Anticollagen Antibodies Following Thermal Trauma (33563)

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The occurrence of antibodies which react with tissue extracts in the sera of convalescent humans and experimental animals following recovery from thermal injury has frequently been reported (1). On the premise that such antibodies were at least partly directed against the proposed "burn toxin," therapeutic use of such sera was recommended (2, 3) but it has failed to gain acceptance (1). It was suggested that immunologic reactions demonstrable with convalescent sera were attributable to antibodies specific for bacteria present on the burn wound, rather than to interaction with tissue breakdown products (4). The present observations indicate that antibodies against skin extracts are in fact induced. Acid extraction, but not aqueous extraction, of normal skin yields a fraction which will react with convalescent sera of rats, whereas the antigenic substances can be obtained by both aqueous and acid extraction of burned skin.

Materials and Methods. Male Holtzman rats, 200–250 g, were given a standard burn of 20% body weight immersion in water at 90° for 20 sec under ether anesthesia (5). Rats were sacrificed by ether anesthesia 24 hr later and the burn area was removed for extraction of antigens. The skin was frozen at −10° until used. Minced normal or burned skin, held in an ice bath, was homogenized in saline. Partial emulsification of burned skin was obtained; normal skin, however, was extremely resistant to mechanical disruption. The supernate obtained by centrifugation at 13,000*g* for 15 min at 5° was used as a crude skin extract. Neutral salt-soluble and acid-soluble collagens were also prepared from minced rat skin and purified by the method of Piez *et al.* (6). Latex particles were passively sensitized with crude skin extracts or with purified collagen preparations solubilized by heating to 45° for 30 min. Twenty-five mg of protein were added to 50 ml of a 0.1% suspension of Bacto-Latex 0.81 (Difco), and resuspended in 100 ml of buffer.

Doubling dilutions of serum were prepared in Bacto RA-buffer and an equal volume of sensitized latex particles added to each tube. After overnight incubation at 37° the tubes were centrifuged at 1000*g* for 10 min and the particles resuspended by gentle agitation.

Absorption spectra of the purified collagen and gelatin between 230 and 290 mμ were determined in a Beckman DU spectrophotometer. Amino acid content was determined in a Spinco amino acid analyzer after autoclaving of the specimen with 6 *N* HCl under nitrogen in a sealed ampoule.

Serum globulins, precipitated by one-third ammonium sulfate saturation, were dissolved in water and dialyzed against Bacto RA-buffer. Serum was separated by chromatography on Sephadex G-200 columns and the various fractions tested against collagen-latex suspensions. Sedimentation constants of the reactive fractions were determined in a Spinco model E analytical ultracentrifuge.

Burned rat sera were absorbed with gelatin, acid-extracted collagen from normal rat skin or acid-extract collagen from burned rat skin. Approximately 10 mg of precipitated protein were used to absorb 1 ml of serum by incubation for 48 hr in the cold followed by centrifugation to remove the precipitate. Salt-extracted collagen was absorbed to latex particles by the above procedure. Burned rat sera and normal human sera were treated with 2-mercaptoethanol by the method of Deutsch and Morton (7).

Results. Sera from normal rats, diluted 1:4, failed to agglutinate latex particles coated with crude saline extracts of burned

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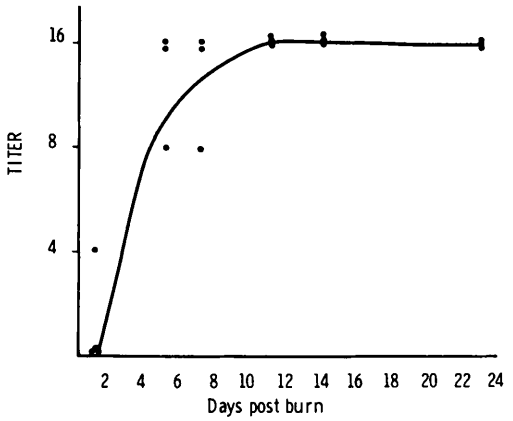

FIG. 1. Antibody to acid-extracted collagen in sera of burned rats.

rat skin. Similarly negative results were obtained from test animals in the first 3 days postburn. However, 4–7 days after injury, agglutination could be demonstrated in increasing titer to a maximum of 1:16, and was shown to persist through the 70-day period of observation.

During the storage of crude skin extract at 5° for prolonged periods, a gelatinous precipitate appeared. Following separation by centrifugation, this material was redissolved by warming to 45° and was used to coat latex particles. These were agglutinated by sera from burned rats but not from normal rats.

Hydrolysis of the gelatinous precipitate with 6 *N* HCl, followed by amino acid analysis, yielded a significant amount of the amino acid hydroxyproline. Since these findings suggested that thermal injury had produced denaturation of skin collagen to a gelatin-like material, latex particles were coated with bovine gelatin and were tested against normal and burned rat sera. Titers of 1:8 were obtained consistently with sera from burned animals, one dilution lower than against the homologous extract. Normal rat sera did not react with gelatin-coated particles. When purified salt-extracted and acid-extracted collagens were used as antigens, titers similar to those obtained with crude skin extract were noted only when acid-extracted collagen was used (Fig. 1). Normal rat sera gave negative results.

Similar results were obtained with pooled sera from normal and burned animals. It was also shown that the agglutinogen could be readily extracted from normal rat skin by acid treatment but not by saline.

Amino acid analysis of the purified acid-extracted collagen revealed a lack of cystine and tyrosine and a high hydroxyproline content. Spectrophotometric examination showed no absorption maxima at 260 and 280 mμ. Since these observations illustrated marked similarity of the acid extract to gelatin, USP, cross-absorption studies were performed which showed that the acid-extract effectively removed antigelatin antibodies (Table I). Conversely, absorption of the serum with gelatin lowered the titer against collagen but did not remove all of the antibody, even after 2 absorptions with relatively large amounts. Similar cross-absorption studies with salt-extracted collagen revealed that particles coated with this preparation, although not agglutinated by serum from burned animals, could nonetheless remove the agglutinin for acid-extracted collagen.

The fact that the agglutinin was precipitated by dialysis against distilled water suggested the presence of a high molecular weight globulin. The globulin fraction obtained by 30% ammonium sulfate precipitation was therefore fractionated on Sephadex G-200 (Fig. 2). When the three fractions obtained were tested against collagen, the ag-

TABLE I. Absorption of Antibody by Bovine Gelatin and Rat Collagen.

Absorbing antigen	Testing antigen	Agglutinin titer
None	Gelatin	1:8
	Acid-ext. collagen	1:16
Bovine gelatin	Gelatin	Neg.
	Acid-ext. collagen	1:8
Bovine gelatin 2×	Gelatin	Neg.
	Acid-ext. collagen	1:8
Acid-ext. collagen	Acid-ext. collagen	Neg.
	Gelatin	Neg.
Salt-ext. collagen	Acid-ext. collagen	Neg.
	Gelatin	Neg.
Latex	Acid-ext. collagen	1:16
	Gelatin	1:8

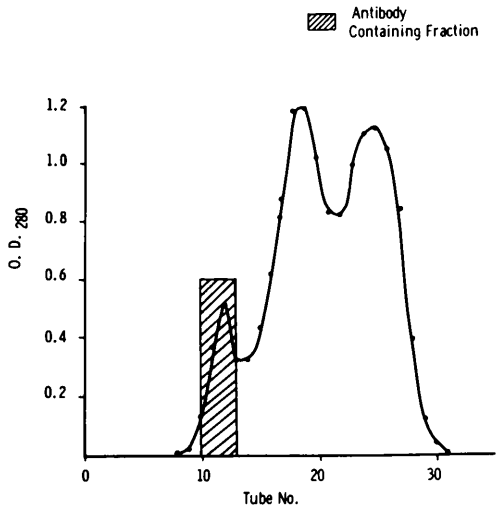

FIG. 2. Gel filtration of burned rat sera on Sephadex G-200.

glutinin was found to be concentrated solely in the first fraction containing the high molecular weight components. Ultracentrifugal analysis revealed two main peaks having S_{20} values of 16.3–16.5 $\times$ 10^{-13} and 4.9–5.0 $\times$ 10^{-13}. Treatment of sera with 2-mercaptoethanol resulted in inactivation of the agglutinating activity.

Sera from normal, nonburned adult humans examined for anticollagen activity contained titers against rat collagen which approximated those found in sera from burned rats. When tested against bovine gelatin, however, they gave consistently higher titers than against rat collagen. The activity of all of several samples tested was destroyed by 2-mercaptoethanol.

TABLE II. Agglutinin Titers of Sera (normal and burn patients) against Bovine Gelatin.

Reciprocal of agg. titer	Normals	Burn patients
128	2[a]	
64	3	
32	6	1
16	1	2
8		5
4		3
<4		4

[a] No. of samples examined.

In view of the occurrence of antibody against gelatin in the sera of normal individuals, it was anticipated that patients recovering from thermal injury would show elevated titers, on the premise that collagen-like material released through thermal trauma would serve as a "booster" mechanism. However, examination of sera from burn patients revealed that titers of sera from burn patients were in fact lower than those of normal sera (Table II). Of those patients from whom sequential sera were obtained (Table III), only two (RE and FE) were early burns and neither of these could be followed for more than 27 days. It is of interest to note, however, that in both instances activity was not detected in the earlier samples. The serum

TABLE III. Anticollagen and Antigelatin Antibody in Serum from Burn Patients.

Patient	Days postburn	3° Burn (%)	Agglutinin titer versus: Rat collagen	Agglutinin titer versus: Bovine gelatin
RE	6	34	Neg.	Neg.
	14		Neg.	Neg.
	22		1:4	Neg.
	28		1:8	1:4
	33		Neg.	Neg.
FE	6	3	Neg.	Neg.
	14		1:4	Neg.
	22		1:8	1:64
	28		1:16	1:32
	33		1:8	1:32
LK	21	13	1:4	1:16
	28		1:8	1:32
	35		1:4	1:16
	40		1:4	1:16
	47		1:4	1:8
	55		1:4	1:32
	62		1:4	1:8
JH	33	12	1:8	1:64
	40		1:4	
	45		1:8	1:32
	52		1:8	1:64
	60		Neg.	1:64
	67		1:4	1:64
	75		1:4	1:128
Pooled normal rat			Neg.	Neg.
Pooled burned rat			1:32	1:16

titer of FE, whose injury was minor (3% third degree) returned to the level found in normal sera by day 22 postburn, whereas that of RE (34% third degree) remained low or negative during the 26-day period of observation. A third patient (LK, 13% third degree), studied from days 21 to 62 postburn, demonstrated fluctuating titers in the lower normal range. Titers obtained on serum samples from a fourth patient beginning on day 33 postburn were within the range of those seen in normal individuals.

Discussion. It was long believed that gelatin was not antigenic and that this lack of antigenicity was attributable to a deficiency of aromatic amino acids (8, 9). Maurer (10) and Grabar (11), however, showed that naturally occurring antibodies to gelatin can be demonstrated in man and other species and that the titer can be increased by immunization. In considering the origin of these antibodies, Maurer was unable to correlate them with gelatin in the diet and suggested that they may be produced as the result of the action of certain enzymes on the collagen of connective tissue, with conversion to a gelatin-like substance which was then antigenic.

Klein *et al.* (12) reported substantial breakdown of collagen following thermal injury and showed that burn patients on a gelatin-free diet excreted large amounts of hydroxyproline-containing peptides in the urine. Hydroxyproline excretion rose immediately following the burn, reached a peak about 15 days postburn, declined sharply in the next 10 days, then slowly in subsequent weeks. Higher than normal values persisted through day 80 postburn.

It is conceivable, therefore, that following severe thermal trauma, breakdown products of collagen of greater complexity than salt-soluble gelatin are released into the circulation and become accessible to immunologically competent cells. That such a mechanism may be operable is suggested by the fact that the agglutinating substance can be extracted readily from burned, but not from normal, rat tissue. In the rat, antigenic stimulation is evident in the appearance of anticollagen antibodies shortly after injury. The occurrence of such antibodies in the sera of normal adult humans may reflect the release of such antigenic moieties following minor burns and other forms of trauma in day-to-day activities.

It was also noted that particles coated with acid-soluble collagen from normal skin were agglutinated, whereas those coated with salt-extracted collagen were not, although the latter would absorb the antibody. The difference in agglutinability of the two products appears to depend on the complexity of the molecule, since their chemical composition is similar. This is in agreement with the view that maturation to the salt-insoluble substance involves the formation of intramolecular cross-links which confer rigidity (13) and probably influence the structure in the direction of antigenicity. Thus, under normal circumstances, the mature, acid-extractable collagen may be considered to be a relatively sequestered antigen, whereas the salt-extractable form, although readily soluble, does not possess the rigidity of structure necessary for good antigenicity.

The antibody elicited in burned rats conforms generally to the characteristics of gamma M globulin in its elution pattern on Sephadex G-200, inactivation by 2-mercaptoethanol, and sedimentation values. Although titers were low, antibody was seen to persist throughout the period of observation. The fact that anticollagen antibodies in patients could not be detected or were present only in low titers infers that collagen breakdown products released by the injury (12) reacted with the antibody *in vivo*. Following healing, when collagen was presumably no longer released in large amounts, antibodies could again be detected. On the basis of the present findings and in view of the nature of the antibody involved, it is doubtful that burned individuals eventually attain titers significantly higher than those found in normal persons.

It is also of interest to note the occurrence of cross-reactions in terms of the source of collagen and of the antibody. In this respect, Rothbard and Watson (14) have noted cross-reactions between the collagens of rat and man and chicken and man. In the present

experiments, sera from burned rats reacted with gelatin of bovine origin, though to a lesser degree than against homologous collagen. In turn, human sera from both normal and burn subjects gave higher titers against bovine gelatin than against rat collagen. It is evident that either some species specificity is retained or that collagen incites the formation of antibodies specific for a variety of configurations, only some of which are removable by the chemically simpler gelatin.

Summary. It was shown by a latex agglutination technique that rats, following thermal injury, are stimulated to produce antibodies to the acid-extractable fraction of collagen in skin. These antibodies do not agglutinate particles coated with the salt-extractable collagen but can be absorbed by this precursor of acid-extractable mature collagen. The anticollagen antibody in rats also reacts with bovine gelatin. An antibody-containing fraction of serum from burned rats was isolated by gel filtration and appears to be a 16.5 S_{20} macroglobulin by ultracentrifugation and is inactivated by mercaptane reduction. Antibodies to rat collagen and bovine gelatin were also demonstrated in the serum of normal humans. In burned humans the agglutinins disappeared following an extensive burn, presumably by reaction with circulating antigen, and reappeared during convalescence.

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