Effect of auranofin on plasma fibronectin, C reactive protein, and albumin levels in arthritic rats

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SUMMARY Auranofin, a member of a class of compounds with disease modifying activity, was given to arthritic rats to determine if it could reverse the abnormal plasma concentrations of fibronectin (Fn), C reactive protein (CRP), and albumin, which were unaffected by treatment with non-steroidal anti-inflammatory drugs (NSAIDs). When auranofin was orally administered for two weeks to adjuvant induced arthritic rats it significantly inhibited swelling of the injected and non-injected paws at doses of 3 and 10 mg/kg. Rocket electroimmunoassay measurement of plasma proteins in normal, arthritic, and auranofin treated arthritic rats indicated that auranofin at 10 mg/kg significantly decreased (by 77%) the abnormally high concentration of arthritic rat plasma Fn, though it had no effect on Fn concentrations when administered to normal rats. CRP, which was raised approximately twofold above normal in arthritic rats, was reduced by 56% after treatment of arthritic rats with auranofin at 10 mg/kg, though CRP concentrations in normal rats were unaffected by auranofin treatment. Depressed albumin concentrations in arthritic rats were significantly enhanced (by 30%) by dosing with 10 mg/kg of auranofin. At the 3 mg/kg dose, auranofin did not significantly change plasma concentrations of Fn, CRP, and albumin in arthritic rats. At a dose of 10 mg/kg, however, auranofin, in addition to inhibiting chronic systemic paw inflammation, also altered abnormal concentrations of plasma Fn, CRP, and albumin in the adjuvant arthritic rat, thus distinguishing auranofin from standard NSAIDs we have previously tested.

Key words: adjuvant arthritis, disease modifying antirheumatic drugs (DMARDs).

Certain gold compounds are classified as disease modifying antirheumatic drugs (DMARDs) based on their ability to slow the progression of joint destruction in rheumatoid arthritis (RA).¹ Adjuvant induced arthritis in rats is a model of disease which shares many of the characteristics of RA² and is therefore useful in identifying compounds which may be beneficial in the clinic.³ When oral gold is administered to adjuvant arthritic rats it significantly decreases paw inflammation.⁴

Paw inflammation in the arthritic rat is accompanied by abnormally high concentrations of plasma fibronectin.⁵ Fibronectin (Fn) is a 440 kilodalton protein found in high concentrations in the synovial

fluid^{6–8} and pannus tissue^{9 10} of patients with RA and the plasma of lupus patients.¹¹ It has opsonic,¹² chemotactic,^{13 14} and adhesive^{15 16} properties, which may contribute to its suggested role in the pathophysiology of some rheumatic diseases.^{17 18} Abnormally high plasma Fn concentrations have been found not only in the arthritic rat⁵ but also in the MRL/MpJ (1pr) lupus mouse.¹⁹ Agents like glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs), which provide symptomatic relief without altering the progression of rheumatic disease,²⁰ are active in decreasing paw swelling in arthritic rats.²¹ Neither glucocorticoids²² nor NSAIDs,²³ however, significantly decrease high Fn concentrations in arthritic rats as measured by rocket electroimmunoassay, though NSAIDs have been reported to lower serum concentrations of the acute phase protein α_1 acid glycoprotein.² In

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another model of inflammation, the carrageenan induced pleurisy macrophage model developed by Ackerman *et al.*,²⁴ NSAIDs also failed to reduce the high concentration of Fn in the rat pleural exudate.²⁵ In contrast, auranofin, as well as other DMARDs, sodium aurothiomalate, chloroquine, and hydroxychloroquine, significantly reduced concentrations of Fn and the number of inflammatory cells in carrageenan induced rat pleural exudates.²⁵

Raised concentrations of acute phase proteins,²⁶ particularly C reactive protein (CRP),^{27 28} have been associated with RA. There is clinical evidence indicating that abnormally high concentrations of CRP in patients with RA cannot be reduced by NSAID treatment, which relieves the symptoms but not the underlying progression of disease.²⁹ DMARDs, however, like auranofin, which are efficacious in the treatment of RA,²⁰ also decrease abnormally high concentrations of CRP in arthritic patients.³⁰⁻³²

In contrast with the typically high concentrations of plasma CRP associated with RA,^{27 28} serum albumin concentrations in patients with RA are typically depressed.^{33 34} Adjuvant arthritic rats also possess abnormally low concentrations of plasma albumin,³⁵ which could not be raised by treatment with standard NSAIDs.³⁶

As raised concentrations of plasma Fn associated with adjuvant arthritis in rats⁵ were reduced after treatment with the DMARD auranofin, but not glucocorticoids²² or NSAIDs,²³ reduction of plasma Fn in arthritic rats and dampening of the acute phase response (typified by alteration of plasma CRP and albumin concentrations) may be characteristic of a class of antirheumatic compounds distinguishable from standard NSAIDs.

Materials and methods

ANIMALS

Male, inbred Lewis rats (approximately 180 g) were obtained from Charles River Laboratories.

INDUCTION AND MEASUREMENT OF ADJUVANT ARTHRITIS

Freund's complete adjuvant was prepared by adding 100 mg of *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, Michigan) to 15.6 ml of squalane oil (Aldrich Chemical Co). The *M tuberculosis* was then ground in a homogeniser (Eberbach Corp, Ann Arbor, Michigan) followed by addition of 1 ml of 0.15 M saline. The mixture was thoroughly emulsified by pulsing for 30 seconds with a Polytron (Brinkman Instruments, Westbury, New York). Each rat was injected in the right hind footpad with 300 µg of *M tuberculosis* in a 0.05 ml

volume. The systemic nature of the disease was assessed by measuring non-injected (left) hind paw swelling 17 days after adjuvant injection and two hours after the final dose of drug. The injected (right) hind paw was also measured at this time. Paw inflammation was measured by obtaining paw volume with a mercury plethysmograph and recording with a polygraph the amount of mercury displaced (ml).

DOSING

Auranofin was delivered orally in a volume of 1 ml/100 g of body weight. The drug was suspended in a 1% solution of warmed (approximately 37°C) gum tragacanth and homogenised by grinding (Eberbach, Ann Arbor, Michigan) before delivery at 3 or 10 mg/kg. Two days after day 1 adjuvant injection daily dosing was begun and continued until the experiment was finished on day 17. Normal and arthritic untreated controls received vehicle alone. Normal non-injected rats treated with auranofin were given drugs orally according to the regimen used for arthritic rats.

PLASMA PREPARATION

Three hours after the animals received the final dose of drug and immediately after paw volume measurements a 0.1 ml sample of blood was obtained by cardiac puncture with a 1 ml syringe and 27 gauge needle. Blood was immediately mixed in microvials with 0.012 ml sodium citrate (18.5 mg/ml), and centrifuged for five minutes in a tabletop centrifuge (Fisher Scientific, Fair Lawn, New Jersey). Whole plasma was removed and assayed for CRP, Fn, and albumin. Repeated freeze thawing was avoided. All experiments were conducted with 10 animals in each group.

PURIFICATION OF PLASMA PROTEINS

Commercially available Cohn fraction 5 purified rat albumin (Sigma Chemical Co, St Louis, Missouri) was used as the albumin standard, and antibody to rat albumin was purchased from Cooper Biomedical Laboratories (Malvern, Pennsylvania). It was necessary to purify large quantities of rat Fn and CRP for use as antigen in obtaining appropriate antibody and for calibration of a rat plasma pool standard.

Purified Fn was obtained by a modification of the affinity chromatography procedure of Weiss and Reddi³⁷ described by us previously.¹⁹ About 200 ml of plasma was applied to a gelatin Sepharose 4B (Pharmacia) column after appropriate ammonium sulphate treatment.¹⁹ The column was extensively washed with a phosphate buffered solution of 1 M saline (PBS), eluted with 4 M urea-trometamol (TRIS) buffer, and dialysed against PBS. The

dialysate was again passed over the gelatin-Sepharose column to purify the Fn further. When the purified Fn was assayed by sodium dodecyl sulphate slab gel electrophoresis it produced a 220 000 molecular weight double band, characteristic of the purified Fn dimer.

Purified CRP was obtained by a single step affinity chromatography procedure that was a synthesis of the methods employed by Young and Williams,³⁸ Nagpurkar and Mookerjee,³⁹ Pontet *et al.*,⁴⁰ and DeBeer *et al.*⁴¹ Approximately 100 ml of rat serum from normal or arthritic rats was passed over a 1 cm×20 cm column of *p*-aminophenyl phosphorylcholine immobilised on agarose (Pierce Chemical Co, Rockford, Illinois). The column was equilibrated and extraneous proteins were eluted with a trometamol (0.02 M)/saline (0.15 M)/Ca⁺⁺ buffer, pH 7.4. The column was stripped of extraneous protein by washing with a trometamol/saline/edetic acid (0.1 M) buffer and re-equilibrated in trometamol/saline/Ca⁺⁺ buffer, pH 7.4. Contamination of CRP by serum amyloid protein was minimised by pooling and concentrating only those fractions from the second half of the CRP elution peak. When this single step procedure was used a 25% yield of CRP was obtained. The purified CRP was free of serum amyloid protein contamination as measured by reduced and unreduced gradient slab polyacrylamide gel electrophoresis gels stained with Coomassie brilliant blue R-250 (Biorad, Rockville Center, New York) or silver stain (Biorad). Purified proteins were measured spectrophotometrically (280 nm) and stored (−70°C) at a concentration ≤3 mg/ml.

PREPARATION OF ANTISERA

Antibody against rat Fn and CRP was prepared by mixing the purified proteins (2 mg/ml) 1:1 with complete or incomplete Freund's adjuvant. On days 1 and 8 a 0.5 ml aliquot of the complete adjuvant (plus Fn or CRP) was injected subcutaneously into each of two sites along the flank of a goat. The procedure was repeated on day 15 using Fn or CRP plus incomplete adjuvant. On day 22 the goat received one subcutaneous and one intramuscular injection of 0.5 ml antigen plus incomplete adjuvant. Ten days later 250–500 ml of whole blood was taken from the jugular vein. Goat, antirat Fn or CRP antibody was obtained from the serum by affinity chromatography.^{19–42} The antibody specificity against Fn or CRP was monitored by immunoelectrophoresis and showed no cross reactivity with rat albumin or C3b or serum amyloid protein.

PURIFICATION OF ANTIBODY

Ten grams of CNBr activated Sepharose 4B (Phar-

macia, Piscataway, New Jersey) was added to a 100 mg of purified Fn or CRP (3 mg/ml). Standard procedures for protein conjugation were followed as described previously.^{19–40} The protein conjugated Sepharose was finally resuspended in degassed PBS (0.01 M, pH 7.4) and poured into a column where it was allowed to equilibrate with PBS. Antibody was precipitated from the goat antiserum with a saturated ammonium sulphate solution. The precipitate was resuspended with 0.01 M PBS (pH 7.2) in one fifth the original 250 ml serum volume. The antibody solution was dialysed in 0.01 M PBS and applied to the affinity column. The column was washed with PBS (pH 7.2) and the antibody to Fn or CRP eluted with 0.2 M sodium acetate. The eluate was immediately dialysed against PBS and stored at −70°C.

MEASUREMENT OF PLASMA PROTEINS

Concentrations of Fn, CRP, and albumin were measured by rocket electroimmunoassay.^{19–43–44} Plasma samples to be tested for Fn or CRP were diluted 1:10 in trometamol-Tricine buffer. Albumin measurement was made on plasma samples diluted 1:150. A series of internal standards was run for each assay. A stock 1 mg/ml solution of rat albumin was diluted 1:4, 1:5, 1:10, and 1:20 to yield standards of 250, 200, 100, and 50 µg/ml respectively. The Fn and CRP standards consisted of a sample from a rat plasma pool diluted with trometamol-Tricine buffer 1:2.5, 1:5, 1:10, and 1:20. The rat plasma pool was originally calibrated against affinity purified rat Fn or CRP. The heights of the sample rocket peaks were compared with the height of the internal standards to determine Fn and CRP concentrations of the sample.

STATISTICS

An analysis of variance and Dunnett's multiple comparison were used to compare all groups (n=10) and to derive significance between groups. Data were expressed as the mean (SEM).

Results

EFFECT OF AURANOFIN ON INJECTED AND NON-INJECTED PAW VOLUME OF ARTHRITIC RATS

Fig. 1 depicts the anti-inflammatory activity of auranofin as measured by its ability to inhibit swelling significantly in injected and non-injected rat paws 17 days after adjuvant injection. Oral drug treatment from day 3 to day 17 with 3 or 10 mg/kg of auranofin resulted in a significant ($p \leq 0.01$) reduction of injected and non-injected arthritic rat paw volumes as calculated using a one tailed

Fig. 1 Effect of auranofin on injected and non-injected paw volume of arthritic rats. On day 1 male Lewis rats (180 g) were injected in the right hind paw with 0.05 ml of complete Freund's adjuvant composed of 100 mg of *Mycobacterium tuberculosis* ground and emulsified in 15.6 ml of squalane oil and 1 ml of saline. From day 3 to day 17 animals were dosed orally once daily with auranofin suspended in 1% gum tragacanth. The volume of drug plus vehicle was calculated so that animals received 1 ml of drug plus vehicle/100 g body weight. Rats in each group of 10 were bled from the heart on day 17 and plasma protein concentrations obtained by rocket electroimmunoassay. Just before bleeding the animals were weighed and assayed for inflammation by measuring the volume of the non-injected left paw and injected right paw using mercury displacement. ** $p \leq 0.01$ compared with untreated arthritic animals. Results are the mean (SEM) of 10 animals per group.

Dunnett's multiple comparison. The percentage inhibition of swelling in the non-injected paw after treatment with 3 or 10 mg/kg of auranofin was 69 and 82% respectively. The same drug regimen inhibited injected paw swelling by 54 and 67% respectively. Treatment with 3 and 10 mg/kg of auranofin resulted in a final weight gain of 1 and 5 g respectively, compared with the final weight of untreated arthritic controls.

EFFECT OF AURANOFIN ON PLASMA Fn IN ARTHRITIC RATS

As shown in Fig. 2 arthritic rats had a significantly higher concentration of plasma Fn than their normal counterparts (619 v 359 $\mu\text{g/ml}$ of Fn). The abnormal concentration of Fn in arthritic rats was significantly ($p \leq 0.01$) reduced after treatment with auranofin at 10 mg/kg but not at 3 mg/kg. Fn concentration was reduced by 77% after treatment with 10 mg/kg of auranofin. Normal animals treated with 10 mg/kg of auranofin did not have significantly different plasma Fn concentrations than untreated controls (aurano-

fin treated normals 338 (7) $\mu\text{g/ml}$ Fn; untreated normals 339 (13) $\mu\text{g/ml}$ Fn).

EFFECT OF AURANOFIN ON PLASMA CRP IN ARTHRITIC RATS

Like the increased plasma Fn level, the CRP concentration was significantly higher in arthritic rats (718 $\mu\text{g/ml}$) than in normal controls (360 $\mu\text{g/ml}$) (Fig. 3). Treatment of arthritic rats with 10 but not 3 mg/kg of auranofin significantly reduced the level of CRP by 56%. Normal animals treated with 10 mg/kg of auranofin did not have reduced CRP concentration (auranofin treated normals 451 (10) $\mu\text{g/ml}$ CRP; untreated normals 450 (9) $\mu\text{g/ml}$ CRP).

Fig. 2 Effect of auranofin on plasma fibronectin (Fn) in arthritic rats. Fn concentrations in plasma test samples were measured by rocket electroimmunoassay. Agarose (630 mg) was dissolved in 63 ml of boiling trometamol-Tricine buffer and cooled to 63°C. Antibody was mixed in liquid gel, which was then poured on a gel bond film (FMC Corp, Rockland, Maine). Wells were punched in the solidified gel, and 10 μl samples of plasma diluted 1:10 in trometamol-Tricine buffer (pH 8.8) were then applied. A series of internal standards was run at each corner of the plate. This consisted of a sample from a rat plasma pool diluted with trometamol-Tricine buffer to 40, 20, 10, and 5% concentrations. The rat plasma pool was originally calibrated against Fn standards purified by affinity chromatography. The gel was run 21 hours on a cooling plate (LKB, Gaithersburg, Maryland) and then dried and stained with Coomassie brilliant blue R-250 (Bio-Rad, Richmond, California). The heights of the sample rocket peaks were compared with the height of the internal standards to determine Fn concentrations of the samples. ** $p \leq 0.01$ compared with untreated arthritic animals. Results represent the mean (SEM) of 10 animals per group.

Fig. 3 Effect of auranofin on plasma CRP in arthritic rats. CRP was measured by rocket electroimmunoassay as described in Fig. 2. ** $p \leq 0.01$ compared with untreated arthritic animals. Results represent the mean (SEM) of 10 animals per group.

Fig. 4 Effect of auranofin on plasma albumin in arthritic rats. Procedures for measuring plasma protein concentrations were identical to those described in Fig. 2 with the following additions. Measurement of plasma albumin was made on plasma samples diluted 1:150 with trometamol-Tricine buffer. The internal standard was a 1 mg/ml stock solution of Cohn fraction 5 albumin (Sigma) diluted 1:20, 1:10, 1:5, and 1:4 with trometamol-Tricine buffer. The antibody to rat albumin was supplied by Cooper Biomedical Laboratories. ** $p \leq 0.01$ compared with untreated arthritic animals. Results represent the mean (SEM) of 10 animals per group.

EFFECT OF AURANOFIN ON PLASMA ALBUMIN IN ARTHRITIC RATS

To determine whether reduction of arthritic rat plasma Fn and CRP by auranofin was due to generalised inhibition of liver protein synthesis, plasma albumin concentrations were measured (Fig. 4). In contrast with the raised levels of Fn and CRP seen in the untreated arthritic rats, plasma albumin concentrations in arthritic rats were depressed compared with normals (6.4 mg/ml v 18.4 mg/ml). Treatment of arthritic rats with auranofin did not reduce the already low concentrations of plasma albumin as would be expected if auranofin were causing generalised inhibition of liver protein synthesis. In the experiment shown in Fig. 4 auranofin at 10 mg/kg significantly enhanced plasma albumin levels by 30%.

Discussion

In this study adjuvant induced arthritic rats had abnormally high plasma concentrations of Fn and CRP and depressed concentrations of plasma albumin. Treatment of arthritic rats with auranofin reduced paw inflammation and significantly altered the abnormal plasma concentrations of Fn, CRP, and albumin.

Fibronectin is a plasma protein normally synthesised in the liver. The high plasma Fn concentrations seen in arthritic rats⁵ may be a result of increased liver synthesis but additionally probably reflect increased *in situ* production in the inflamed joints,⁸ as synovial fluids from patients with RA contain a higher concentration of Fn than the plasma. Systemic vasculitis, characteristic of severe arthritis,⁴⁵ may also contribute to the high concentrations of plasma Fn in arthritic rats as large amounts of cryoprecipitates associated with Fn have been detected in the serum of patients with RA and vasculitis.⁴⁶ In the case of the rat model, with its severe and widely disseminated disease and relatively small blood volume, high plasma Fn concentrations may be partially a result of synthesis from the inflamed tissue. In humans arthritis is rarely allowed to progress to the degree of severity seen in the arthritic rat so that plasma Fn concentrations in humans would be expected to be normal¹⁹ in all but the most severe cases of RA. One of the effects of a DMARD such as auranofin may be its ability to dampen the overactivity of the inflamed joint and vascular tissue, resulting in a reduced concentration of plasma Fn in rats, and possibly a reduced synovial fluid Fn concentration in humans.

Unlike Fn, the acute phase protein CRP is produced only in the liver.¹⁷ Although there is no active synthesis at the site of inflammation, CRP is generally accepted as a useful clinical measurement

of disease activity.²⁶⁻²⁸ The present study supports the use of CRP as a marker of arthritic disease, as the results indicate that plasma CRP concentrations are significantly raised in arthritic rats and can be reduced by treatment with auranofin.

As the liver accounts for all or part of the synthesis of CRP and Fn it was important to establish that the auranofin mediated reduction of plasma Fn and CRP was not due to generalised inhibition of protein synthesis by the liver. Clinical results indicate that a subnormal concentration of plasma albumin is associated with RA.³³⁻³⁴ Data from this laboratory^{35,36} and others⁴⁸ indicate that arthritic rats also possess abnormally low concentrations of plasma albumin. Although these low concentrations may be due to increased catabolism of albumin,⁴⁹ there is evidence that a low plasma albumin concentration is an indicator of liver dysfunction.⁵⁰⁻⁵² If auranofin reduced plasma Fn and CRP merely by inhibiting protein synthesis in the liver then plasma albumin concentrations in arthritic rats treated with auranofin would also be expected to be low. Auranofin treatment, however, reversed the low albumin concentrations seen in arthritic rats. In view of the inverse relation between the ability of auranofin to lower plasma CRP and Fn concentration while at the same time raising plasma albumin concentration it is unlikely that auranofin is causing generalised inhibition of protein synthesis in the liver. Although 30 mg/kg of auranofin has been cited as the minimum lethal dose in rats,⁵³ the 10 mg/kg dose used in our study resulted in no lethality or weight loss. Results of a three month toxicology study indicated that the only side effect of dosing with 12 mg/kg of auranofin was a less than average weight gain.⁵⁴ This supports our data showing that treatment of normal animals with 10 mg/kg of auranofin did not alter plasma Fn or CRP concentrations. Thus it does not appear that reduction of Fn and CRP in arthritic rats is due to non-specific toxicity. The alteration of Fn,⁵⁵ CRP,⁵⁶ and albumin⁵⁷⁻⁵⁸ production may be under regulation by one or more cytokines. Hepatocyte stimulatory factor⁵⁹ has been reportedly involved in the induction of CRP in human hepatoma cell lines,⁵⁶ while we and others have evidence that high concentrations of splenic interleukin-1 in adjuvant arthritic rats³⁶ can be significantly reduced after treatment with auranofin.⁶⁰

We have also recently shown that abnormal concentrations of plasma Fn, CRP, and albumin in the adjuvant arthritic rat are not altered by NSAID treatment.³⁶ Thus measurement of these acute phase proteins in the adjuvant arthritic rat may be useful in differentiating potential antirheumatic compounds from standard NSAIDs.

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