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

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This is the third report in a series on the inflammatory mediators and modulators released in organ culture from skin lesions of various ages, which were produced *in vivo* in rabbits by the military vesicant, sulfur mustard (SM). It describes the electrophoretic protein fractions and trypsin-inhibitory capacities of the various culture fluids and the amounts of α_1-proteinase inhibitor and α-macroglobulin proteinase inhibitors in these fluids. 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 fractions of serum at *all* stages of lesion development and healing. The γ-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

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were 80–90% of serum origin. The trypsin-inhibitory capacity (TIC) per milligram protein in the culture fluids of *healing* lesions was markedly less than the TIC per milligram 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_1-PI) (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, Western blots, specific antibodies, and the immunoperoxidase technique. The levels of both free and proteinase-complexed α_1PI and 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 unbounded 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. (Am J Pathol 1987, 126:148–163)

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 (SM) 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] eg, α_1-proteinase inhibitor (α_1PI) (formerly called α_1-antitrypsin), α_1- and α_2-macroglobulin (the αMs), C1 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. (Alpha$_1$-macroglobulin 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 SM lesions (produced *in vivo* in the skin of rabbits). These experiments also identify α_1PI and the αMs as major sources of the trypsin-inhibitory capacity (TIC) of these culture fluids.

Materials and Methods

Production and Organ-Culture of SM Lesions

SM (1% in methylene chloride) was received from the United States Army Medical Research Institute of Chemical Defense at Aberdeen Proving Ground, Maryland. With a Hamilton syringe, 7.5 μl of the SM solution was applied, at various times, to many sites on the clipped back and flanks of rabbits,[3] so that 2-hour 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 (1) for obtaining 1.0-sq cm biopsy specimens of these SM lesions, (2) for organ-culturing them in serum-free medium RPMI 1640, and (3) for preparing glycol methacrylate-embedded tissue sections (for histologic studies) were described in the first paper of this series.[3]

One-Dimensional Gel Electrophoresis

Agarose Gel Electrophoresis (to measure the albumin, α_1-, α_2-, and γ-globulins in the culture fluids)

The composition of the proteins in the culture fluids was determined by slab-type gel electrophoresis.[7] One milliliter of each sample was concentrated 10–20-fold by ultrafiltration with an Amicon CS-15 filter (Amicon Corporation, Lexington, Mass). Then, 3–4 μl of the sample was placed on a thin sheet of agarose gel (Corning Agarose Universal Gel Films, Cat. No. AC470100, Fisher Scientific, Pittsburgh, Pa) and electrophoresed in the Corning Electrophoresis Starter System (Cat. No. 09-529-201C, Fisher) for 25 minutes at 100 V in a 0.05 M sodium barbital buffer (pH 8.6) (Cat. No. AC470180, Fisher). The gels were stained for 20 minutes in 1% Amido Black 10B (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, Tex), and the results are represented in Figure 1.

Sodium Dodecyl Sulfate—Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Undiluted SM lesion culture fluids (8, 4, 2 μl) or rabbit serum (1 μl of a 1 : 40 dilution) were mixed with

15 μl 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 hour at 37 C and then electrophoresed with a 25 m A current for 45 minutes at 23 C in a Mini-Vertical Slab Cell (Cat. No. 165-1700, Bio-Rad Laboratories, Richmond, Calif) 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 hour 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 minutes at 23 C in 25 mM Tris buffer (pH 8.6), 200 mM glycine, and 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-milliliter samples of culture fluids from SM lesions and of serum (diluted 1:4) were centrifuged at 5000*g* for 10 minutes. One part of the supernate was mixed with three parts of the diluent. The diluent contained 2% SDS, 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 minutes, 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). 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), 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 Dannenberg et al[3]), and 20.0 μg of protein was applied to the first dimension of the acrylamide gel (about 4 μl for the serum samples and 10–50 μl for the culture samples). Isoelectric focusing was conducted at 700 V for 17 hours. The second dimension of the acrylamide was electrophoresed at constant 0.06 ampere for 3.5–4.0 hours. 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) 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 A for 3 hours in a transfer chamber (Cat. No. TE 42, Hoefer Scientific Instruments, San Francisco, Calif) 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 V for 16 hours. 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_1PI was supplied by Dr. Friedrich Kueppers, Department of Medicine, Temple University (Philadelphia, Pa). It 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, Ill). It, too, was produced in goats, with purified αM in 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 hour at 37 C on a rocking platform. Then they were similarly incubated 1–4 hours at 37 C in goat antisera against α_1PI or α_1M–α_2M, 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) was diluted 1 : 100 in blocking solution and incubated with the appropriate blots for 1 hour 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 4-chloro-1-naphthol in 4 ml of methanol and 16 ml of PBS. The substrate solution was activated by adding 20 μl of 30% H_2O_2, and the color reaction was allowed to develop for 15 minutes 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) had a specific activity of 2.3 μCi/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., St. Louis, Mo).

Trypsin Solution

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

Procedure

The culture fluids were diluted with RPMI 1640 to make a protein concentration of 30 μg/ml, and 100 μl was placed in a 12 × 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 Dannenberg et al[3]). Buffer (355 μl of 0.056 M Tris, pH 8.0) and trypsin solution (20 μl) were added and mixed with the culture fluids. After 10 minutes at room temperature, the tubes were placed in an ice bath, and ^{14}C-casein (25 μl working substrate) was added. Then the solutions were incubated by shaking for 90 minutes 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 hours.) 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 minutes. 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 minutes for ^{14}C radioactivity in a Beckman LS 7500 liquid scintillation counter (Beckman Instruments, Inc., Irvine, Calif).

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 micrograms of trypsin inhibited by 1.0 ml of culture fluid from 1.0-sq cm skin biopsy specimens cultured in 2.5 ml.

The calculations were made according to these formulas:

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

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

A straight-line relationship existed between 20% and 80% inhibition of the 0.05 μg of trypsin used. Our experiments were performed in this range. When 0.078 μl of rabbit serum was diluted to 100 μl with RPMI 1640, 0.05 μg of trypsin was inhibited by 50%. This amount of serum (0.078 μl) 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 *t* test was used. We first examined the data 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 γ-globulin—were identified in the culture fluids by agarose gel electrophoresis. When compared with *normal skin*, the *peak* (1-day) *lesions* showed a 2.8–4.8-fold *increase* in the concentration of protein in each fraction (Table 1).

When compared with 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 γ-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 is 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% ($3.0 \div 4.7 \times 100\%$) of the α_1-globulin fraction of serum protein. Such percentages for first-day culture fluids are 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 significantly 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).

Table 1—Concentrations of the Major Electrophoretic Fractions of First-Day Culture Fluids From Normal Skin and From Peak and Healing SM Skin Lesions*

Electrophoretic fraction	Source of culture fluid: Normal skin (μg/ml)	Peak (1-day lesions (μg/ml)	Healing (6-day) lesions (μg/ml)	Peak lesions concentration/ normal skin concentration	Healing lesions concentration/ peak lesion concentration
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
γ-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

*The skin explants, 1.0 sq cm in size, were cultured in 2.5 ml of supplemented RPMI 1640 (see Dannenberg et al[3]). The culture fluids were electrophoresed in 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 sera from 6 rabbits were fractionated for preparation of this table. These sera had a mean protein concentration of 60.6 ± 1.1 mg/ml. If the total protein in the culture fluids of 1-day lesions were translated into a serum equivalent, the 1-day SM lesions (1.0 sq cm) would contain about 35% serum by weight.

The *P* values for 1-day versus 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 γ-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 *t* test was used.

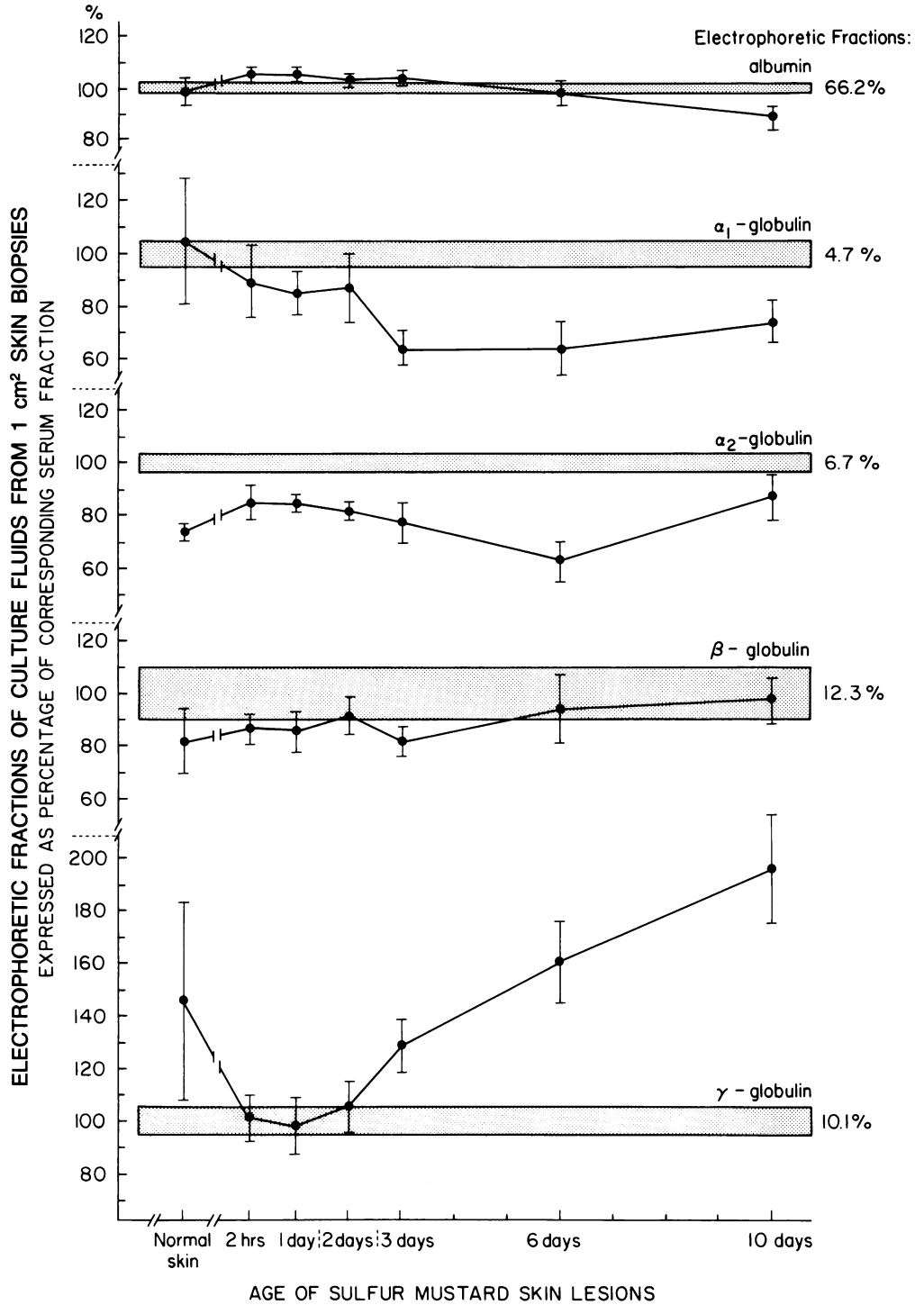


Figure 1 — Electrophoretic fractions of first-day culture fluids from 1.0-sq cm dermal SM skin lesions of various ages from 6 rabbits. On the left, the albumin, α_1-globulin, α_2-globulin, β-globulin, and γ-globulin fractions of the protein in first-day culture fluids are expressed as a percentage of the corresponding fraction of the serum protein from these 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 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 γ-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 t test was used.

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). Alpha$_2$-macroglobulin is the major proteinase inhibitor in the serum α_2-globulin fraction (see Discussion).

In contrast, the γ-globulin fraction of the protein in culture fluids from normal skin and from 3-, 6-, and 10-day SM lesions was significantly *larger* than the corresponding fraction of serum protein (Figure 1). However, during the *acute* phases of the lesions (at 2 hours and on Days 1 and 2), this γ-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 Dannenberg et al[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 1) normal skin, 2) a 1-day SM lesion (Figure 2), and 3) a 6-day SM lesion, from each of three rabbits. Because of the relatively low amount of protein loaded onto the gel (2.0 μg), most of the silver-stained protein could be resolved as distinct spots, 300–500 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 and in those containing culture fluids of 1-day lesions. Because 2.0 μg of protein was applied in each case and because 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. We evaluated these images 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: 46% 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.

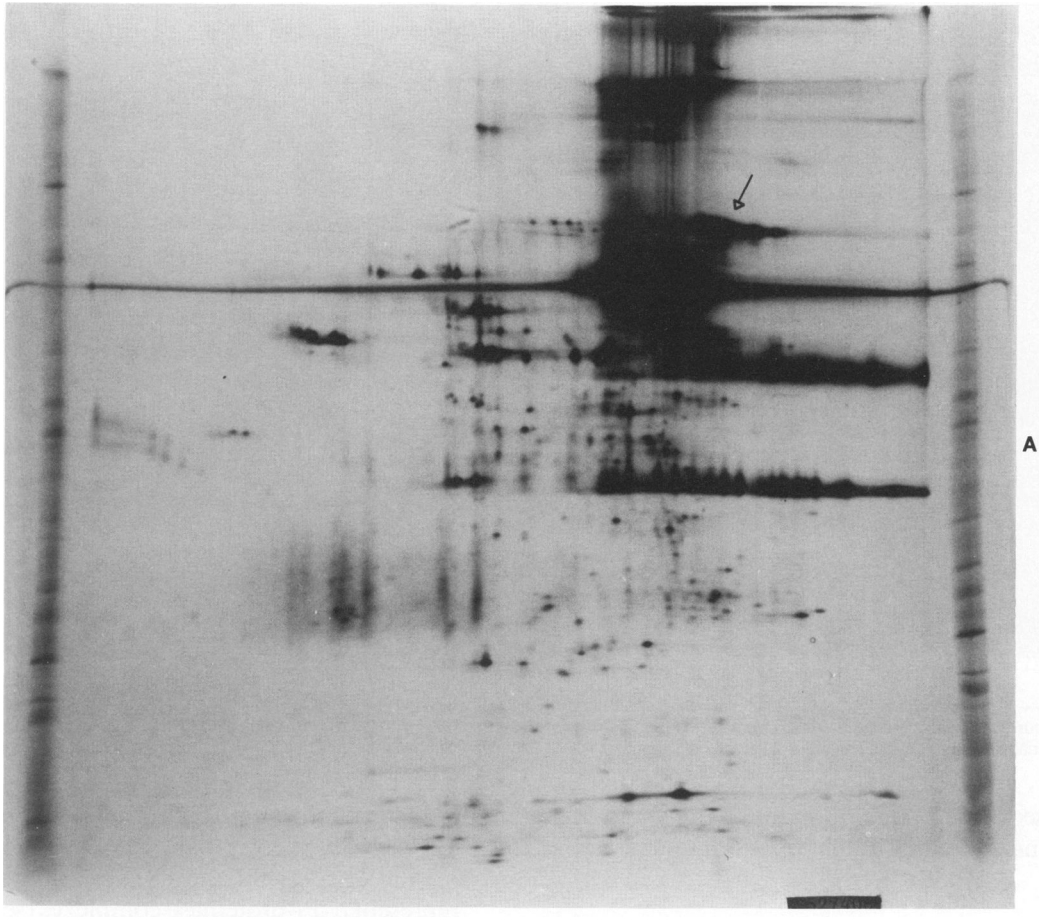
No attempt was made to enumerate the family of spots representing polydispersed albumin, because their protein concentration was too high. In all culture fluids, the pattern of the albumin spots resembled that found in serum. About two-thirds of the total protein in every culture fluid was serum albumin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

Western Blots to Identify α_1PI and the αMs 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_1PI and with an antibody specific for the two rabbit

Figure 2A—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, where the location of a given spot on the gel is represented by an ellipse and the amount of protein in the spot (ie., 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" 10–15 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.



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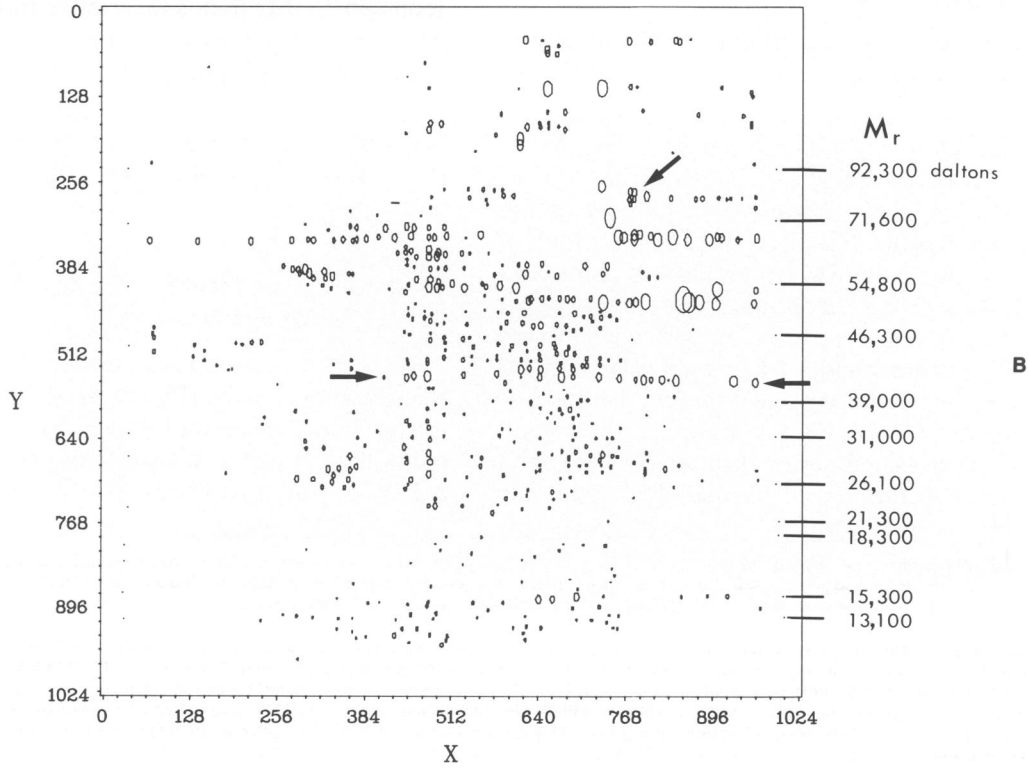
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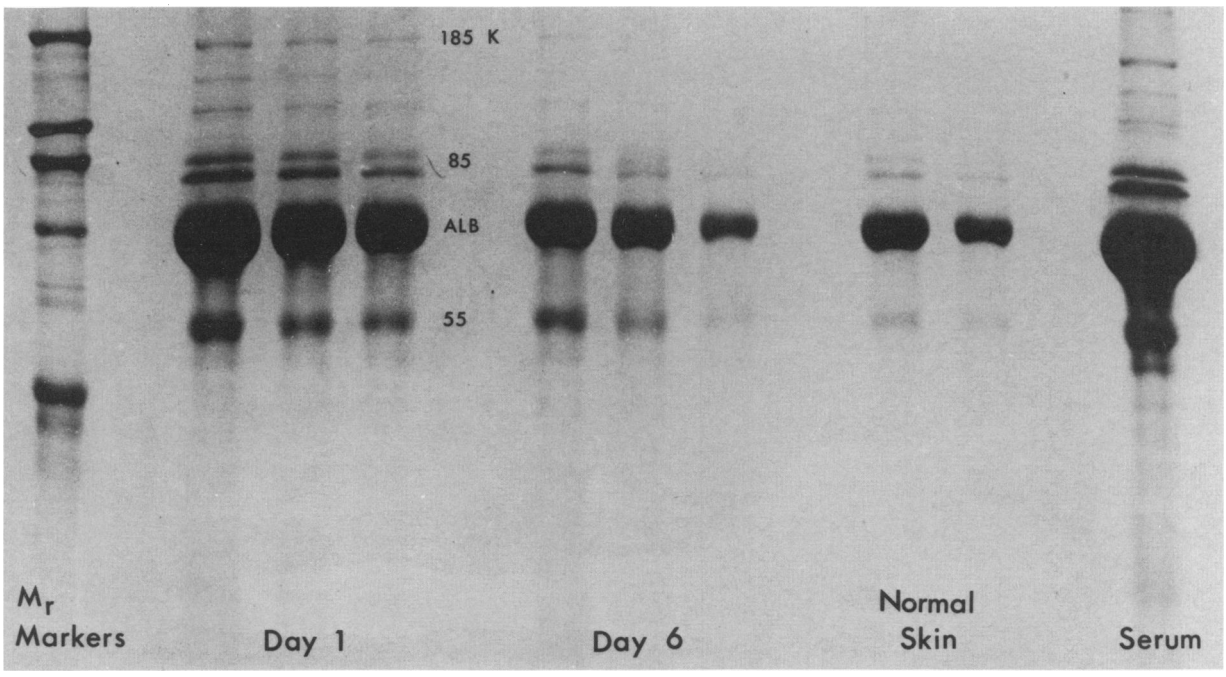

Figure 3—A polyacrylamide gel slab, containing prestained high molecular weight (Mr) markers (Catalog No. 6041SA, Bethesda Research Laboratory, Gaithersburg, Md), undiluted culture fluids from 1- and 6-day SM lesions (8, 4, and 2 μl) and from normal skin (8 and 4 μl), and serum (1 μl, diluted 1 : 40), after one-dimensional electrophoresis and staining with Coomassie blue. The 65K band is serum albumin (ALB). SDS-PAGE with mercaptoethanol.

αMs (see Materials and Methods). In the rabbit, both α_1M and α_2M are proteinase inhibitors (see Discussion).

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 Mr band probably represents free α_1PI.[21] The 71,000 and 88,000 Mr bands, probably represent α_1PI-proteinase complexes (see Laurell and Jeppsson[22]). (Mr stands for relative molecular mass.) All three α_1PI bands were most intense in gels prepared from peak lesions, less intense in gels prepared

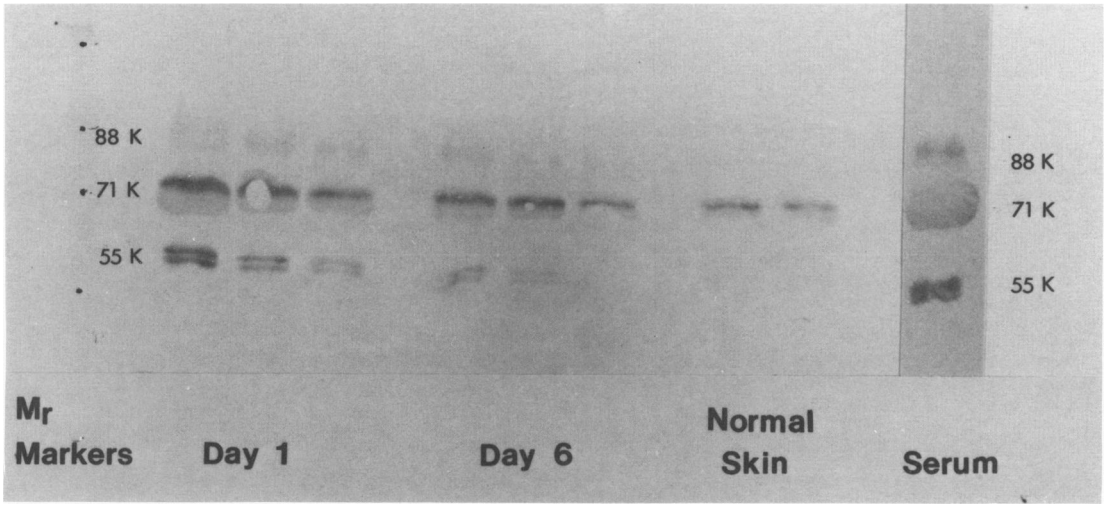

Figure 4—A Western blot of an acrylamide gel, containing first-day SM lesion culture fluids and serum in the amounts described in Figure 3, stained by immunoperoxidase technique utilizing an antiserum specific for rabbit α_1PI. The 55K bands probably represent free α_1PI. The 71K and 88K bands probably represent α_1PI complexed with proteinases, since treatment with SDS and mercaptoethanol does not dissociate such complexes.[46] In other Western blots, the two 55K bands were fused into one band. The 8-, 4-, and 2-μl samples on the gel (see Figure 3) enabled us to estimate the relative concentrations of α_1PI in culture fluids from 1-day and 6-day lesions and from normal skin. They were in a ratio of about 9 : 3 : 1 with 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.

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 accurate 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* $\alpha_1M-\alpha_2M$ (and the immunoperoxidase technique) was a band of about 85,000 Mr (Figure 5). The next major area stained by the αM antiserum consisted of multiple bands of lower Mr. A 185,000 Mr 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 Mr components when treated with SDS under reducing conditions.[21,23,24]

A simplified (but reasonable) interpretation of these results would be 1) that the 85,000 Mr band contained αM that had bound (or complexed with proteinases, 2) that the 185,000 band contained αM that had not bound proteinases, and 3) that the bands of lower Mr contained hydrolytic products of both free αM and proteinase-complexed αM (see Discussion). The ratio of the 85,000 Mr 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 Woessner et al[25] and Pula et al[26]), associated with the remodeling of connective tissue. In all culture fluids, at least half of the αM was present in the gels as the 85,000 Mr band. In other words, considerable amounts of αM were complexed with proteinases (see Discussion).

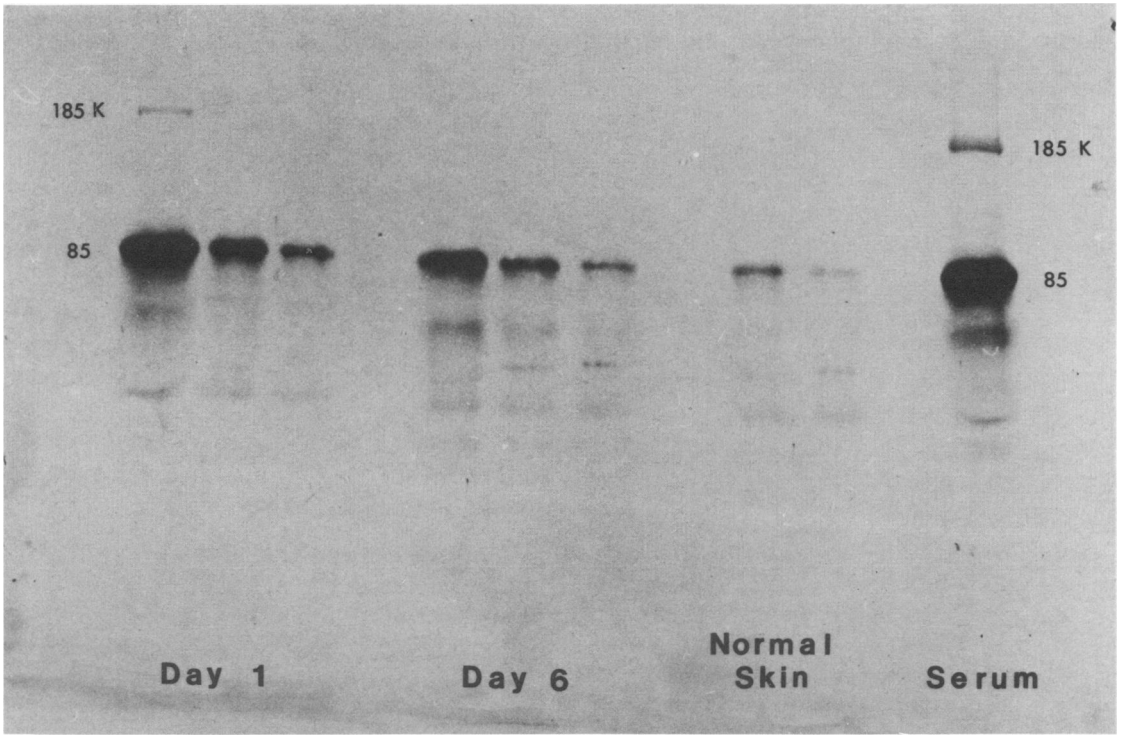

Figure 5—A Western blot of an acrylamide gel, containing SM lesion culture fluids and serum in the amounts described in Figure 3, stained by the immunoperoxidase technique utilizing an antiserum specific for rabbit $\alpha_1M-\alpha_2M$. The 85K band is thought to be a subunit of αM that had been split 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 by 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 convalently bound should not migrate within the 85K band. The 185K band represents αM that had not been complexed with a proteinase. Bands of 68K and below probably represent fragments derived from spontaneous autolytic cleavage of αM and αM-proteinase complexes. The 8-, 4-, and 2-μl samples on the gel (see Figure 3) enabled us to estimate the relative concentration 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.

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

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, and 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 lesions, which are known to contain large amounts of serum[3,4] (see Discussion).

Trypsin Inhibitory Capacity (TIC) of First-Day Culture Fluids of Rabbit SM 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).

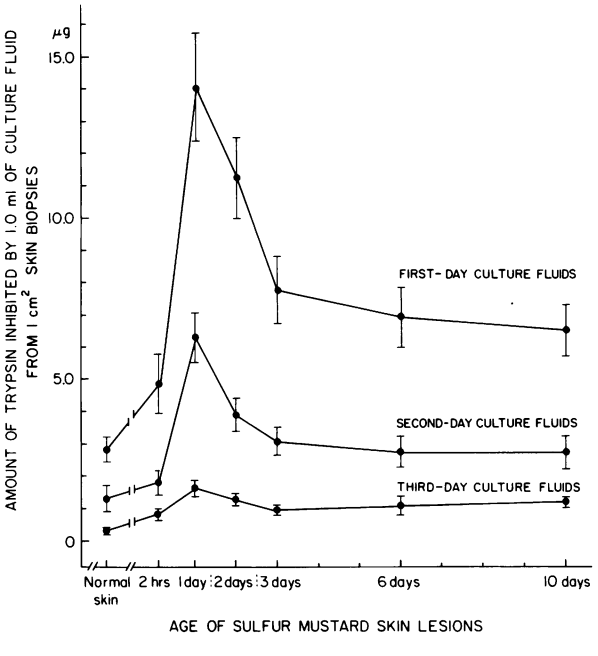

Figure 6 — Trypsin inhibitory capacity in first-, second- and third-day organ culture fluids of 1.0-sq cm dermal SM lesions of various ages from 6 rabbits. The means and their standard errors are depicted. The trypsin inhibitory capacities of organ culture fluids from 1-, 2-, 3-, 6-, and 10-day SM lesions 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-sq cm skin biopsy was cultured in 2.5 ml of RPMI 1640.

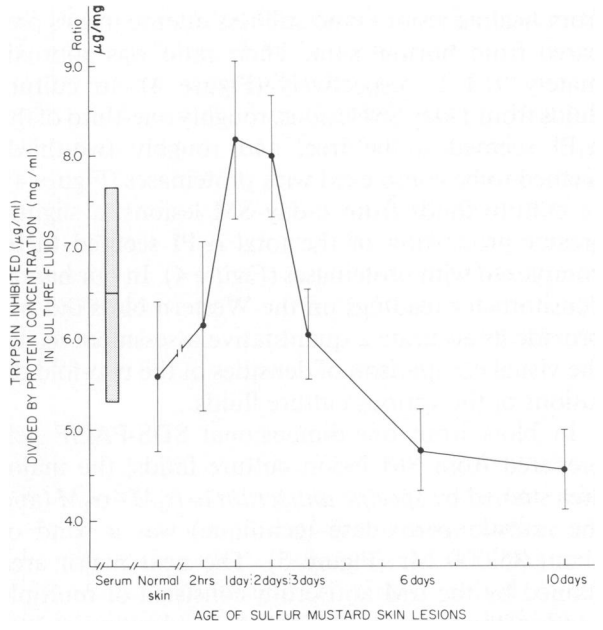

Figure 7 — Micrograms of trypsin inhibited per milligram protein in sera and in first-day culture fluids of 1.0-sq cm dermal SM lesions of various ages from six rabbits. The means and their standard errors are depicted. Culture fluids from 1- and 2-day (peak) lesions showed greater trypsin inhibitory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

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

TIC of Second- and Third-Day Culture Fluids of Rabbit SM Lesions of Various Ages

We evaluated the second- and third-day fluids in order to determine whether the explants themselves produced trypsin-inhibitory substances. Since most of the unbound "serum" inhibitors would be extracted by the first-day culture fluids, an increase in inhibitor levels per milligram 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 Dannenberg et al[3]) and also reduced trypsin inhibitory capacities (Figure 6). The "TIC/milligram protein" pattern in the second- and third-day culture fluids resembled the pattern in first-

day culture fluids (shown in Figure 7). That is, the second- and third-day culture fluids of peak lesions showed the *same* TIC per milligram protein as serum, and such culture fluids from 6- and 10-day lesions showed less, not more, TIC per milligram protein (data not shown).

These findings suggest 1) that the cells in these inflammatory lesions did not synthesize and release large amounts of proteinase inhibitor(s), and 2) that serum was the major source of the extracellular inhibitor(s) present.

TIC of Rabbit Serum

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

Discussion

Developing and healing rabbit dermal 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 80–90% of the total protein was similar to that in serum (Figures 1 and 2). Serum albumin was the main component.

The α_1PI and the α_1M and α_2M in these culture fluids were measured by means of Western blots, specific antibodies, and the immunoperoxidase technique. The amount of these inhibitors (per milligram protein) in the lesion culture fluids was 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 Harada et al[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]

Normal Skin

In the extracellular fluids (ie, 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 smaller than that of serum, and the γ-globulin fraction was greater than that of serum (Figure 1).

Dermal SM Lesions

Greater amounts of each serum protein fraction were present in the extracellular fluids of *developing and peak lesions* than were present in normal skin and healing lesions (see Dannenberg et al[3] and Harada et al[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 γ-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, probably because the α_1PI–proteinase complexes left the α_1-globulin fraction, possibly entering the β-globulin fraction (see Ohlsson[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 (Mr) of 725,000 daltons, probably did not extravasate into the lesions (or normal skin) as readily as did α_1PI, a 55,000-dalton protein.[21]

γ-Globulin

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 Dannenberg et al[3]). In developing and peak lesions, this ground substance seemed to be in a "sol" state (see Dannenberg et al[3]). When electrophoresed, the γ-globulin that is eluted from the *fixed* "gel" state by the culture fluids should migrate normally, but the γ-globulin that is bound to *unfixed* "sol" state

ground substance should appear in the culture fluids as a complex. This complex should not migrate with the γ-globulin fraction, but migrate more slowly (perhaps with the albumin fraction, where its low percentage would not be noticed).

The host's serologic defense (antibodies) against infectious agents resides almost entirely within the circulating γ-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.

TIC and α_1PI and αM in Culture Fluids

In the plasma of normal rabbits, α_1PI accounts for about 86% of the TIC[34] (see Kueppers et al[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 Berne et al[35] and Starkey[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 Berne et al[35] and Panrucker and Lorscheider[39]).

Sera from normal rabbits and sera from rabbits bearing multiple dermal SM lesions had the same TIC per milligram protein: 10.2 ± 0.8 and 9.1 ± 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 Got et al[36]).

In culture fluids from SM lesions, the total TIC and TIC per milligrams 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 Harada et al[4]) and partly due to combining with proteinases associated with the remodeling part of healing (see Woessner et al[25] and Pula et al[26]) and their subsequent clearance.

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 Mr fragment that apparently results from proteinases splitting a peptide bond in the "bait" region of the 185,000 Mr subunits.* The 85,000 Mr 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 Harpel,[23] Wang et al,[24] and Harpel[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 Mr of about 68,000. These lower Mr bands were probably fragments of free and proteinase-complexed αM derived from spontaneous autolytic cleavage.[23,41,42] Such fragments could be formed both *in vivo* in the blood and lesions and *in vitro* in the skin explants and during the SDS-PAGE procedures.

The existence of these lower Mr bands in the SDS gels makes 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 Mr suggests that a substantial amount of the αM was present as αM-proteinase complexes (Figure 5).

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)

*A conformational change occurs when the "bait" region of the 185,000 Mr subunit of α_2M 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 high molecular weight. (The proteinase will still hydrolyze small peptide substrates up to 8000–10,000 Mr[20,45] and the terminal regions of larger proteins, such as trypsinogen, plasminogen, and fibrinogen.[21] A percentage (8–61%, depending on the proteinase[42]) also becomes irreversibly bound when an αM internal γ-glutamyl thioester of cysteine reacts nonenzymatically with a lysyl side chain of the proteinase to form a covalent bond.[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, affect the catalytic action of the bound proteinase on small peptide substrates.[21,23]

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 6000–8000 Mr peptide from native α_1PI, which subsequently undergoes rearrangement and (covalent) acyl bond formation with the hydroxyl group of the serine in the proteinases's active site (see Heimburger[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 oxidants, 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]

than we had reason to expect from published studies, in which somewhat different techniques had been employed (see Ohlsson[53] and Ohlsson et al[54]).

Our results with αM-proteinase complexes are, however, in agreement with the unpublished results of experiments by Drs. Doina Ganea and Katherine L. Knight at 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 Ohlsson[53] and Ohlsson et al[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 Starkey and Barrett[20] and Harpel[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 capacity (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), 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 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 proteinases 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.

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 in a constant state of being 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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