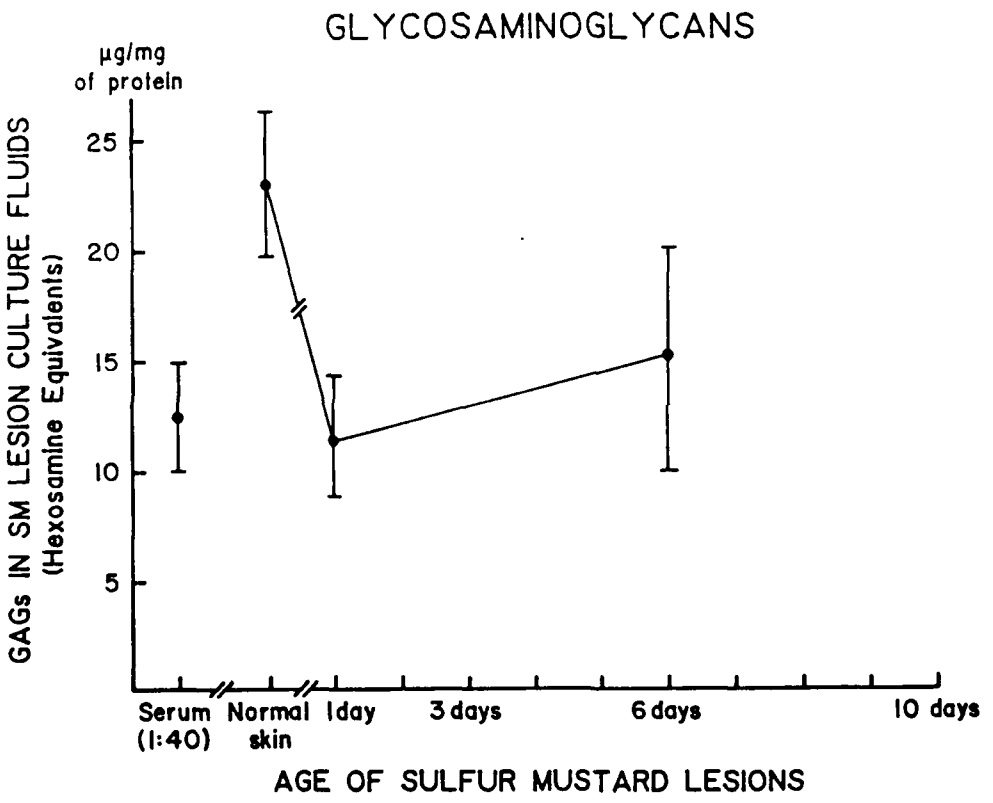


Figure 4. Glycosaminoglycan concentration (ug per mg of protein) in serum, and in organ-culture fluids from normal skin, 1-day (peak) SM lesions and 6-day (healing) SM lesions. The GAG concentration in 1-day lesions was significantly lower than that in normal skin ($P < 0.01$), probably because of the large amount of extravasated serum in these lesions.



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PERSPECTIVES

On this basic research

Our studies are just a few among many possible studies on the mediators and modulators regulating SM injury, inflammation and repair. As is characteristic of science, these studies call forth as many questions as they have answered. Some of these questions are listed here as a guide for further research.

What are the roles of each of the numerous proteinases in SM lesions? Which ones cause injury? Which ones stimulate repair? Are they self-regulatingμ e.g., do active proteinases cause vascular leakage so that larger quantities of serum inhibitors enter the lesions? If so, do proteinases produce this effect by directly acting on the vascular endothelium, or do proteinase-inhibitor complexes or the products of proteinase action (peptide fragments) do so? What is the role of various proteinases in macrophage and fibroblast activation and in the epithelial cell and endothelial cell proliferation associated with repair? Proteinases are known to control cell proliferation.

In addition, the hydrolysis of proteins, the hydrolysis of nucleic acids, lipids and polysaccharides must play a role in SM inflammation and repair. Phlogistic agents are known in each group.

The state and composition of the ground substance is important (see Chapter 1). It preferentially binds certain inflammatory mediators and modulators and not others. Its πsolπ state allows a rapid influx of phagocytes, and a faster exchange of regulating substances. Its πgelπ state is an important stabilizing factor and a likely stimulator of repair.

Phagocytes produce oxidants which may further injure tissues. The extravasated serum contains antioxidants, e.g., ceruloplasmin and haptoglobin, which protect tissues. The control of such oxidants needs to be investigated.

What receptors exist on the various cells in the SM lesions? Almost all mediators and modulators act by combining with specific receptors, and such receptors are up-regulated and down-regulated during different stages of the inflammatory and repair processes. Research on such receptors is in its infancy, and yet such information can lead (and has led) to many an effective therapeutic agent.

On treatment of skin lesions caused by sulfur mustard

We strongly recommend more interaction between medically oriented basic research scientists and the clinicians who set the guidelines for therapy of sulfur mustard casualties. Such interactions could provide a better understanding of current therapeutic procedures and suggest new ones. For example:

(a) the application of cold to the skin soon after SM exposure could reduce the amount of injury. (A Freon spray can could be carried into the battlefield.) If cold therapy is successful, the basic scientist could find out why it works. Less DNA damage? Less vascular leakage? Less infiltration by leukocytes (and, therefore, less of their damaging oxidants)?

(b) The application of heat later in the therapy could hasten healing. If such heat therapy is successful, what is its basis? More rapid growth of epidermal cells? Fibroblast stimulation? Faster angiogenesis? Greater amounts of serum and their proteinase inhibitors?

(c) The use of wet or dry dressings could have a rational basis, if we found out whether they increased or decreased beneficial mediators (growth factors) released from the lesion crusts. (By bringing them into solution, wet dressings could either remove them or stimuate their entry into the injured tissues.)

* * * * * * *

In summary, SM injury, inflammation and repair is a complex process. Yet like any other injurious process, it should be subject to therapeutic control. Understanding of the basic mechanisms involved should lead not only to a more precise use of existing therapies but also to the development of new types of therapy.

PUBLICATIONS RESULTING FROM THE STUDIES DESCRIBED IN THIS REPORT

1. Dannenberg AM Jr, Pula PJ, Liu LH, et al. Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced in vivo by sulfur mustard. I. Quantitative histopathology; PMN, basophil and mononuclear cell survival; and unbound (serum) protein content. Am J Pathol 121: 15-27, 1985.

2. Harada S, Dannenberg AM Jr, Kajiki A, Higuchi K, Tanaka F, Pula PJ. Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced in vivo by sulfur mustard. II. Evans blue dye experiments that determined the rates of entry and turnover of serum protein in developing and healing lesions. Am J Pathol 121: 28-38, 1985.

3. Harada S, Dannenberg AM Jr, Vogt RF Jr, et al. Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced in vivo by sulfur mustard. III. Electrophoretic protein fractions, trypsin-inhibitory capacity, α_1-proteinase inhibitor and α_1- and α_2-macroglobulin proteinase inhibitors of culture fluids and serum. Am J Pathol 126: 130-145, 1987.

4. Tanaka F, Higuchi K, Dannenberg AM Jr, et al. Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced in vivo by sulfur mustard. Chemotactic activities for granulocytes and monocytes. (in preparation)

5. Kajiki A, Higuchi K, Nakamura M, Liu LH, Pula PJ, Dannenberg, AM Jr. Estimates of cellular and serum sources of extracellular lysosomal enzymes in developing and healing inflammatory lesions, induced in rabbit skin by sulfur mustard: (a) Acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme (and lactic dehydrogenase) released by organ-cultured lesions, (b) histochemical-biochemical correlations, and (c) lysosomal enzymes released by cultured granulocytes, macrophages and fibroblasts. J Leukocyte Biol 43: 104-116, 1988.

6. Higuchi K, Kajiki A, Nakamura M, Harada S, Pula PJ, Scott AL, Dannenberg AM Jr. Proteases released in organ culture by acute dermal inflammatory lesions produced in rabbit skin by sulfur mustard: Hydrolysis of synthetic peptide substrates for trypsin-like and chymotrypsin-like enzymes. Inflammation 12: 311-334, 1988.

7. Woessner JF Jr, Higuchi K, Kajiki A, Pula PJ, Dannenberg AM Jr. Proteoglycanase and collagenase released in organ culture by acute dermal inflammatory lesions produced by sulfur mustard. (Submitted to J. Invest. Dermatol. and tentatively accepted)

8. Pula PJ, Ruppert CL, Dannenberg AM Jr, et al. Hexosamine-containing and hydroxyproline-containing extracellular matrix components released in organ culture by acute dermal inflammatory lesions produced by sulfur mustard. (Submitted to J. Invest. Dermatol. and tentatively accepted)

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