Abnormal Sulfhydryl–Disulfide Interchange in Serum of Rats with Adjuvant Arthritis: Correction by Anti-inflammatory Agents (34242)

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Serum protein sulfhydryl (SH)–disulfide (SS) interchange reactions may play important roles in protein conformation and binding, blood clotting, and cell division (1), phenomena which are markedly influenced by inflammatory states (2–4). Therefore, it was of interest when Gerber and co-workers (5) reported that addition of nonsteroidal anti-inflammatory agents accelerated the interchange reaction between human serum protein SH groups and the S–S linkage of 5,5′-dithiobis (2-nitrobenzoic acid) (DNBA) to yield a colored product, 5-thio-2-nitrobenzoic acid (NBA). This finding suggested that antiphologistic agents may somehow influence the exposure, reactivity or number of SH groups present in serum and may exert at least a portion of their anti-inflammatory activity by such a mechanism.

When we explored the SH–SS interchange reaction using rat serum, little or no acceleration was observed when anti-inflammatory agents were added *in vitro* or administered to the animals. However, a clear reduction in the reaction rate was evident in serum obtained from rats with adjuvant-induced polyarthritis as opposed to that of intact rats. This phenomenon and its reversal by steroidal and nonsteroidal anti-inflammatory agents are reported below.

Materials and Methods. A. Induction of inflammation. Polyarthritis was induced by intradermal injection of a suspension of *Mycobacterium butyricum* (1 mg/0.05 ml of heavy mineral oil) into the tails of Sprague-Dawley rats according to a slight modification of the method described by Glenn *et al* (3). Severity of the arthritic lesions (which appear in 10–15 days) was graded using a scoring system of 1+ = detectable, 2+ = moderate, 3+ = severe for each inflamed paw. The individual scores thus could range from 0 (no lesions) to 12+ (severe lesions of all four paws). Blood was drawn by heart puncture on days 22–23, except in a time study experiment where blood was obtained at 2, 4, 7, 14, and 22 days.

Other types of inflammation studied for comparison were the carrageenin paw edema (6), croton oil paw edema (7), turpentine pleuritis (8), egg white-induced anaphylactoid reaction (9), and granuloma pouch (10). The inflammatory states were induced in rats essentially according to the procedures described in the literature cited. Briefly, 0.1 ml of 1% carrageenin or 0.1 ml of 1% croton oil was injected intradermally in the plantar region of the hind paws. The former animals were bled at 4 hr while the latter were bled at 30 hr. To induce pleuritis, 0.1 ml of turpentine was injected into the pleural cavity. Blood samples were obtained 3 hr later. Granuloma pouches were prepared by injecting 25 ml of air subcutaneously in the dorsal region. Two-tenths ml of 1% croton oil was injected into the pouch. Blood was obtained 5 days later.

B. Administration of anti-inflammatory compounds to arthritic rats. Indomethacin (0.3 mg), phenylbutazone (50 mg) or 3-chloro-4-cyclohexyl-α-methyl phenyl acetic acid "CMPA" (0.3 mg) were administered orally as aqueous suspensions. Dexamethasone (0.05 mg) was administered orally as an oil solution. Treatments were started on day 7 after adjuvant injection and continued daily for 14 days. Blood was drawn on days 22–23 (*i.e.*, 24–48 hr after the last treatment).

C. Determination of SH–SS interchange reaction. Only fresh serum samples were used because the rate of the interchange reaction was decreased when samples were stored overnight at refrigerator temperature. Immediately after the fresh serum was harvested, 1 ml was pipetted into each of two 40-ml round bottom tubes. To one tube (the blank) was added 1.5 ml of 0.1 *M* phosphate buffer, pH 7.4. This blank was used to zero the spectrophotometer (Beckman DB) at 440 mμ. To the other tube was added 1.0 ml of the phosphate buffer and 0.5 ml of DNBA[1] solution (5). The reaction was timed by stopwatch or registered on a Beckman recorder. In initial experiments the optical density (OD) at 2 min was used as the final value. Subsequently it became apparent that the change in OD between 1 and 2 min yielded more consistent data. The increase in OD was strictly linear during this time interval, and thus the change in OD occurring between 1 and 2 min was adopted as the routine measure of the SH–SS interchange reaction.

In a recent paper (11) dealing with measurement of tissue SH groups, the importance of pH was stressed. We investigated the influence of pH using a pool of fresh normal rat serum. The pH of the 0.1 *M* phosphate buffer was adjusted to include a range from 7.4 to 8.3. Although the intensity of the DNBA-induced color increased with increasing pH, the change in OD between 1 and 2 min remained constant (Fig. 1). Therefore, we chose to continue to run our interchange reactions at pH 7.4: (a) higher pH might alter the availability of serum SH groups; (b) the readings at pH 7.4 were on the most useful portion of the OD scale.

D. Inhibition of SH–SS interchange reaction by "SH-reagents". Serum from control and arthritic rats and a glutathione standard (30 μg) were prepared in phosphate buffer and reacted with DNBA in the usual way. Duplicate samples were pretreated with SH inhibitors iodoacetamide or *N*-ethylmaleimide (each 1.33 m*M*). The interchange reaction was in each case severely impaired by these reagents (Fig. 2).

[1] The DNBA solutions were freshly prepared on each test day by dissolving 2.576 mg of DNBA in 20 ml of the phosphate buffer.

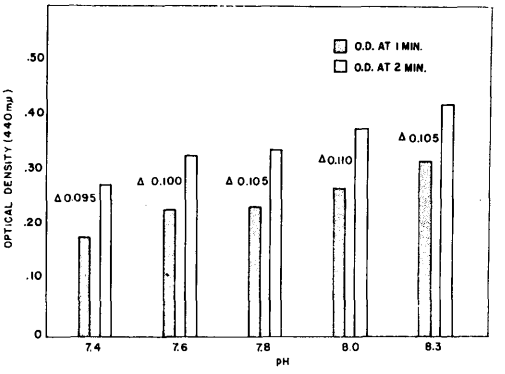

FIG. 1. Effect of pH on SH–SS interchange reaction of rat serum with DNBA (△OD, 1–2 min): buffer = 0.1 *M* phosphate. Note that increasing pH increases the OD readings but does not influence the *change* in OD between 1 and 2 min.

Results. A. Comparison of rat and human serum SH–SS interchange. Direct comparison of the SH–SS interchange of normal rat versus normal human serum revealed that the reaction proceeded much more rapidly and plateaued earlier in the case of the rat serum (Fig. 3). Arthritic rat serum showed very little change over the 10-min observation period. Addition of phenylbutazone (65 μ*M*) *in vitro* accelerated the interchange in human

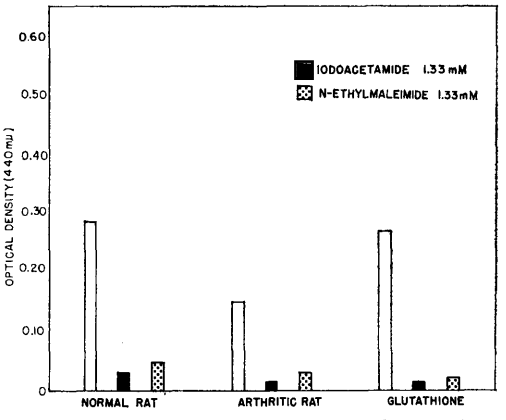

FIG. 2. Inhibition of the SH–SS interchange of rat serum and DNBA by addition of sulfhydryl reagents; interaction time was 2 min. Duplicate serum samples were mixed with 0.1 *M* phosphate buffer with or without 1.33 m*M* iodoacetamide or *N*-ethylmaleimide prior to reacting with DNBA.

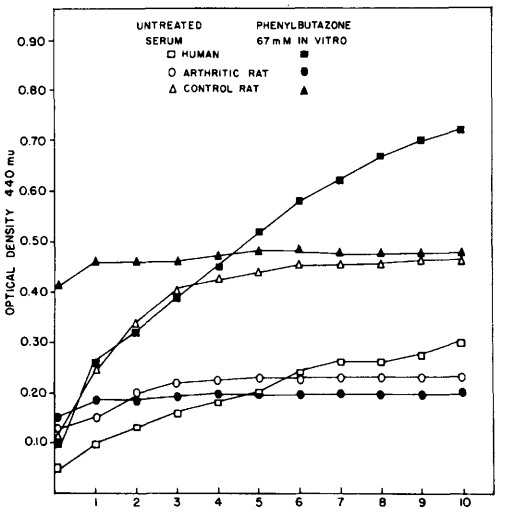

FIG. 3. Effect of anti-inflammatory compounds; comparison of rat and human SH–SS interchange reactions and influence of phenylbutazone added *in vitro*.

serum (Fig. 3) confirming the work of Gerber *et al.* (5). The results with normal or arthritic serum were more difficult to interpret as the OD at 0 time was elevated in the serum samples which contained phenylbutazone and no further increase was seen during the 10-min period of observation (Fig. 3). This is thought to be an artifact due to a slight turbidity which developed when phenylbutazone was added to rat serum. (As shown below, phenylbutazone did not alter the SH–SS interchange rate of serum when administered to the rats *in vivo*).

B. SH–SS interchange reaction during development of adjuvant-induced polyarthritis in rats. In comparison with serum of normal rats, the SH–SS interchange reaction of arthritic rat serum obtained 22 days after adjuvant administration was severely depressed (Fig. 4). This depression could be detected in some animals 96 hr after adjuvant injection and progressively worsened until day 22 (Fig. 4). Gross arthritic lesions first appeared between days 10 and 15, so the impaired SH–SS interchange reaction preceded visible signs of the adjuvant disease. When indomethacin (0.5 mg/day) was administered orally to a similar group of arthritic rats from day 0 to 21, the SH–SS interchange reaction was intermediate between that of the untreated controls and the arthritic animals (Fig. 4). Their lesions were likewise less severe than those of arthritic rats which did not receive therapy.

C. Specificity of the abnormal SH–SS interchange reaction. When blood was obtained from rats which had been subjected to various other inflammation-inducing procedures, the SH–SS interchange reaction did not differ significatly from control (Fig. 5). Thus, rats with turpentine pleuritis, egg white-induced anaphylactoid shock, croton oil or carrageenin-induced paw edema or croton oil-induced granuloma pouches exhibited essentially normal serum SH–SS interchange reactions. Only rats with adjuvant-induced arthritis exhibited the severe depression in the interchange reaction. Furthermore, adjuvant-treated animals which did not develop gross arthritic lesions did not show a significant decrease in the interchange reaction (Fig. 5).

D. Influence of steroidal and nonsteroidal anti-inflammatory compounds on SH–SS interchange reaction in arthritic rats. Neither indomethacin, phenylbutazone, CMPA, nor dexamethasone administered for 14 days to normal rats influenced the SH–SS interchange reaction of their sera with DNBA (Fig. 6). However, when these same compounds were administered from days 7 to 21

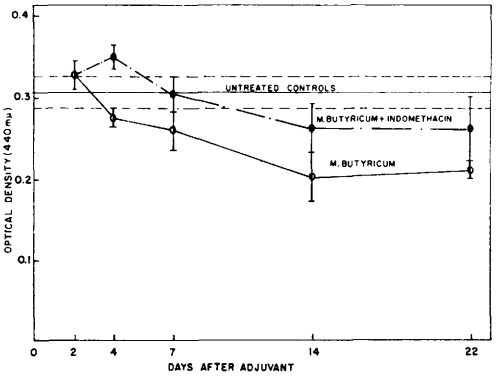

FIG. 4. Sulfhydryl–disulfide interchange between normal or arthritic rat serum and DNBA; time course of depression of the serum SH–SS interchange reaction in adjuvant-treated rats and its partial prevention by indomethacin (0.3 mg/day). Five to 10 rats from each group were killed at each time interval. Reaction time of serum with DNBA = 2 min.

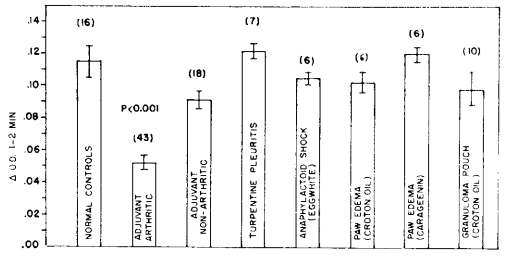

FIG. 5. The SH–SS interchange reactions with DNBA of serum samples obtained from rats with various types of experimental inflammation. Only the adjuvant arthritic rats differed significantly from control. Adjuvant injected rats, which failed to develop lesions, had only a slightly impaired interchange. Number of animals given in parentheses.

after adjuvant injection they not only reduced the number and severity of arthritic lesions but restored the serum SH–SS interchange reaction partially or completely to normal (Fig. 6). The correlation between the severity of the arthritic lesions and the depression of the SH–SS interchange reaction was 0.77, which was significant at $p<0.01$.

Discussion. Our data suggest that the impaired SH–SS interchange reaction of arthritic rat serum may offer a useful parameter for the evaluation of steroidal and nonsteroidal anti-inflammatory compounds. The depression of the interchange correlated well with the severity of the adjuvant disease. That the abnormal interchange reaction could actually be detected prior to the development of gross arthritic lesions suggests that it may represent one of the underlying biochemical aberrances which lead to lesion formation. From a practical standpoint the early detection of the abnormal SH–SS interchange might permit a drastic reduction in the time required to assess the value of anti-inflammatory compounds in the adjuvant arthritis test. The abnormal reaction under investigation was not detected in serum obtained from animals bearing other types of standardized experimental inflammations. It might be argued that these other tests are considerably shorter than is the adjuvant arthritis test, and that a chronic inflammation might be required to bring about this serum change. However, it has been pointed out that the response is seen in the adjuvant treated rats in 4 days. The granuloma pouch test was run for 5 days and no abnormal SH–SS interchange was observed. Other serum abnormalities such as the appearance of an abnormal α-2-glycoprotein described by Bogden *et al.* (7, 12) appear in several types of inflammatory states and thus are not as specific as the abnormal SH–SS interchange reaction. Furthermore, abnormal α-2-glycoprotein synthesis is *stimulated* by glucocorticoids (7) and thus would not appear to provide a valid assessment of steroidal anti-inflammatory agents. The abnormal serum SH–SS interchange reaction is corrected by both steroidal and nonsteroidal anti-inflammatory compounds and thus would appear to offer a more useful as well as more specific parameter for the assessment of such agents.

Summary. Using the reagent 5,5′-dithiobis(2-nitrobenzoic acid), a sulfhydryl–disulfide (SH–SS) interchange reaction was observed and characterized in serum of normal rats and rats with adjuvant-induced arthritis. The SH–SS interchange reaction was severely retarded in the latter group of animals but could be restored essentially to normal by appropriate therapy with steroidal or nonsteroidal anti-inflammatory agents. The defect in SH group availability was detectable as early as 4 days after adjuvant injection and well before the appearance of gross arthritic lesions (days 10–15). The ab-

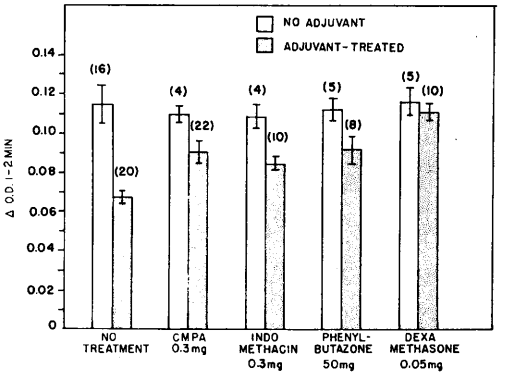

FIG. 6. Effect of anti-inflammatory compounds on serum SH–SS interchange reaction in normal and arthritic rats. Treatments were given orally daily for 14 days (days 7–21 postadjuvant in the arthritic rats). Number of rats per group given in parentheses.

normal serum SH–SS interchange reaction was specific for rats with adjuvant arthritis and did not appear in adjuvant-treated rats which failed to develop arthritis or in a variety of other experimentally induced inflammatory states.

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