Conditions of Reactivation of Arthus-Type Inflammation by Thiol Compounds.* (29265)

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In earlier studies(1,2), we presented a new technique for reactivating the Arthus reaction; a single intradermal injection of reduced glutathione (GSH) into the healing site induces the rapid development of local cutaneous lesions similar to the original reaction. The mechanism of this phenomenon could be explained by the increased protease activity of euglobulin preparations extracted from the lesion. It was shown that the increase in the enzyme activity itself is explicable by the reactivation of the SH-dependent protease responsible for the original reaction and which is inactivated during healing. The positive but less marked results obtained with cysteine were also explicable in the same terms. Negative results were obtained with other reducing agents, such as L-ascorbic acid, 6,8-disulfhydryl-octanoic acid and thiosulfate.

In further studies(3,4,5), we found that the increased susceptibility of the healing site is due to the presence of the protease in readily activable form.

This paper deals with further correlation between the ability of thiol compounds to reactivate the Arthus reaction and their effect on the protease extracted from healing sites.

Materials and methods. 1) *Induction of Arthus reaction.* Adult male albino rabbits (2.0-2.3 kg) were sensitized with 3 subcutaneous injections of 5 ml bovine serum every other day. On the eleventh day after the last injection, the precipitin titer was measured in the serum of each animal by the ring test (6). Animals showing precipitin titers of 2^6-2^7 were given 4 intradermal injections of 0.1 ml of antigen (4% bovine serum albumin Armour in saline) into the clipped flanks. Macroscopically, the reactions began in 2 hours, were maximal in 22-24 hours, and then declined in intensity. Under the conditions of these experiments, the inflammatory reactions usually disappeared completely 12-14 days after the challenging injection. The intensity of reaction was graded following the method previously described(2). 2) *Injection of thiol compounds.* Commercial preparations (Wako Chemical) of glutathione (GSH) and cysteine were used after verification of their reduced state by amperometric titration(7). Amounts of 0.3 ml of 0.1 M GSH or 0.1 M cysteine in physiological saline were injected intradermally in the center of 2 of the healing sites clipped 24 hours prior to injection. The intradermal injections of these substances were made at intervals varying from 2 to 15 weeks after the challenging injection. The opposite sites received the same volume of saline. GSH or cysteine also was tested in virgin sites of the skin of sensitized animals. 3) *Extraction of euglobulin preparations from Arthus lesions.* Healing skin was excised immediately after the animals were killed. The skin was finely cut and frozen at —80°C. The pieces of frozen skin were cut into slices about 50 μ thick and the slices dehydrated by repeated treatment with cold acetone. Skin acetone powder was extracted for 4 hours with 10 volumes of M/15 phosphate buffer, pH 7.4 and the suspension filtered. From the extracts the euglobulin preparations were fractionated with ammonium sulfate. The protein fractions were used in 0.15 M NaCl solution. Further details of the preparation were given by Udaka(8). 4) *Protease assay.* This was performed essentially according to Kunitz(9). One ml of 2% casein solution and 1 ml buffer were mixed and kept at 37°C for 5 minutes before adding 1 ml of protein solution and 1 ml of 0.15 M NaCl. Protein concentration of the preparations was calculated from their nitrogen content estimated by Kjeldahl's micro-method. The preparations were maintained at pH 7.1, at buffer concentration of 0.05 M, and

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TABLE I. Macroscopic Reactions to Injection of Thiol Compounds into Healing Arthus Sites in Rabbit's Skin.

Animal groups No.	Compounds injected ml	Original Arthus, 24 hr old*	Age of lesion at injection† weeks	Maximal reaction after injection*	Duration of reaction hr
1–6	GSH; 0.3	+++±	2	+++‡	96–120
11–16	" "	+++±	3	++‡	72–86
21–24	" "	+++±	4	+§	48–72
31–34	" "	+++±	5	+§	36–48
41–44	" "	+++±	6	±‖	12–18
51–58	" "	+++±	9–15	—	3–4 ¶
61–63	Cysteine; 0.3	+++±	2	±‖	14–18
71–73	" "	+++±	4	—	0

* Mean intensity of inflammation in rabbits of each group; there was little difference in reaction intensity in different sites of the same rabbit. Injection of thiol compounds into virgin sites or of saline into healing sites induced no reaction.

† Days after challenging injection. The original Arthus reaction completely disappeared in 12-14 days.

‡ Reactions at 8 hr. § Reactions at 6 hr. ‖ Reactions at 4 hr. ¶ Mild edema but not erythema observed.

ionic strength of 0.25. After incubation at 37°C for 30 minutes, 5 ml of 6% trichloroacetic acid was added. The optical density at 276 mμ of the filtrate was estimated with a Beckman spectrophotometer model DU, using the filtrates from the controls as blanks. In the controls, the protein solution was added after the trichloroacetic acid.

Results. Reactivation of healing Arthus skin sites by thiol compounds. As shown in Table I, the intensity of reactivation of the Arthus reaction decreased with increasing age of the original lesions. Intradermal injections of 0.3 ml GSH into a 2-week-old healing site induced marked inflammatory reactions in every instance; diffuse edema and erythema appeared in 2 hours, becoming maximal in 7-9 hours, and persisting to some extent for 96-120 hours. The reactions developed and regressed more rapidly than the original Arthus lesions. At the height of the reaction, moderate hemorrhage was observed in the center of the lesion. Definite but less marked inflammation occurred after injection of 0.3 ml GSH into 6-week-old healing sites; mild edema and erythema became detectable in 2 hours after injection, and lasted 12-18 hours. However, similar injection of GSH into 9-15 week old healing sites induced only a negligible edematous reaction. Injection of saline in healing sites or GSH in normal untreated sites was ineffective. The observations indicate that reactivation of the healing sites by GSH occurs only within 6 weeks after the challenging injection, *i.e.* 4 weeks after healing.

Less marked inflammation occurred in 2-week-old healing sites injected with 0.3 ml cysteine; the edema and erythema appeared in 3 hours after injection, and lasted only 18 hours. Little reaction was induced by the same amount of cysteine injected into 4-week-old healing sites.

Activation of euglobulin protease by thiol compounds. One ml of euglobulin solution was mixed with 1.0 ml 0.15 M NaCl containing GSH or cysteine at 10^{-3} or 10^{-4} M. The mixture was kept for 30 minutes at room temperature after which the protease activity was assayed. Table II indicates that the euglobulin fraction extracted from healing Arthus sites shows little protease activity. Addition of thiol compounds, however, produces a marked increase in proteolysis. The protease extracted from a 2-week-old healing Arthus site is activated by GSH or cysteine far more significantly than that of the normal untreated sites but activation of the protease of 9-week-old healing sites is almost indistinguishable from that of the protease of normal untreated sites. In other words, the activability of the protease of healing sites seems correlated with the age of the lesion. Protease activity of the activated euglobulin preparations was maximal at pH 7.1. This is identical with the optimum pH of the pro-

TABLE II. Activation of Euglobulin Protease by Thiol Compounds.

Compounds added	Euglobulin of normal site	Euglobulin of healing Arthus sites* 2 wk old	3 wk old	6 wk old	9 wk old
GSH					
0	.019	.024	.020	.023	.020
10^{-4} M	.034	.085	.083	.063	.035
10^{-3} M	.054	.133	.117	.082	.052
Cysteine					
0	.020	.022	.022	.023	.020
10^{-4} M	.026	.069	.059	.054	.028
10^{-3} M	.049	.121	.098	.079	.050

Estimated from optical density at 276 mμ; ionic strength 0.25; pH 7.1. Values recorded represent means of several determinations on the enzymes extracted from 5 animals of each group. Euglobulin 1.0 ml (0.95 mg N/ml); GSH and cysteine each 0.1 ml.

* Days after challenging injection.

tease responsible for the original and GSH-reactivated Arthus lesions(2,3,8). The euglobulin protease is distinct from the autolytic proteases which are active in a more acid pH range. It is thermolabile; its activity is lost at 70°C for 30 minutes or at 100°C for 5 minutes.

Discussion. Intradermal injection of GSH into healing sites of Arthus reaction produces the development of local cutaneous lesions similar to those of the original reaction. This reactivation can probably be explained by the characteristic susceptibility to GSH of the SH-dependent protease extracted from the healing sites. The enzyme is responsible for the original reaction and is inactivated during healing. The enzyme extracted from the healing sites and activated by GSH or cysteine, when injected into normal skin, induces changes similar to those caused by the extract of reactivated lesions(3,4).

We described previously(8,10-13) that the activity of the SH-dependent protease increases simultaneously with the development of the original Arthus reaction and decreases as inflammation subsides owing to the local increase of a specific polypeptide inhibitor. It is possible that GSH acts on the polypeptide inhibitor since it has been shown that this substance loses its activity under the influence of reducing agents(5). It was assumed, therefore, that higher susceptibility to GSH of the protease in the healing sites may be concerned with the ease of inactivation of the inhibitor by GSH. The susceptibility to GSH of the protease from healing sites decreases with increasing age of the lesion and 9 weeks after the challenging injection (*i.e.* 7 weeks after complete healing) it is completely abolished. This can be correlated with the fact that intradermal injection of GSH into 9-week-old healing sites induced little reaction. It should be noted that although cysteine is less active *in vivo,* no difference in the *in vitro* effects of GSH and cysteine was observed under our experimental conditions.

Cochrane *et al*(14) showed that a repeated injection of antigen into healing Arthus sites induces reactions similar to the original lesion and ascribed the effect to the concentration of antigen-antibody complex in the walls of blood vessels. However, the mechanism of reactivation demonstrated in the previous(1,2) and present experiments is clearly different and not related to the antigen-antibody reaction.

In more recent studies(18), we found that reactivation of healing sites by GSH can also be produced after thermal injury or Shwartzman reaction. This reactivation is also associated with increased protease activity in the euglobulin preparations extracted from the lesions. Negative results, however, were observed in healing skin sites after croton oil and ultraviolet induced injury. Our observations suggest that tissue injuries may be divided into 2 groups: a) lesions characterized by reactivation of healing site by GSH; b) lesions unaffected by GSH which are due to the activation of a non-SH protease as we observed in radiation injury(19). The observations with GSH or cysteine may explain the recurrence frequently observed in human allergic disease such as rheumatic heart disease and rheumatoid arthritis. GSH is an important hydrogen carrier and its amount may vary under different physiological or pathological conditions(16,17). Since its local increase may be related to recurrence of the disease, it would be desirable to find experimental conditions which can induce local in-

crease of GSH in various tissues.

Summary. Intradermal injection of GSH into healing sites of Arthus phenomenon produces a reactivation of the inflammatory process having the same aspect as the original reaction. The interval during which reactivation occurs is correlated with the susceptibility to GSH of a protease extracted from the healing sites, which decreases with the age of the lesion. Cysteine is less active *in vivo* than GSH but no difference was observed between the *in vitro* effects of the 2 thiol compounds.

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Effect of Endotoxin upon Total Iron-Binding Capacity of the Serum. (29266)

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The iron-binding property of transferrin is specific for this particular protein and is not exhibited by any of the other proteins of serum(1). It follows, therefore, that the capacity of serum for transport of iron is limited by its content of transferrin. This is called the total iron-binding capacity (TIBC).

A decrease in TIBC has been shown to occur during infections in humans and dogs (2), during turpentine inflammation in rats (3) and in protein deficiency in swine(4) as well as such diseases as hemolytic or aplastic anemia and malignant conditions(4). In some of these conditions the decrease in TIBC seems to be a part of a general change in plasma proteins(5). There is also some evidence that the balance between tissue oxygen supply and demand can be a regulating factor of the TIBC(6).

It was recently shown that when endotoxins were injected into normal rats a rapid decline in plasma iron concentration was produced(7). This decrease in plasma iron was not due to a more rapid loss of iron from the plasma, but seemed instead to be related to reutilization of iron from recently destroyed red blood cells(8). It was therefore of interest to see what effect the endotoxins had upon TIBC.

Materials and methods. Female Holtzman rats, 50 to 70 days old, weighing 180-200 g were used in these experiments. Lipopolysaccharide from *Escherichia coli* 055:B5 was obtained from Difco Laboratories, Detroit, Mich. (Lot No. 460830).

Plasma bound iron and unsaturated iron-binding capacity (UIBC) were determined by the method of Schade *et al*(9). The bound iron and the UIBC were added to give the TIBC. The TIBC was also checked by the