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THE REDUCTION OF EXPERIMENTALLY INDUCED INFLAMMATION BY SULFHYDRYL COMPOUNDS

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Abstract—The sulfhydryl compounds acetylcysteine and acetylpenicillamine were found to reduce the severity of induced dermal inflammation in rabbits when the compounds were administered in repeated doses. Treatment with acetylcysteine and salicylate given concomitantly resulted in an anti-inflammatory response which was equal to that obtained with thiosalicylate and was significantly greater than that obtained with either acetylcysteine or salicylate given individually.

SULFHYDRYL compounds decrease inflammation in a variety of experimental test systems,[1–4] and evidence pointing to a physiological relationship between steroid hormones and sulfhydryl compounds has been reported.[5] However, separate from an anti-inflammatory function, the role of sulfhydryl groups in connective tissue disease became of added importance when it was found that serum sulfhydryl levels were depressed in patients with rheumatoid arthritis,[6, 7] Earlier, Shipton and Parr[8] reported that the injection of cysteine intramuscularly produced dramatic clinical improvement in two patients with severe rheumatoid arthritis. Subsequently, Henry and Holley[9] found that penicillamine reduced the latex fixation titer of sera from patients with rheumatoid arthritis, and Dresner and Trombly[10] obtained similar results when penicillamine was added to high-titer rheumatoid sera or fed to patients with rheumatoid arthritis. Griffen *et al.*[11] administered DL-penicillamine orally to seven patients, and in five of these there was a significant decrease in rheumatoid factor; clinical improvement coincided with the decrease in titer. Lorber also found[12] that the oral administration of D-penicillamine to a patient with rheumatoid arthritis reduced the rheumatoid factor titer and produced favorable clinical response.

Adrenocortical steroid treatment of patients with rheumatoid arthritis has been shown to elevate depressed serum sulfhydryl levels.[6] Similarly, Lorber *et al.* observed[7] that in a patient with systemic lupus erythematosus, treatment with prednisone resulted in an increase in serum sulfhydryl and improvement in the clinical condition.

Thiol compounds in general have been found to modify the chemical structure of many macroglobulins[13–15] and other disulfide-containing proteins and peptides presumably by reducing susceptible disulfide bonds to sulfhydryl groups.[16] Consequently, various thiol compounds were tested for activity in reducing an induced dermal inflammation in rabbits in order to evaluate these compounds as possible anti-inflammatory agents.

* Deceased.

MATERIALS AND METHODS

Male rabbits of the New Zealand strain, weighing between 1·4 and 2·6 kg, were maintained in separate cages and supplied food and water *ad libitum* for at least 1 week prior to an experiment. Depilatory agents were not used since they contain thiol compounds and would possibly modify the induced inflammation. All test compounds were dissolved in pyrogen-free distilled water, adjusted to pH 7·2 ± 0·1 with sodium hydroxide, and sodium ion concentration was adjusted to 0·154 M by the addition of sodium chloride.

Dexamethasone-21-phosphate and hydrocortisone acetate were purchased from Sharp Merck & Dohme; benzoid acid, salicylic acid, and thiosalicylic acid (*O*-mercaptobenzoic acid) from Distillation Products Industries; DL-methionine from Matheson Coleman & Bell; L-proline from Mann Research Laboratories, Inc.; and thiazolidine-4-carboxylic acid (thioproline) from Nutritional Biochemicals. Only thiosalicylic acid required purification by recrystallization before use.

Test compounds were given by i.v. (ear vein) or subcutaneous (hindleg) injection, or orally by stomach tube. Treatments were administered twice daily (8 a.m. and 4 p.m.) until the desired dose had been given; single doses were administered in some cases. Treatments with test compounds were completed 1 hr before the intradermal injection of the standardized inflammatory agent at each of four sites along one side of the back or abdomen; equivalent volumes of saline were injected at corresponding sites along the other side of the midline to serve as control sites. Three to 15 mg of rose bengal or Evans blue (both from Allied Chemical Corp.) in saline was then injected i.v. to distinguish the inflamed sites. Inflammation was quantitated by measuring the increased serous fluid accumulation at the inflamed site, according to the method of Ungar *et al.*[17] Animals were sacrificed approximately 3 hr after injection of the inflammatory agent; their skins were then removed and stapled hair-side-down on a board. The inflamed and saline-injected areas were excised with a metal punch (15/16-in. Arch hole punch) and weighed on a Roller-Smith balance to the nearest mg. Fluid accumulation was determined by comparing the weight of an inflamed area of skin with that of a corresponding saline-injected area (paired comparison). In agreement with the observation of Berg,[18] a more uniform inflammation was obtained on skin from the back than from the abdomen.

Dog serum or bovine serum fraction V (Pentex Inc.) used as an inflammatory agent was lyophilized and stored at −20°. Prior to use these agents were dissolved in saline and standardized to produce an inflamed area 20–22 mm in diameter (corresponding to a skin weight of approximately 350 mg) when 0·2 ml was injected intradermally into normal rabbits. Reduction in inflammation was calculated by comparing inflammatory sites in animals receiving test compounds with those of animals receiving saline. Statistical significance was determined by the *t* test or *F* test.[19]

Inflammation was also quantitated in earlier experiments by measuring in inflamed areas of skin the increased accumulation of i.v. injected radioactive rose bengal-^{131}I (Volk Radiochemical Co. or Abbott Radio-Pharmaceuticals).[20] Skin samples were excised as described above and counted in test tubes in a Baird Atomic (model 810) bench-type scintillation detector. Injections containing 12–36 μc gave satisfactory results with this instrument. Since the skin-weight method was found to be as accurate but less time consuming than the rose bengal-^{131}I method, the former was used for most of the assays.

RESULTS

Standardization of anti-inflammatory procedure with corticosteroids

The i.v. administration of dexamethasone-21-phosphate to rabbits 1 hr before the induction of the inflammation with dog serum resulted in a 39 per cent reduction in inflammation ($P < 0.01$) with a steroid dose of 3·0 mg/kg, and 51 per cent ($P < 0.01$) with a dose of 6·0 mg/kg (Table 1). Hydrocortisone acetate at doses of

TABLE 1. THE REDUCTION OF INDUCED DERMAL INFLAMMATION BY CORTICOSTEROIDS*

Compound	Dose (mg/kg)	Inflammation (cpm)	Reduction in inflammation (%)	P
Control (saline)		1370 ± 34 (8)		
Dexamethasone-21-phosphate (IV)†	3·0	841 ± 46 (8)	39	<0·01
Dexamethasone-21-phosphate (IV)	6·0	655 ± 73 (8)	51	<0·01
		(mg)		
Control (saline)		334 ± 18 (18)		
Hydrocortisone acetate (SC)‡	2·5	315 ± 19 (8)	6	N.S.
Hydrocortisone acetate (SC)	5·0	294 ± 50 (12)	12	N.S.

* A single injection of the test compound was given 1 hr before the inflammatory agent (dog serum). Dexamethasone was studied by the ^{131}I-rose bengal procedure, whereas the skin-weight method was used for hydrocortisone. In all tables the results are presented as the mean ± standard error; the number of skin tests is indicated in parentheses; N.S. = not significant.

† Intravenous.

‡ Subcutaneous.

2·5 and 5·0 mg/kg reduced inflammation 6–12 per cent, which was not statistically significant in either case. These results are consistent with the known relative activities of these corticosteroids.

The anti-inflammatory activity of sulfhydryl and non-sulfhydryl sulfur compounds

The effect of acetylcysteine, acetylpenicillamine, and methionine given s.c. 1 hr before the inflammatory agent (dog serum) is presented in Table 2. With a single dose of 1·5 m mole/kg none of these compounds produced a significant reduction in inflammation. Three doses of each compound, administered 25, 17, and 1 hr before the inflammatory agent, resulted in a 29 per cent reduction in inflammation ($P < 0.01$) for the acetylpenicillamine treatment but no significant changes after treatment with the other two compounds. Five doses, given 49, 41, 25, 17, and 1 hr before the inflammation was induced, did not further enhance the effect of acetylpenicillamine but resulted in a 23 per cent reduction in inflammation due to acetylcysteine. Methionine, even after five doses, did not reduce the induced inflammation.

The anti-inflammatory activities of salicylate and thiosalicylate administered subcutaneously

The subcutaneous administration of sodium salicylate (Table 3) at doses of 0·75 and 1·5 m-mole/kg reduced inflammation due to bovine fraction V 29 and 31 per cent respectively ($P < 0.01$). Sodium benzoate actually increased the inflammation. Sodium thiosalicylate with a single dose of 0·75 m-mole/kg reduced inflammation only 19 per cent (not statistically significant), whereas at 1·5 m-mole/kg it produced a 55 per cent reduction in inflammation ($P < 0.01$).

The anti-inflammatory activity of sulfhydryl compounds administered orally

The oral administration of acetylcysteine at a dose of 1·0 m-mole/kg did not significantly reduce the dermal inflammation due to dog serum until eight doses had been given (34 per cent; $P < 0{\cdot}01$), as shown in Table 4. However, at 2·0 m-mole/kg

TABLE 2. THE EFFECT OF MULTIPLE DOSES OF SULFHYDRYL COMPOUNDS ON INDUCED DERMAL INFLAMMATION

Compound*	No. of doses	Inflammation (cpm)	Reduction in inflammation (%)	P
Control (saline)	1	7970 ± 274 (24)		
Acetylcysteine, (1·5 m mole/kg)	1	7670 ± 618 (8)	4	N.S.
	3	7140 ± 409 (8)	10	N.S.
	5	6120 ± 488 (8)	23	<0·01
Acetylpenicillamine, (1.5 m mole/kg)	1	8100 ± 414 (8)	0	N.S.
	3	5620 ± 283 (8)	29	<0·01
	5	5600 ± 340 (8)	30	<0·01
Methionine, (1·5 m mole/kg)	1	7260 ± 561 (8)	9	N.S.
	3	7680 ± 662 (8)	4	N.S.
	5	8770 ± 531 (4)	0	N.S.

* Compounds were given daily by subcutaneous injection at 4 p.m. and 8 a.m. The last treatment was given 1 hr before injection of the inflammatory agent (dog serum). Inflammation was quantitated by the ^{131}I-rose bengal procedure.

TABLE 3. THE REDUCTION IN INFLAMMATION AFTER SUBCUTANEOUS INJECTION OF SALICYLATES

Compound*	Dose (m mole/kg)	Inflammation (mg)	Reduction in inflammation (%)	P
Control (saline)		334 ± 18 (18)		
Salicylate	0·75	238 ± 11 (7)	29	<0·01
	1·5	228 ± 19 (10)	31	<0·01
Benzoate	1·5	421 ± 63 (8)	−26†	N.S.
Thiosalicylate	0·75	272 ± 26 (6)	19	N.S.
	1·5	150 ± 13 (10)	55	<0·01

* The compounds were injected 1 hr before injection of the inflammatory agent (bovine fraction V). Inflammation was assayed by the skin-weight method.

† Increased degree of inflammation.

the reduction in inflammation was 20 per cent with one dose (not statistically significant), 30 per cent with two doses ($P < 0{\cdot}05$), and 32 per cent with four doses ($P < 0{\cdot}05$). In a separate experiment, acetylpenicillamine given orally at a dose of 1·6 m-mole/kg for two doses also significantly reduced the inflammation by 54 per cent. The latter value is somewhat higher than that previously obtained with

acetylpenicillamine given subcutaneously and may be due either to the different methods of administration or to the fact that the inflammation was measured by the skin-weight method in this experiment, whereas the ^{131}I-counting procedure was used in the previous one.

TABLE 4. THE EFFECT OF ORAL ADMINISTRATION OF ACETYLCYSTEINE ON INDUCED DERMAL INFLAMMATION

Dose (m moles/kg)	No. of doses*	Inflammation (mg)	Reduction in inflammation (%)	P
Control (saline)		377 ± 22 (16)		
1·0	1	412 ± 15 (12)	0	N.S.
1·0	2	373 ± 22 (16)	1	N.S.
1·0	4	343 ± 42 (16)	9	N.S.
1·0	8	247 ± 26 (12)	34	<0·01
2·0	1	302 ± 29 (16)	20	N.S.
2·0	2	265 ± 24 (16)	30	<0·05
2·0	4	255 ± 16 (12)	32	<0·05

* Acetylcysteine was administered daily at 8 a.m. and 4 p.m. by stomach tube. The final treatment was given 1 hr before injection of the inflammatory agent (dog serum). Inflammation was assayed by the skin-weight method.

The anti-inflammatory activity of acetylcysteine combined with salicylate

It was shown in Table 3 that when salicylate was given s.c. at doses of 0·75 and 1·5 m-mole/kg, the inflammation was reduced 29 and 31 per cent respectively. However, thiosalicylate at 1·5 m-mole/kg reduced the inflammation 55 per cent. Consequently, it was of interest to determine whether the concomitant addition of salicylate and sulfhydryl compound would have activity greater than that achieved with either compound alone. The results of such an experiment are presented in Table 5.

TABLE 5. COMPARISON OF THIOSALICYLATE TREATMENT WITH CONCOMITANT ACETYLCYSTEINE–SALICYLATE TREATMENT*

Treatment	Dose (m mole/kg)	No. of doses	Inflammation (mg)	Reduction in inflammation (%)	P
Control (saline)		1	344 ± 22 (28)		
Acetylcysteine	2·0	2	323 ± 26 (20)	6	N.S.†
Salicylate	1·5	1	277 ± 16 (19)	20	<0·05‡
Thiosalicylate	1·5	1	223 ± 18 (19)	35	<0·01
Acetylcysteine + salicylate	2·0 + 1·5	2 + 1	218 ± 14 (20)	37	<0·01‡

* Salicylate and thiosalicylate were given by subcutaneous injection 1 hr before injection of the inflammatory agent (bovine fraction V). Acetylcysteine was given orally by stomach tube 17 hr and 1 hr before the inflammatory agent. Inflammation was assayed by the skin-weight method.

† Not significant compared to control group receiving saline.

‡ The difference between the salicylate group and the group receiving salicylate plus acetylcysteine was statistically significant at the 0·05 level.

Salicylate given s.c. in a single dose of 1·5 m-mole/kg reduced inflammation (to bovine fraction V) 20 per cent ($P < 0{\cdot}05$); acetylcysteine given orally (two doses of 2 m-mole/kg each) reduced inflammation 6 per cent (not significant). The combined administration of salicylate (s.c.; 1·5 m-mole/kg) and acetylcysteine (oral; two doses of 2·0 m-mole/kg each) reduced inflammation 37 per cent. Similarly, thiosalicylate produced a 75 per cent greater reduction in inflammation than was achieved with salicylate *per se* (−35 compared to −20 per cent).

DISCUSSION

There is reason to believe that the various types of allergic and anaphylactoid responses are elicited by some common mechanism.[21] It appears that the allergic type of response may be the result of a cellular excitation in the course of which such mediators as histamine, serotonin, etc. are released. The mechanism is commonly believed to be a sudden increase in membrane permeability. There has been a trend in recent years to revise the classic permeability concept, as evidence has accumulated indicating that excitation is accompanied by a structural rearrangement of certain cell proteins, resembling that occurring in reversible denaturation.[22] Such a rearrangement, besides being able to activate enzymes, may modify the distribution of electrical charges in the cytoplasm and thus affect the attachment of inorganic and organic ions.[23]

It has been observed that the release of histamine following the injection of dextran is insulin dependent.[24] This phenomenon supports the idea that the cell must be in an excited state when an allergic or anaphylactoid response occurs, since insulin can maintain a high degree of electrical polarization in muscle cells by increasing potassium content and decreasing sodium concentration in the cell.[25, 26] Insulin, however, has no effect on histamine release induced by compound 48–80, a potent histamine liberator. This can be correlated with the observation that dextran releases potassium from the cell,[27] whereas 48–80 is without effect on this ion.[28]

The number of reactive –SH groups also increases when a cell is in an excited state.[29] Since many cellular proteases show –SH dependence, this increase in reactive protein –SH groups may be a factor in the activation of these enzyme systems.[21] The activated cellular proteases may then damage directly the capillary endothelium and bring about the characteristic vascular permeability observed in anaphylactoid responses,[30] and they may also act directly on structural protein without necessarily inducing the release of inflammation mediators.[31]

Protease activation is a general response[32] observed in many types of tissue damage such as trauma[33] or thermal[34] injury. Protease activation may therefore be a generalized response induced by any factor that disturbs the homeostatic balance in the cell.[35] In terms of the final mechanism, a specific allergic reaction differs from other cellular injuries and responds to injuries only in the state of sensitivity of the individual to the stimulus.[21]

Many groups of compounds may inhibit cellular proteases. Of the nonspecific inhibitors, citrates inhibit anaphylactoxin formation[36] and the release of histamine from rabbit blood.[37] Herberts investigated a number of synthetic thiols and thioethers which not only inhibited lung protease but also prevented anaphylactic shock in the guinea pig.[38, 39] Certain drugs such as salicylates, pyrazolone derivatives (aminopyrine, phenylbutazone), and other antipyretic drugs were shown to counteract experimental

allergic reactions and to inhibit plasma proteases *in vitro* at concentrations similar to those found in the circulating blood of subjects treated with these compounds.[40]

The anti-inflammatory hormones as a group are among the most active anti-allergic agents known. Ungar *et al.*[41] observed an increased antiprotease activity in the serum of animals treated with ACTH or cortisone. Synthetic steroids such as methylprednisolone and triamcinolone also showed good correlation between clinical activity of the compound and its capacity to increase serum antitryptic activity.[42]

Many of the compounds that can inhibit cellular protease activity are also inhibitors of other enzyme systems. Whitehouse[43] has reported that anti-inflammatory steroids, salicylates, synthetic thiol compounds, phenylbutazone, chloroquine, and the active congeners of phenylbutazone and chloroquine are potent inhibitors of polysaccharide sulfation and of acetate and glucose incorporation into polysaccharides. He also observed that potency of inhibition was correlated with the ability of the compounds to form complexes with metal ions and with their lipophilic character. Steggle *et. al.*[44] reported that salicylic acid and its thiol and hydroxyl congeners inhibited serum glutamic-pyruvic transaminase.

The results presented in this report suggest several important points. Sulfhydryl compounds are in general weak anti-inflammatory compounds, but their anti-inflammatory activity can be readily demonstrated if the compounds are given in repeated doses. In this respect, chloroquine, which has been shown to be a useful agent in the control of rheumatoid arthritis, must be given for a long period before benefit becomes evident. Also, the anti-inflammatory response to salicylate could not be enhanced by increasing the dose above 0·75 m-mole/kg; however, the concomitant administration of acetylcysteine significantly reduced the inflammation to a greater extent than that resulting with either compound alone. Consequently, treatment of persons with rheumatoid arthritis by a combination of salicylate and acetylcysteine may be more effective than treatment with salicylate alone. In this respect, it may be possible to lower the effective dose of salicylate to less toxic levels. However, from a completely different aspect, unrelated to the anti-inflammatory mechanisms discussed thus far, sulfhydryl compounds such as acetylcysteine may reduce the rheumatoid factor in rheumatoid arthritis from an abnormal macroglobulin to smaller more nearly normal globulins. Rheumatoid factor possibly acts as a foreign protein in the tissues and joints; degradation of this molecule thus could remove one of the factors leading to inflammation. Studies with sulfhydryl compounds in rheumatoid arthritis indicate, however, that clinical improvement does not occur for several weeks after initiation of treatment.[12] Therefore, it may be postulated that abnormal globulins which are produced in these patients are not immediately reduced by the sulfhydryl compounds *in vivo*; however, new synthesis of rheumatoid factor may cease, and improvement can occur as the already formed circulating macroglobulins are metabolized. Thus sulfhydryl compounds may be of use in the treatment of rheumatoid arthritis via three mechanisms, of which only the first two were studied in this investigation: (1) direct effect on the inflammatory process; (2) potentiation of salicylate activity; and (3) reduction of rheumatoid factor.

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