The results confirm the view that a low level of surface activity of the surfactant may have a role in the pathogenesis of pneumonia.

LITERATURE CITED

1. M. V. Barinova, "The state of the surfactant under certain experimental conditions and in chronic nonspecific diseases of the human lungs," Author's Abstract of Candidate's Dissertation, Moscow (1971).
2. I. S. Bubnova, in: Current Problems in Pulmonology [in Russian], Leningrad (1975), pp. 58-60.
3. A. M. Zakhar'evskaya and I. N. Anichkov, Byull. Éksp. Biol. Med., No. 6, 62 (1952).
4. G. N. Dudnikova, Byull. Éksp. Biol. Med., No. 2, 17 (1973).
5. E. N. Nesterov, G. V. Kobozev, and G. A. Zavarzina, Byull. Éksp. Biol. Med., No. 2, 120 (1974).
6. M. Abrams, J. Appl. Physiol., 21, 718 (1966).
7. A. Galowaisky and S. Giammona, Am. Rev. Resp. Dis., 2, 336 (1972).
8. E. M. Scarpelli, Triangle, 10, 47 (1971).
9. V. Somerson et al., Science, 171, 66 (1971).

INHIBITORS OF TRYPSIN-LIKE PROTEOLYTIC ENZYMES AS SUBSTANCES PREVENTING THE DEVELOPMENT OF SECONDARY NECROSIS IN BURN WOUNDS

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A biochemical, histological, and histochemical study was made of the effect of contrykal, an inhibitor of proteolytic enzymes, on the healing of experimental burn wounds in rats. Healing of burn wounds (flame burns covering 20% of the body surface) in animals untreated with contrykal was found to be accompanied by the development of secondary necrosis, by a marked inflammatory reaction, and by increased activity of certain proteases and peptidases. Administration of contrykal prevented the development of secondary necrosis, which is evidently associated with a reduction in the activity of the tissue proteolytic enzymes.

KEY WORDS: *contrykal; proteases; necrosis; healing of burn wounds.*

A distinguishing feature of the burn wound is its gradual deepening through the formation of secondary necrosis. The mechanism of development of secondary necrosis is linked with a disturbance of the circulation in the region of the burn, hypoxia, the inflammatory reaction, and destruction of the tissues beneath the primary necrotic zone. Destruction of the underlying tissues may be caused by proteolytic enzymes. Besides evidence of a decrease in their activity in the zone of burns [4, 5, 8], there are also indications of subsequent activation of proteases [7] in leukocytes [9] and in the wound discharge; the increase in proteolytic activity in the wound exudate is usually accompanied by autolysis of the grafts and their rejection [12]. These circumstances have led some workers to use inhibitors of proteolytic enzymes in the treatment of burns [2, 6]. To undertake a detailed study of this

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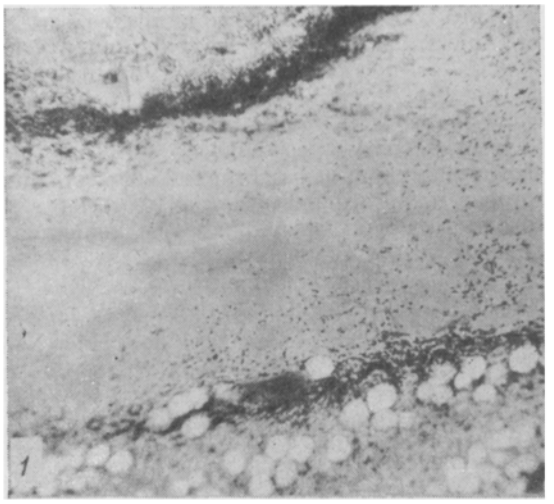

Fig. 1

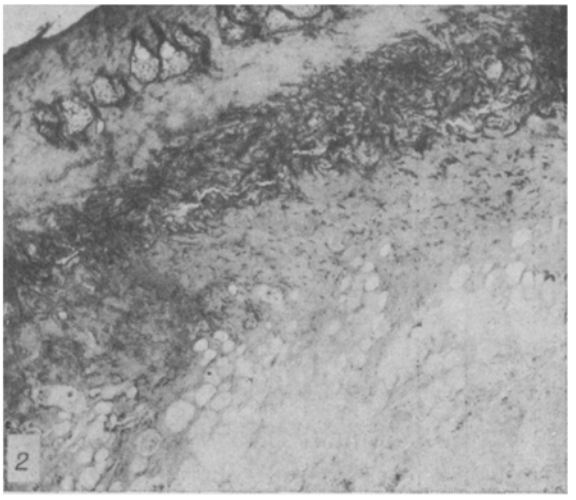

Fig. 2

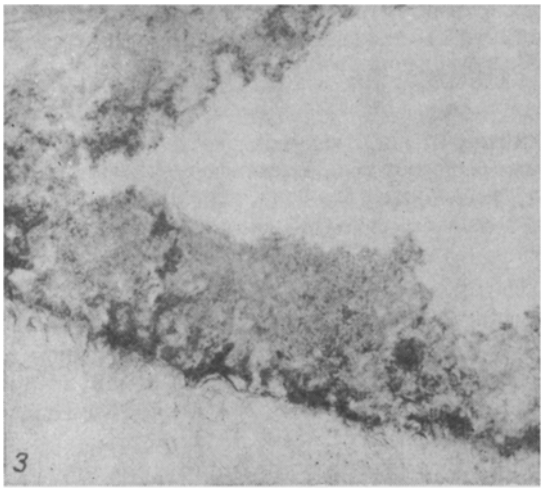

Fig. 3

Fig. 1. Secondary deepening of zone of burn necrosis on 14th day after trauma; two demarcation barriers can be seen in a mass of coagulated collagen; the second demarcation barrier lies in the substance of the muscular layer. Here and in Fig. 2, hematoxylin—eosin, 80 ×.

Fig. 2. Superficial necrosis of skin on 14th day after trauma in animals receiving contrykal; zone of necrosis bounded by a thin demarcation barrier.

Fig. 3. Separation of scab in animals treated with contrykal on 22nd day after burn trauma; considerable alkaline phosphatase activity visible in remnants of demarcation barrier lying beneath scab. Gomori's method, 80 ×.

problem the action of contrykal, an inhibitor of trypsin-like enzymes, on the course of wound healing and on activity of the proteolytic enzymes was investigated in different parts of burn wounds.

EXPERIMENTAL METHOD

Experiments were carried out on 73 male albino rats weighing from 150 to 200 g. A flame burn covering 20% of the body surface was inflicted on the rats. During the first week after burning 25 animals received an intraperitoneal injection of contrykal (from VEB Arzneimittelwerke), a trypsin inhibitor with kallikrein, in a dose of 60,000 antitrypsin units/kg body weight.

TABLE 1. Changes in Enzyme Activity in Skin of Burned Rats Treated with Protease Inhibitor, Contrykal

Test object	State of skin or tissue, days	Cathepsin D		Cathepsin B		Trypsin-like proteases		Leucine aminopeptidase		Lactate dehydrogenase	
		without inhibitor	with inhibitor	without inhibitor	with inhibitor	without inhibitor	with inhibitor	without inhibitor	with inhibitor	without inhibitor	with inhibitor
Skin or scab	Intact	18,2±1,9	—	5,0±0,8	—	2,4±0,5	—	25,9±4,4	—	2929±455	—
	After burning										
	1 day	9,9±0,4*	—	0,3±0,1*	—	0,16±0,5*	—	12,4±1,9*	—	784±131*	—
	7 "	9,5±1,6*	10,2±1,4	2,5±0,8*	3,5±1,1	1,3±0,3*	1,8±0,5	10,6±1,9*	5,9±1,4†	1108±76*	1191±269*
	14 "	9,3±1,1	13,5±1,9†	3,0±1,5	2,8±1,4	1,6±0,8	0,4±0,1†	7,3±1,5*	7,4±4	849±22*	650±20*
	21 "	18,6±4,6	28,9±3,7†	1,9±1,2*	1,5±0,8	7,0±2,4*	0†	9,7±3,7†	14,9±5,2	772±84*	1100±222*
Subcutaneous tissues	Intact	9,5±0,7	—	1,8±1	—	3,6±0,4	—	14,0±1,9	—	5082±120	—
	After burning										
	1 day	7,1±1*	—	0,2±0,1*	4,1±0,5	1,0±0,5*	—	7,3±0,8*	—	952±93*	—
	7 "	14,0±2,7	19,1±3,9	2,0±0,7	2,5±0,7	1,6±0,4*	3,2±0,7†	17,7±3,3	12,2±3	926±181*	903±92*
	14 "	17,4±1,4*	14,1±1,7	2,3±0,5	2,5±0,4	1,5±0,2*	0,4±0,2†	13,9±0,9	9,7±1,4†	769±97*	596±59*
	21 "	25,0±4,7*	16,5±4,5	1,9±0,5	—	3,8±0,3	1,7±0,4†	19,8±9	20,3±5	1066±236	700±101

Legend. Activity of cathepsin D expressed in μg tyrosine/mg protein; activity of cathepsin B, trypsin-like proteases, and leucine aminopeptidase in μg nitrogen/mg protein; lactate dehydrogenase activity given in Wroblewski units.

*Significant ($P < 0.05$) differences from normal (intact tissues).

†Significant differences from burned rats not receiving inhibitor.

Biochemical investigations of tissue from the region of the wound were carried out 1, 7, 14, and 22 days after trauma. Morphological investigations of tissue from the wound region were carried out 7, 14, and 22 days after trauma. The burned skin with the subcutaneous tissue and a strip of adjacent undamaged skin was fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections were stained with hematoxylin–eosin and by Van Gieson's method. Activity of alkaline and acid phosphatases (by Gomori's and the azo-coupling method) and of ATPase (by the method of Padykula and German) was determined in fresh frozen sections. Saline extracts were prepared from intact and burned skin, from areas bordering on the burned skin, and from tissue beneath the scab for biochemical investigation. Activity of the proteolytic enzymes [1] cathepsins D and B, trypsin-like proteases active in a weak alkaline medium, and leucine aminopeptidase, and also of lactate dehydrogenase — the total activity and the isozyme spectrum [11] — was determined in the extracts.

EXPERIMENTAL RESULTS

A dry scab, brown in color, was found on the wound surface of the burned animals not receiving contrykal 7 days after burning. In some animals a region of wet necrosis was present at the periphery of the scab. Microscopic examination revealed an extensive region of deep necrosis of the epidermis and subjacent layers of the dermis, affecting part of the muscles, with coagulation and picrinophilia of the collagen fibers. In regions of tissue beneath the zone of necrosis marked disturbance of the circulation was observed: congestion, stasis, and solitary thrombi in the large vessels.

Beneath the necrotic region marked infiltration with leukocytes could be observed; in some areas, especially at the periphery of the wound, it assumed the character of a demarcation barrier. High alkaline phosphatase activity and moderate ATPase activity were found in the leukocytes.

The surface of the burn wound 14 days after injury was covered by a very dense dark brown scab, joined to the underlying tissues. Peripheral separation of the scab was observed and continued during the following week. Beneath the zone of necrosis a powerful demarcation barrier formed. In these areas disintegration and lysis of the necrotic tissue were observed, with resorption by macrophages here and there. High acid phosphatase activity was detected in the macrophages and alkaline phosphatase activity in the leukocytes of the demarcation barrier. In some areas, the formation of a second demarcation barrier was clearly distinguishable, evidence of the development of secondary necrosis (Fig. 1). Disturbances of the circulation in the tissues beneath the zone of necrosis (principally fatty areolar tissue and the muscular layer) were more marked than at the previous stage of the investigation. In many of the large vessels the thrombi were seen to separate into layers. Well-marked leukocytic infiltration was present in the muscle tissue forming the floor of the wound.

The process of scab separation was taking place very slowly 22 days after burning, the primary demarcation barrier still remained, but alkaline phosphatase activity in its leukocytes was reduced. Very low activity of this enzyme was observed in the leukocytes of the partly preserved secondary demarcation barrier. Disturbances of the circulation in tissues lying beneath the demarcation barrier were just as clearly defined as in the previous periods.

A brown scab, moderately dense but without suppuration, could be seen on the surface of the burn wound 7 days after injury in the rats receiving contrykal. No region of wet necrosis was present.

Histological investigation revealed that in rats receiving contrykal the zone of necrosis affected only the epidermis and stratum papillare of the dermis. Only moderate disturbances of the circulation, mainly in the form of congestion of the vessels and capillaries, were observed in the deep layers of the dermis. No definite demarcation barrier was present. Small groups of leukocytes were observed beneath the zone of necrosis. The leukocytes had high phosphatase activity.

The scab in rats treated with contrykal was more mobile on the 14th day after burn trauma than in the early period of observation and it was thinner. Areas of separation and of peripheral epithelization could be seen at its borders. The zone of necrosis was bounded by a clearly defined demarcation barrier, which possessed a number of special features: It was thin, apparently "rudimentary" in nature, and its leukocytes were concentrated around the

capillaries (Fig. 2). Separation of the scab took place slowly, just as in the control experiments. Disturbances of the circulation in the tissues beneath the zone of necrosis were mainly in the form of congestion of the vessels. Stasis was an isolated event. In five cases there was no secondary demarcation barrier, and in the other 20 cases it was much less well defined than in the control (it was smaller, thinner, always concentrated in the substance of the reticular layer, without spreading to the muscular layer). Discrete groups of leukocytes could be seen in the substance of the reticular layer. The scab had separated over much of its extent 22 days after burning in animals treated with contrykal, and separation was proceeding even more rapidly than in the untreated animals (Fig. 3). A growing thin layer of newly formed epithelium and granulations could be seen beneath the separating scab.

Biochemical studies showed a change only in lactate dehydrogenase activity in the intact skin throughout the period of investigation: It fell in animals both treated and not treated with contrykal (Table 1).

Activity of the proteolytic enzymes in the untreated animals during the first day after burning was sharply reduced both in the zone of the burn and in the adjacent and underlying tissues. The activity of certain proteolytic enzymes (trypsin-like proteases) was back to normal after 7 days, and the activity of leucine aminopeptidase and that of cathepsin D in the zone adjacent to the scab and in the tissues beneath it were 50-100% higher than normally. A progressive increase in proteolytic activity was observed 22 days after burning.

Activity of the proteolytic enzymes in the scab of the treated rats was much reduced throughout the period of investigation and in the tissue border surrounding the scab and in tissues beneath the scab protease activity was restored to normal later or was increased less than in rats not treated with inhibitors.

During wound healing, periods could thus be distinguished during which inhibition of proteolytic activity in the zone of the burn was followed by activation of several proteases and peptidases, mainly at the periphery of the scab and in the tissues beneath it. After administration of the inhibitor, no increase was observed in the activity of the above-mentioned enzymes.

It can tentatively be suggested that the development of secondary necrosis under these circumstances was prevented by inhibition of infiltration by leukocytes and reduced activity of proteolytic enzymes of leukocytic origin.

It could be concluded from this investigation that burn wounds distinguished by the presence of an extensive zone of primary necrosis and by the formation of secondary necrosis require specific treatment. Under these conditions, in the early period of treatment there is evidently good reason to administer inhibitors of the trypsin–kallikrein system in order to inhibit the inflammatory reaction and reduce tissue destruction. During the formation of granulations and separation of the necrotic tissues, the tactics of treatment must be appropriately modified.

LITERATURE CITED

1. A. N. Alekseenko, in: Modern Methods in Biochemistry [in Russian], Vol. 2, Moscow (1968), p. 115.
2. T. L. Zaets, M. I. Dolgina, L. I. Muzykant, et al., Éksp. Khir., No. 5, 49 (1974).
3. T. L. Zaets and M. A. Zaidenberg, Patol. Fiziol., No. 5, 2 (1974).
4. A. Beloff and R. Peters, J. Physiol. (London), 103, 461 (1945).
5. W. Greuer, Dtsch. Med. Wschr., 74, 1205 (1949).
6. L. Kozlowski and L. Kilninger, in: Third International Congress on Research in Burns, Abstracts, Bern (1971), p. 144.
7. L. Lewis, J. Peters, and A. White, Br. J. Pharmacol., 42, 437 (1971).
8. M. Malik, Br. J. Pathol., 52, 345 (1971).
9. J. Niedworor and E. Spychalski, Acta Physiol. Pol., 23, 987 (1972).
10. E. Werle, J. Trauschold, and H. Haendle, Ann. N.Y. Acad. Sci., 146, 464 (1918).
11. F. Wroblewski and J. S. La Due, Proc. Soc. Exp. Biol. (New York), 90, 210 (1955).
12. W. Zietkiewierz, Pol. Tyg. Lek., 27, 557 (1972).