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Research Article

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The Mechanism of Action of a Single Dose of Methylprednisolone on Acute Inflammation In Vivo

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ABSTRACT A model system for the study of inflammation in vivo has been developed using the 16-h polyvinyl sponge implant in the rat. This system allows for simultaneous measurement of in vivo chemotaxis, volume of fluid influx, and fluid concentrations of lysosomal and lactic dehydrogenase (LDH) enzymes. In addition, the enzyme content of inflammatory fluid neutrophils may also be determined.

A parallel time course of neutrophil and lysosomal enzyme influx into sponge implants was observed. This was characterized by an initial lag phase and a rapid increase between 5 and 16 h.

The origin of supernatant LDH and lysosomal enzymes was studied with anti-neutrophil serum to produce agranulocytic rats. Inflammatory fluid in these rats was almost acellular and contained decreased concentrations of beta glucuronidase (−96%) and LDH (−74%).

In control rats all of the supernatant beta glucuronidase could be accounted for by cell death and lysis, as estimated from measurements of soluble DNA. Only 15-20% of the LDH activity could be accounted for on the basis of cell lysis. The remainder was derived from neutrophil-mediated injury to connective tissue cells.

Large intravascular doses of methylprednisolone markedly inhibited neutrophil influx into sponges and adjacent connective tissue. Secondary to decreased neutrophil influx, fewer neutrophils were available for lysis, and lysosomal enzyme levels in inflammatory fluid decreased.

No evidence for intracellular or extracellular stabilization of neutrophil lysosomal granules by methylprednisolone was found.

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INTRODUCTION

Corticosteroids have been studied extensively in respect to their effects on the acute inflammatory reaction.

In various in vivo models of acute inflammation they have been shown to inhibit neutrophil accumulation in injured tissue. This has been demonstrated in the rat after carrageenin injections into the paw (1) by polyvinyl sponge implantation (2), and by subcutaneous injection of air and carboxymethylcellulose (3). In man, a similar effect has been noted with Rebuck skin windows (4, 5) or Senn plastic skin chambers (6). Steroid-treated mice have shown decreased amounts of tissue neutrophils after subcutaneous injections of cotton dust and *Staphylococcus aureus* (7).

Paramethasone (1 mg/kg orally) (1), methylprednisolone (30 mg/kg i.v.) (2), cortisol (2-3 mg/kg i.v.) (4), prednisone (0.5-1.5 mg/kg daily orally) (5, 6), and prednisolone (100 mg/kg i.p.) (7) were among the drugs used in these in vivo studies. Tissue fluid levels of these steroids were not measured.

In vitro exposure of neutrophils to high concentrations of hydrocortisone succinate (100 μg/ml) or methylprednisolone (50 μg/ml) inhibited rabbit neutrophil migration in Boyden chambers (8). However, Borel was unable to inhibit rabbit neutrophil chemotaxis in vitro at hydrocortisone concentrations of 100 μg/ml (9). He has stated that higher in vitro concentrations were cytotoxic. Ketchel, Favour, and Sturgis, using human neutrophils, showed inhibition of migration in capillary tubes after exposure of cells to 2-10 μg/ml of hydrocortisone (10). Perper, Sanda, Chinea, and Oronsky (1) have shown decreased chemotaxis in Boyden chambers of neutrophils isolated from steroid-treated rats.

In vivo studies of lysosomal enzyme release from inflammatory areas have involved the injection of carrageenin or nystatin into the paws of rats. A perfusion technique was then used to collect inflammatory fluid for enzyme assays (11). Dexamethasone (100 μg/kg, orally) and beta methasone (100 μg/kg, orally) decreased lysosomal enzyme levels in perfusates. Prednisolone (1 mg/kg orally) had no effect. Lysosomal enzymes have also been extracted by homogenization of rat paws afflicted with adjuvant arthritis. Hydrocortisone (20 mg/kg, orally) decreased enzyme levels in these extracts (12).

In vitro studies have failed to show steroid protection of human neutrophil lysosomal membranes from thermal or detergent injury (13).

Slight protection of guinea pig neutrophil lysosomes against hypotonic lysis has been demonstrated in vitro by Ignarro and Colombo (14). Goldstein has shown that steroids inhibited release of lysosomal enzymes from cytochalasin B-treated human neutrophils in vitro (15).

The implantation of polyvinyl sponges for 16 h has been shown to be useful for the study of in vivo chemotaxis (16). We have extended the usefulness of this model by making simultaneous measurements of sponge fluid neutrophil content, volume, protein, and soluble enzyme concentrations, and the intracellular enzyme contents of fluid neutrophils. We have used this system to study the effects of antineutrophil serum (ANS)[1] and a single injection of methylprednisolone (1-60 mg/kg) on these parameters.

These studies made possible the evaluation of the relative quantitative importance of methylprednisolone effects on neutrophil chemotaxis, lysosomal granule stabilization, and lysosomal enzyme secretion in the mediation of its anti-inflammatory effect.

METHODS

Materials

Drugs. Methylprednisolone sodium succinate in mix-o-vials with diluent was a gift of the Upjohn Company (Kalamazoo, Mich.). Nembutal sodium was puchased from Abbott Laboratories (North Chicago, Ill.).

Chemicals. Phenolphthalein monoglucuronic acid, sodium pyruvate, and NADH were products of the Sigma Chemical Co. (St. Louis, Mo.). [U-^{14}C]Tyrosine (404 mCi/mmol) and [*carboxyl*-^{14}C]dextran (1.28 mCi/g) were purchased from New England Nuclear (Boston, Mass.). Benzidine and trypan blue were products of Matheson Coleman & Bell (East Rutherford, N. J.). Triton-X-100 was purchased from the Rohm and Haas Co. (Philadelphia, Pa.). Yeast transfer RNA was a product of Schwarz/Mann Div., Becton, Dickinson & Co., (Orangeburg, N. Y.). Calf thymus DNA was a product of General Biochemicals (Div., North American Mogul Products Co., Chagrin Falls, Ohio). Electrophoretically pure deoxyribonuclease I was purchased from Worthington Biochemical Corp. (Freehold, N. J.) and pronase B (nuclease-free) was a product of Calbiochem (San Diego, Calif.). All other chemicals used were of reagent grade.

Animals. Female Wistar rats (wt range 180–210 g) were used in all experiments. They were purchased from the Charles River Breeding Laboratories, Inc. (Wilmington, Mass.).

Sponges. Polyvinyl sheets, dry- or formaldehyde-packed, were obtained from the Unipoint Co., High Point, N. C. Dry sponges were cut to a uniform 1.3 × 1.3 × 0.3-cm size (dry wt 30–32 mg) and washed in tap water by 35–50 soakings and squeezings. The sponges were then washed in filtered deionized water in the same manner, before sterilization by boiling in deionized water for 30 min before transfer to 0.15 M NaCl. Before implantation, wet sponges were dried by compression between two sterile cloth towels. A residual of 40–50 μl of 0.15 M saline was present in each of these sponges at the time of implantation. This was determined by weighing 20 sponges dry and after compression, as described. Air-dried, ultraviolet light-sterilized sponges were also used in some experiments.

ANS and control serum. Preparation and assay. Rabbit ANS was prepared by a modification of a method described by Simpson and Ross (17). Rat neutrophils were prepared from peritoneal exudates produced by injection of 30 ml of 3% proteose peptone. The cells were collected in an equal volume of heparinized (10 U/ml) 0.15 M NaCl and then centrifuged at 700 *g* for 10 min at 25°C. Red cells were removed by one or two cycles of hypotonic lysis, and the neutrophil pellet was washed three times with 9 ml of phosphate-buffered saline (PBS) (0.14 M NaCl, 0.01 M phosphate buffer, pH 7.3). Two New Zealand rabbits (wt 3 kg) were each immunized with 1 × 10^8 cells mixed with 2 ml of complete Freund's adjuvant. Each dose was given at four subcutaneous sites. Weekly boosters of 1 × 10^8 cells in PBS were given for 5 consecutive wk. In one rabbit the ANS agglutination titer at 5 wk was 1:640 and its cytotoxic titer 1:1,280. This animal was used as a source of ANS. Agglutination titers were measured as described by Simpson and Ross (17) and cytotoxic assays as described by Simpson and Ross (18) and modified by Miller and Wilson (19). ANS and normal rabbit serum (NS) were absorbed as described by Simpson (17), Millipore-filtered (Millipore Corp., Bedford, Mass.), and frozen at − 20°C in 2.5-ml aliquots before use.

Neutrophil lysosomal granule sonicates. Rat neutrophils were obtained from 18-h proteose peptone peritoneal exudates. From 2 × 10^8 neutrophils, lysosomal granules were prepared according to Chodirker, Bock, and Vaughan (20). These granules were suspended in 0.15 M NaCl and lysed by ultrasound (Branson Sonifier, Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) at a setting of speed 3 for 10 s. The Millipore-filtered sonicate was assayed for beta glucuronidase (21), LDH (22), acid ribonuclease (23), cathepsin D (24), and protein (25).

Sponge model system techniques

Sponge implantation was accomplished as previously described (16), except that three sponges were implanted on either side of the midline in the lower thoracic and lumbar regions.

[1] *Abbreviations used in this paper:* ANS, antineutrophil serum; IF, sponge inflammatory fluid; LDH, lactic acid dehydrogenase; NS, normal serum; PBS, phosphate-buffered saline; WCEE, white cell equivalent of enzymes.

Sponge removal. Sponges were removed through an extension of the original incision, with Nembutal (Abbott Laboratories) for anesthesia (16). They were placed in preweighed beakers and reweighed to determine fluid content. Cultures were obtained from all sponge sites. Data from infected rats were discarded. Sponge capsular connective tissue was biopsied, fixed in 10% formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sponges were squeezed manually with gloved fingers to obtain fluid free of skin enzymes. The fluid from three sponges was pooled, giving two samples for analysis from each rat.

Experimental schedules

Time studies. The rats were divided into four groups of six to eight animals. Sponges were removed from the first group at 5 h and from the others at 11, 16, and 21 h, respectively.

Methylprednisolone studies. Rats were divided into groups of six to eight animals. Methylprednisolone was injected as an intracardiac bolus 5 min before sponge implantation. Sponges were removed at 16 h. Group dosages were 0, 1, 10, 20, 30, and 60 mg/kg.

ANS-treated rats. Agranulocytosis was produced in six rats given 0.5 ml of ANS i.p. every 4 h for five doses. All rats had neutrophil counts of less than 100/mm^3 before sponge implantation. A control group was treated with an identical schedule of injections of NS. Sponges were removed in both groups 16 h after implantation. All fluid cultures on agranulocytic and control rats were negative.

Lysosomal enzyme-containing sponges were implanted into four agranulocytic rats rendered neutropenic as described above. Two enzyme-containing sponges were implanted in the left lumbar area and two 0.15 M NaCl-containing sponges in the right lumbar area of each rat. Separate incisions were made on each side. Sponges were removed at 16 h. Each sponge contained 300–400 μl of lysosomal enzyme solution. The enzyme solutions used contained no LDH activity. The lysosomal enzyme concentration of this solution was beta glucuronidase, 3 U/ml (4×10^6/ml); Cathepsin D, 0.1 U/ml (5×10^6/ml); and acid ribonuclease, 14 U/ml (3.5×10^6/ml). The numbers in parentheses indicate the cell numbers required to supply these enzyme concentrations, as determined from assays of intact peritoneal neutrophils.

Assays of sponge fluid

Aliquots were taken from each pool for total and differential cell counts (Wright-Giemsa stained smears). Total counts were done by hemocytometer and checked by Coulter automatic counting (Coulter Electronics Inc., Hialeah, Fla.). Squeezed sponges were fixed in 10% formaldehyde and processed for histologic study as described. Fluid content per sponge was determined by weighing.

Centrifugation and dilution of sponge fluid. An aliquot of each pool (100–400 μl) was centrifuged at 700 *g* for 20 min at 4°C after addition of 10 μl of [^{14}C]dextran (30,000 cpm). Supernates were removed and diluted 1:10 with PBS. Pellets were lysed in PBS and 0.1% Triton-X-100 in a volume of 1–1.5 ml.

Assays of inflammatory fluid and blood

Aliquots of diluted supernates and cell lysates were assayed for protein (25), hemoglobin (26), and for beta glucuronidase (21, 27), LDH (22), and acid ribonuclease (23). Triton-X-100 was added to supernates to a concentration of 0.1% to measure the enzyme content of lysosomal granules present in this fraction. Aliquots of undiluted supernate were combined with an equal volume of 10% trichloroacetic acid (TCA) at 4°C. DNA internal standards were added to duplicate aliquots. The precipitates were washed three times with cold 5% TCA with centrifugation at 20,000 *g* for 5 min after each wash. The precipitates were then extracted with 5% TCA for 1 h at 70°C and DNA was determined (28).

A blood sample was obtained from each rat immediately after sponge removal. Aliquots were used to measure hematocrits, hemoglobins, white counts, and differential counts. Heparinized plasma was obtained, as well as a 1:10 hemolysate of whole blood in distilled water for determination of enzymes, hemoglobin, and protein, as described above. The hemolysate was used after centrifugation at 1,500 rpm for 15 min.

The data obtained from inflammatory fluid, plasma, and hemolysate assays were inserted into a computer program based on the equations presented below.

Equations

Calculation of inflammatory fluid (IF) supernate enzyme concentrations.

$$E_{SC} = E_{ST} - (E_{SP} + E_{SR}) \quad (1)$$

E_{SC}, corrected IF supernatant enzyme concentration; E_{ST}, total IF supernatant enzyme concentration; E_{SP}, plasma-derived supernatant enzyme concentration; E_{SR}, hemolysed red cell-derived supernatant enzyme concentration, all in units per milliliter. Eq. 1 corrects the total enzyme activity of supernatant fluid for contributions due to enzyme activity derived from plasma and hemolysed red cells. The Eqs. 2–5 are used to calculate the terms E_{SP} and E_{SR} used in Eq. 1. R_p and C are calculated by dividing the hemoglobin concentration of plasma and of inflammatory fluid supernate, respectively, by the hemoglobin content of a microliter of red cells.

$$E_{pc} = E_{PT} - (R_p \times E_R) \quad (2)$$

E_{pc}, corrected plasma enzyme concentration in units per milliliter; E_{PT}, total plasma enzyme concentration in units per milliliter; R_p, microliters of red cells per milliliter of plasma; E_R, enzyme concentration of red cells in units per microliter of red cells.

$$E_{SP} = E_{pc} \times \frac{\text{(IF protein, mg/ml)}}{\text{(serum protein, mg/ml)}} \quad (3)$$

Gel electrophoresis of plasma and inflammatory fluid proteins (29) gave identical patterns. This indicated that there was no differential diffusion of plasma proteins into the sponge fluid. This provided the rationale for the use of this simple ratio to calculate the plasma contribution (E_{SP}) to total supernatant enzyme activity.

$$E_R = \frac{E_{HT} - E_{PT} \times 10^{-3} \times (1 - \text{HCT}/100)}{\text{HCT}/100} \quad (4)$$

E_{HT}, total enzyme concentration of hemolysate of whole blood in units per microliter; HCT, hematocrit in percent.

$$E_{SR} = C \times E_R \quad (5)$$

C, microliters of hemolysed red cells per milliliter of inflammatory fluid supernate.

Calculation of intracellular enzyme concentrations of inflammatory fluid neutrophils. An aliquot of each inflammatory fluid pool was centrifuged for 20 min at 700 *g* in an International Centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) at 4°C after addition of 10 μl of [^{14}C]dextran (30,000 cpm). The supernate was removed and the neutrophil pellet was lysed in 1.5 ml of PBS and 0.1% Triton-X-100. [^{14}C]dextran counts of neutrophil lysate and supernate were used to calculate the volume of supernatant present in the lysate fraction (Eq. 7). Lysate hemoglobin concentration was used to calculate the volume of red cells present in this fraction (Eq. 8). Eq. 6 corrects the cell lysate activity for enzyme content derived from supernatant and intact red cells. Eqs. 7 and 8 calculate the terms on the right side of Eq. 6.

$$E_{NC} = E_{NT} - (E_{NS} + E_{NR}) \quad (6)$$

E_{NC}, corrected IF neutrophil enzyme concentration of the neutrophil lysate fraction; E_{NT}, total enzyme concentration of the neutrophil lysate fraction; E_{NS}, supernate-derived enzyme concentration in the neutrophil lysate fraction; E_{NR}, intact red cell-derived enzyme concentration in the neutrophil lysate fraction; all in units per milliliter.

$$E_{NS} = \frac{\mu\text{l supernate}}{\text{ml neutrophil lysate}} \times (E_{ST} \times 10^{-3}) \quad (7)$$

$$E_{NR} = \frac{\mu\text{l red cells}}{\text{ml neutrophil lysate}} \times E_R \quad (8)$$

$$E_{NIC} = \frac{E_{NC} \times V_{NL}}{T_N} \quad (9)$$

V_{NL}, volume of neutrophil lysate; T_N, total number of cells in the lysate in millions, based on cell counts done on the inflammatory fluid before centrifugation and lysis; E_{NIC}, intracellular enzyme content of neutrophils in units per 10^6 cells.

Estimation of supernatant enzyme activity dependent on the presence of neutrophils in the inflammatory fluid.

$$E_{ND} = E_{SC} - E_{SCA} \quad (10)$$

E_{ND}, enzyme concentration in the IF supernate dependent on the presence of neutrophils, in units per milliliter; E_{SCA}, mean enzyme concentration in the IF supernate of agranulocytic rats. These fluids are almost acellular and the activity probably is derived from local connective tissue sources.

$$\text{WCEE} = \frac{E_{ND} \times V_N}{E_{NIC}} \quad (11)$$

V_N, volume of fluid for sponge pool N. WCEE, the white cell equivalent of the enzyme content of supernatant fluid (number of cells) is a derived parameter. It is a theoretical estimate of the number of white cells whose death and lysis would be required to provide the excess enzyme found in supernatant fluids of control rats when compared to agranulocytic rats. This excess activity is dependent on the presence of neutrophils but is not necessarily due entirely to neutrophil lysis. An alternative source is neutrophil-induced injury to local tissue cells with enzyme release. WCEE as calculated attributes the total amount of excess enzyme to neutrophil lysis.

Additional studies

Studies of the effect of sponge compression on the mechanical breakage of cells with possible enzyme release were done. Sponge fluid was obtained from 16-h implants by compression, and duplicate aliquots were taken for cell counts and enzyme assays. The fluid was then absorbed into fresh dry sponges and resqueezed. All assays were repeated. Decreased cell concentration and increased supernatant LDH were used as indicators of cell lysis.

[^{14}C]Serum protein fluxes in sponges in inflammatory areas were studied. Five rats were given 5 μCi of [^{14}C]-tyrosine i.p. 4 days and 1 day before the experiment. The [^{14}C]tyrosine labeled the serum proteins and was not circulating as free [^{14}C]tyrosine at the time of the study.

10 sponges were implanted in each of four rats with radioactive serum proteins. 10 sponges were also implanted in three unlabeled rats. At 16 h supernates were obtained by centrifugation from each group.

Dry sterile sponges were used to absorb ^{14}C-labeled inflammatory fluid proteins (300–400 μl). These sponges were placed in the inflamed subcutaneous tissue space of the unlabeled rats. Sponges filled with a similar volume of unlabeled inflammatory fluid were placed in the labeled rats. Sponges were removed from each rat at 1-h intervals up to 4 h. Fluid from all sponges were assayed for [^{14}C]protein content, specific activity (cpm/mg protein) and for enzyme content. Sponge capacity is about 600 μl of 0.15 M NaCl.

Enzyme studies. DNase I, 200 U in 1 ml of 0.05 M Tris acetate buffer, pH 7, 0.05 M magnesium sulfate was added to 1 ml of inflammatory fluid supernate. Incubation was carried out at 37°C for 2 h. DNA content at zero time and after incubation was determined as described above. Pronase (2 mg/ml in Tris acetate buffer 0.02 M, 0.005 M $CaCl_2$) in a volume of 0.05 ml was added to 1 ml of supernate and incubation was carried out for 2 h at 45°C. Then pronase was inactivated by heating to 80°C for 3 min. After it cooled, DNase was added as above. DNA was determined on aliquots after treatment with DNase alone, with pronase alone, or with pronase followed by DNase.

RESULTS

The time-course of neutrophil and enzyme influx into sponges implanted in normal rats. Neutrophils began to appear in sponge inflammatory fluid after 2 h of implantation. The relatively slow influx observed during the first 5 h was accelerated during the interval between 5 and 16 h (Fig. 1A). Results have been expressed for both neutrophils and enzymes as content per sponge (i.e., sponge volume X concentration of neutrophils or enzymes). Changes in sponge contents of beta glucuronidase (Fig. 1B) and acid ribonuclease (Fig. 1C) showed a similar time course to neutrophil influx. Initially, there was a slow accumulation of these enzymes with a more rapid increase in enzyme content between 5 and 16 h. LDH activity increased linearly from zero time until 16 h and did not show an initial lag period (Fig. 1D).

No significant change in neutrophil or enzyme content occurred between 16 and 21 h. 50% or more of

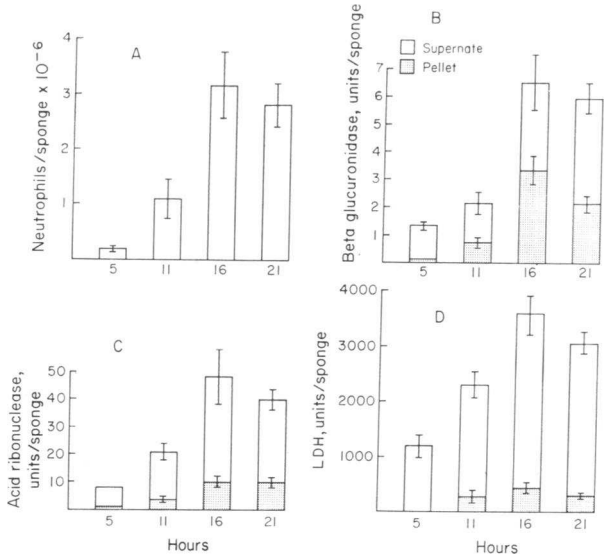

FIGURE 1 Changes in total neutrophil (A), beta glucuronidase (B), acid ribonuclease (C), and LDH (D) content of sponges in control rats over a 21-h period after sponge implantation. Vertical bars represent ±1 SEM. Hatched areas indicate the portion of enzyme activity present in intact cells.

the total sponge enzyme content at all times studied was found in the supernatant fraction.

Sponge fluid volume changed most rapidly between 0 and 5 h with a more gradual but significant rise thereafter (Fig. 2A). Protein concentrations in supernatant fluid were at maximum levels by 5 h and did not change over the next 16 h (Fig. 2B).

Fig. 3 displays the changes in peripheral blood neutrophil and total leukocyte counts in these rats as a function of time after implantation. During the first 5 h a marked fall in the total leukocyte concentration occurred. During this period the rate of fall of circulating lymphocytes was greater than that of neutrophils. After an initial fall, neutrophil levels rose to zero time values at 16 h. During the 16–21-h interval when sponge neutrophil and enzyme contents showed no change, peripheral neutrophil concentrations fell to their lowest levels. The initial fall in neutrophil levels was most likely due to influx into the inflammatory zone. In the experiments described below sponges were removed at 16 h because neutrophil and enzyme contents had reached a plateau at this time.

Supernatant enzymes and their origins. 16-h inflammatory fluid supernate was found to contain concentrations of LDH, beta glucuronidase, and acid ribonuclease that were 17, 13, and 6 times higher, respectively, than the levels of these enzymes in plasma. Supernatant fluid protein concentration was only 0.63 of that of plasma. Since all supernatant enzyme concentrations had already been corrected for plasma and red cell origins by Eqs. 1–5, the source of these high enzyme levels observed was most likely the neutrophil and the adjacent connective tissue. Sponges were capable of rapidly absorbing enzymes from the surrounding inflammatory area.

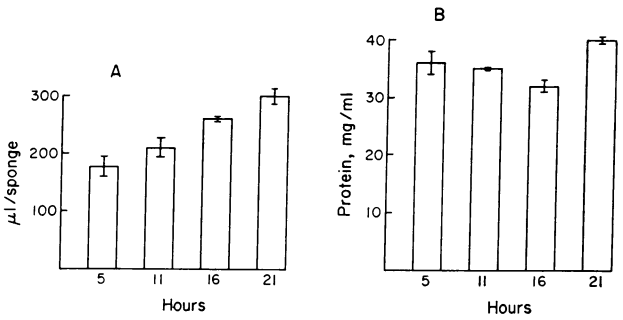

FIGURE 2 Changes in fluid volume (A) and protein concentration (B) in sponges from control rats removed over a 21-h period. Vertical bars represent ±1 SEM.

The ability of sponge implants to retain [^{14}C]proteins is demonstrated in Fig. 4A and to absorb inflammatory fluid proteins rapidly is shown in Figs. 4B and 4C. When [^{14}C]proteins were implanted in sponges in the inflamed subcutaneous connective tissue of unlabeled rats, no loss of radioactive proteins occurred during the first 3 h (Fig. 4A). The specific activity of the [^{14}C]proteins in these sponges fell rapidly because of the rapid sponge uptake of unlabeled proteins (Fig. 4B). Fig. 4C shows a rapid uptake of [^{14}C]protein and LDH when sponges were implanted in labeled rats.

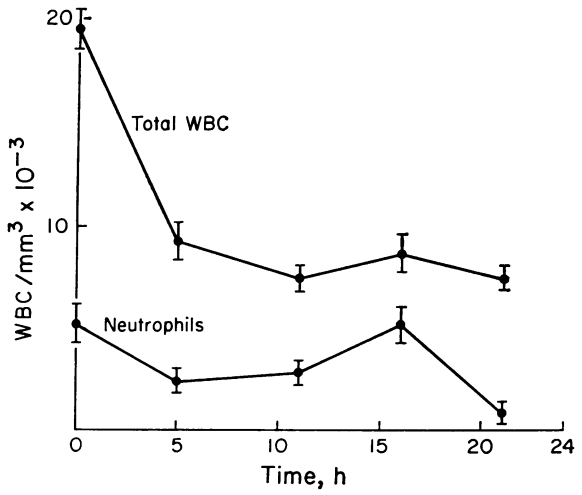

FIGURE 3 Changes in peripheral blood counts in control rats after sponge implantation during the first 21 h. The major difference between total white cells and neutrophils is made up predominantly of lymphocytes.

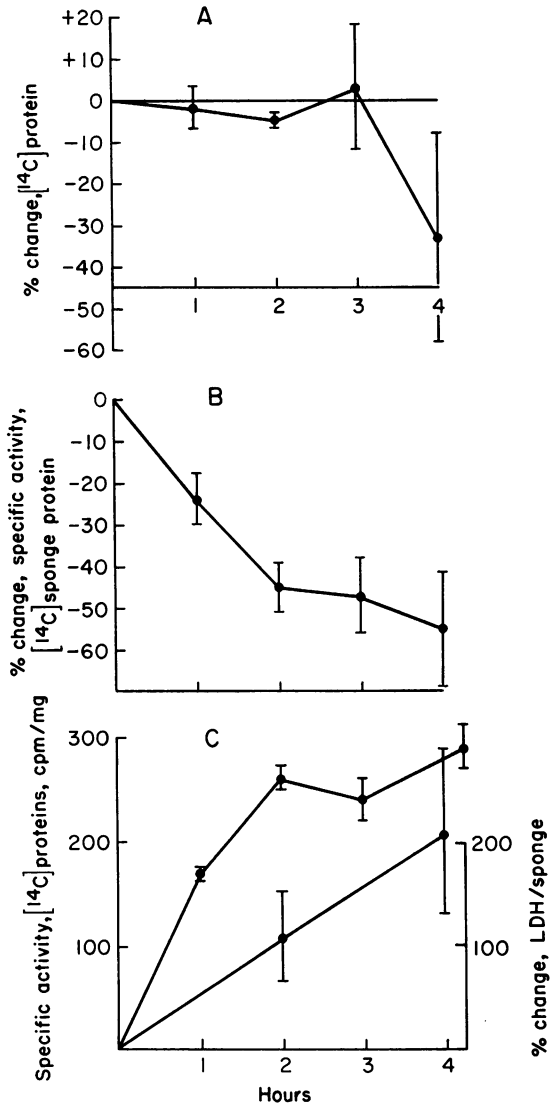

FIGURE 4 (A) The percent change in total [^{14}C]protein content of sponges implanted with labeled inflammatory fluid proteins in the inflammatory connective tissue of unlabeled rats for 4 h. The initial number of [^{14}C]protein counts implanted with each sponge varied in a range of 2,445–4,382 cpm. (B) Decrease in specific activity of the same sponges shown in A due to influx of unlabeled inflammatory fluid proteins present in the area of implantation over a 4-h period. The initial specific activity of the implanted [^{14}C]-inflammatory fluid protein was 410 cpm/mg protein. C. Rise of [^{14}C]protein specific activity in sponges containing unlabeled inflammatory fluid implanted in the inflammatory connective tissue of [^{14}C]protein-labeled rats for 4 h. LDH content (lower line) of these sponges also rises by absorption of locally present enzyme. Vertical bars indicate ±1 SEM.

ANS-treated rats were used to estimate the contribution of neutrophils to the supernatant enzyme activity present at 16 h. Table I*a* shows that ANS almost completely inhibited all neutrophil influx. Without neutrophils, the supernatant LDH fell 74%, beta glucuronidase 96%, and acid ribonuclease 72%, when compared to control animal fluids. Although enzyme concentrations showed neutrophil dependence, no effect on fluid volume or protein concentration was found in the agranulocytic rats. All residual enzyme activity in these rats must have come from the adjacent connective tissue cells, since there were no neutrophils.

The neutrophil itself or its interaction with local connective tissue cells were the possible sources of neutrophil-dependent supernatant enzyme activity in normal rats. The latter possibility was explored by implantation of sponges containing soluble lysosomal enzymes or saline into agranulocytic rats. An increase in sponge fluid LDH levels above the values found on the saline-implanted side was sought as evidence of local tissue injury. No difference in LDH levels between the lysosomal enzyme and saline sides was found (Table I*b*), indicating a lack of effect of soluble lysosomal enzymes.

Implantation of lysosomal enzymes is not equivalent to the tissue and fluid influx of neutrophils that occurs in normal rats. The failure of lysosomal enzyme implants to cause LDH release from the adjacent connective tissue of agranulocytic rats should be interpreted with caution.

An attempt was made to estimate the role of neutrophil death and lysis as a source of supernatant enzymes. The neutrophil-dependent portion of supernatant enzyme activity was estimated by subtracting the mean concentration of each enzyme in agranulocytic rats from the corresponding concentrations of each enzyme in fluid samples from control rats. From the sponge volume, the amount of neutrophil dependent enzyme per sponge could be estimated for each of the three enzymes (Table II). This amount of enzyme was then divided by the intracellular enzyme content of 10^6 neutrophils (Eq. 11).

The resulting parameter, WCEE, was a theoretical estimate of the number of neutrophils that would have had to die and release their enzymes into the supernatant fluid to account for the amount of neutrophil-dependent enzyme present.

If the supernatant LDH resulted only from neutrophil lysis, then 17.5×10^6 neutrophils/sponge would be required to provide the amount of LDH found per sponge. On the other hand only 3×10^6 neutrophils would be required to account for the amount of beta glucuronidase observed. Measurement of the quantity of soluble DNA present in supernatant fluid showed concentrations of 60-70 μg/ml or 20-25 μg/sponge. This was equivalent to the remnants of $3 - 3.5 \times 10^6$ nucleated cells/sponge and agreed with the neutrophil

TABLE I

Rats	n	Neutrophils Mean	Neutrophils SEM	Neutrophils Mean	Neutrophils SEM	Fluid Mean	Fluid SEM	Inflammatory fluid protein Mean	Inflammatory fluid protein SEM	LDH Mean	LDH SEM	LDH Change	Beta glucuronidase Mean	Beta glucuronidase SEM	Beta glucuronidase Change	Acid ribonuclease Mean	Acid ribonuclease SEM	Acid ribonuclease Change
		$10^3/mm^3$		10^6/sponge		µl/sponge		mg/ml		U/ml		%	U/ml		%	U/ml		%
a. Inflammatory Fluid Cell and Enzyme Concentrations in Agranulocytic and Control Rats																		
Normal (NS)	4	11.1	1.8	4	0.7	359	14	33	0.9	10,895	468		16.1	1.4		99	8	
Agranulocytic rats (ANS)	6	0.03	0.01	0.01	0.01	316	16	31	0.7	2,808	852	−74	0.58	0.28	−96	28	6	−72
b. Effect of Neutrophil Lysosomal Enzymes on Sponge Fluid Enzyme Levels in Agranulocytic Rats‡																		
Right lumbar implants Control, saline sponges								31.5	0.6	2,521	482		1.3	0.4		74.3	11	
Left lumbar implants Lysosomal enzyme sponges								29.4	1	2,254	470	−11	1.6	0.3	+23	78	16	+5

All data were from fluid obtained at 16 h. Abbreviations: NS, normal serum-injected rats; ANS, antineutrophil serum-injected rats.

lysis estimate based on beta glucuronidase. This data suggests that supernatant beta glucuronidase may have arisen entirely by neutrophil lysis, while less than 20% of supernatant LDH originated from this source. The remainder was probably released by injured connective tissue in the inflammatory area. Fluid DNA was present in the form of DNase-susceptible DNA (62%) and nucleoprotein (38%). No endogenous DNase activity in sponge fluid was demonstrated.

The effect of methylprednisolone on sponge neutrophil and enzyme content. When rats received doses of 10 mg/kg or more of methylprednisolone, a significant decrease of neutrophil influx into the sponges occurred. At a dose of 30 mg/kg neutrophil chemotaxis was reduced by 82% (Fig. 5A). Fluid accumulation was not affected until doses in excess of 30 mg/kg were given. The effect on fluid influx amounted to less than a 20% decrease (Fig. 5B). Sponge content of soluble beta glucuronidase (Fig. 5C) and acid ribonuclease (Fig. 5D) decreased at doses of 20 mg/kg and higher and paralleled the decrease in sponge neutrophils.

LDH levels did not fall after steroid administration (Fig. 5E), in contrast to the significant decrease seen in agranulocytic rats (Table I*a*). At 10 mg/kg a significant fall of LDH was noted but this may represent only experimental variation.

The effect of methylprednisolone on neutrophil lysosomal stabilization. The intracellular contents of lysosomal enzymes were not elevated in inflammatory fluid neutrophils isolated from methylprednisolone-treated rats (Table III). If this drug had an effect on neutrophils to decrease secretion, or "regurgitation" losses, then higher intracellular contents of lysosomal enzymes should have occurred.

No significant difference in lysosomal enzyme content occurred between control neutrophils and steroid neutrophils at a dose of 1 mg/kg. At this dosage, beta glucuronidase levels in neutrophils were decreased. This may have been due to stimulation of secretion or intracellular protein degradation. The elevated intracellular level of LDH seen at a dose of 30 mg/kg could be due to increased synthesis in precursor cells or to decreased intracellular degradation.

Treatment of supernate from steroid-injected and control rats with 0.1% Triton-X-100 released nearly equal amounts of granule-associated beta glucuronidase from each group. Control rats showed latent enzyme equal to + 12% while fluids from rats given 30 mg/kg

TABLE II

Calculated Neutrophil-Dependent Sponge Soluble Enzyme Content and WCEE‡*

	No methylprednisolone				30 mg/kg methylprednisolone			
	Neutrophil-dependent enzyme		WCEE		Neutrophil dependent enzyme		WCEE	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	U/sponge		$\times 10^{-6}$		U/sponge		$\times 10^{-6}$	
LDH	2,413	336	17.6	2.4	2,725	573	11.4	2.4
Beta glucuronidase	3	0.4	3	0.4	1.3	0.1	1.4	0.1
Acid ribonuclease	28	5	8	1.4	13	0.2	2.8	0.05

* Eq. 10, Methods section.

‡ Eq. 11, Methods section.

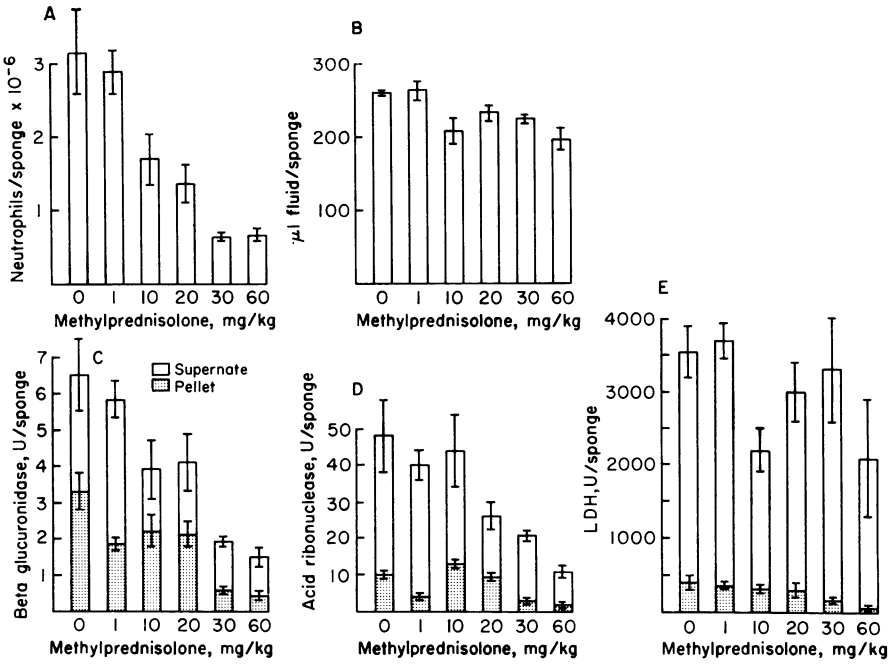

FIGURE 5 (A) Total number of neutrophils per sponge, (B) total volume of fluid per sponge, (C) total amount of beta glucuronidase per sponge, (D) total amount of acid ribonuclease per sponge, (E) total amount of LDH per sponge in 16-h implants from rats given different dosages of methylprednisolone. Vertical bars are ±1 SEM. Enzymes are designated as supernatant or cell-associated in C, D, and E.

of methylprednisolone showed a 13% increase in activity. Failure to find more free granule activity in fluids from steroid-treated rats indicated a lack of effect of methylprednisolone on the "stabilization" of extracellular lysosomal granules.

Fig. 6 provides some evidence that the fluid cell counts reflect events occurring in the adjacent connective tissue. Neutrophil counts of histologic sections of connective tissue capsules in direct contact with the surface of specific sponges was strongly correlated with the neutrophil concentrations in fluids of these sponges ($r = 0.803$). Rats receiving more than 20 mg/kg methylprednisolone all had sponge fluid neutrophil counts below $4 \times 10^3/mm^3$ and constitute all the points below this value in Fig. 6.

Peripheral blood neutrophil counts obtained at 16 h were not decreased in steroid-treated or control rats. Decrease in the circulating neutrophil pool was not the basis of the decreased in vivo chemotaxis of neutrophils into sponge implants in methylprednisolone-treated rats.

Studies for possible system artifacts. Mechanical breakage of neutrophils with enzyme release did not

TABLE III

*Effect of Methylprednisolone on Sponge Fluid Neutrophil Intracellular Enzyme Content**

Dose	LDH			Beta glucuronidase			Acid ribonuclease		
	M	SEM	P‡	M	SEM	P‡	M	SEM	P‡
mg/kg				*U/10⁶ cells*					
0 (control)	137	13		1.1	0.13		3.5	0.5	
1	125	17	NS	0.63	0.04	0.001	1.5	0.5	0.001
10	152	17	NS	1.2	0.1	NS	3.6	1	NS
20	202	67	NS	1.7	0.3	NS	3.9	1.5	NS
30	239	53	0.05	0.9	0.1	NS	4.7	0.6	NS

* Observations made on cells removed at 16 h after implantation.

‡ Probability as determined by Student *t* test.

occur after fresh inflammatory fluid was absorbed into dry sponges and then recollected by sponge compression. Single neutrophils or clumps were not adherent to the sponge matrix when histologic sections of squeezed sponges were examined. Such sections from control rats showed 7-10 neutrophils/10 oil fields and 1-2 neutrophils/10 oil field from rats treated with doses of methylprednisolone in excess of 20 mg/kg.

Correction of supernatant and cell lysate enzyme activities. It was most important to correct supernatant enzyme concentrations for contaminating plasma enzyme activity. The magnitude of the correction varied with the enzyme. For LDH the plasma correction was -3.3%, while for ribonuclease it amounted to -22%. Corrections of supernate for enzyme activity released from hemolyzed red cells were less than -1% for all enzymes. Supernate contamination of the cell lysate was most important, amounting to 47% for LDH. Red cell enzymes accounted for less than 1% of the lysosomal enzyme activity in the neutrophil lysate.

DISCUSSION

This model system has provided data that have helped in understanding the origin of inflammatory fluid enzymes and the mechanism of action of high doses of methylprednisolone.

Neutrophil influx and the appearance of soluble supernatant enzymes in sponge fluid followed a similar time course in normal rats. This was because supernatant enzyme activity resulted in large part from either neutrophil lysis or neutrophil-mediated tissue injury. Some supernatant enzyme activity still was present in agranulocytic rats, and injured tissue cells were the most likely enzyme source in these animals.

The calculation of WCEE was an attempt to estimate the magnitude of white cell lysis required to produce the neutrophil-dependent enzyme activity found in sponges. Soluble DNA present in the sponge supernatant fluid gave positive evidence of neutrophil lysis and death. Estimates of neutrophil lysis based on beta glucuronidase and DNA content of sponges were in good agreement. A large amount of LDH could not be accounted for on the basis of DNA-estimated cell lysis. It is likely that the source of this excess LDH was enzyme leakage from injured connective tissue cells. An alternative hypothesis for the different estimates of neutrophil lysis required to produce the amounts of LDH, beta glucuronidase, and DNA found in supernatant fluid would depend on different rates of local destruction or removal of these molecules. Since there was no detectable DNase activity, DNA would have had to diffuse from the sponges six times faster than LDH after cell death. This is unlikely because of the large size of DNA relative to LDH.

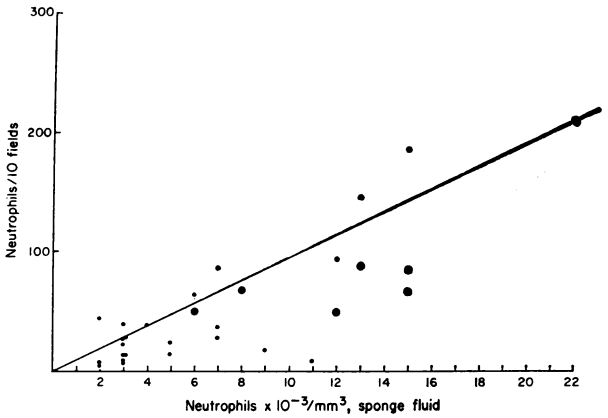

FIGURE 6 Neutrophil counts of hematoxylin and eosin-stained sections of connective tissue capsules of 16-h sponge implants were plotted against the neutrophil concentration of the fluid contained in the corresponding sponges. Double circles indicate counts from control rats. All of the points with low neutrophil concentrations (less that 4,000/mm^3) are from rats treated with methylprednisolone in doses of 20–60 mg/kg. The higher counts are from control rats or rats given 1 mg/kg.

The high LDH concentrations in supernatant fluids from steroid-treated rats relative to fluid from agranulocytic rats was of interest. In the agranulocytic rats no neutrophil-mediated tissue injury or lysis could occur. In the methylprednisolone-treated rats, neutrophil influx was decreased by over 80% at doses of 30 mg/kg or higher. However, the neutrophils that did enter the sponges and tissue were still capable of cell lysis and of producing connective tissue cell injury. The higher LDH content of neutrophils isolated from steroid-treated rats may also have contributed to the higher levels of supernatant LDH activity found in these animals.

Doses of methylprednisolone of 20 mg/kg or higher given immediately before sponge implantation had a marked inhibitory effect on neutrophil influx into sponge implants. Decreased lysosomal enzyme contents of sponge fluid paralleled the decreased influx of neutrophils. Lower levels of these enzymes were probably secondary to the decreased numbers of neutrophils available for lysis and enzyme release. The individual neutrophil can be thought of as a lysosomal enzyme-carrying unit. With fewer of such units entering the inflammatory area, decreased amounts of lysosomal enzymes are carried in. The primary effect of methylprednisolone was to decrease neutrophil influx into the inflammatory zone.

Inhibition of neutrophil chemotaxis in vivo by corticosteroids has been described in skin pouches by Ishikawa, Mory, and Tsurufuji (3), in the [^{51}Cr]neutrophil rat paw assay (1) and by use of Rebuck skin

windows in humans (4. 5). In vitro studies of neutrophil migration in Boyden-type chambers have given conflicting results. Ward reported inhibition of chemotaxis by hydrocortisone (8), while Borel reported no effect (9). Inflammatory area perfusates from rat paws injected with nystatin have shown increased levels of lysosomal enzymes that are markedly reduced by neutrophil-depleting methotrexate pretreatment or steroids (11). No simultaneous quantitative studies of in vivo chemotaxis were made in this model.

The mechanism of steroid inhibition of neutrophil chemotaxis in vivo is unknown. Some qualitative explanations, such as decreased stickiness to capillary walls, preservation of endothelial cells, and direct effects on the neutrophil, have been suggested (8, 30).

No "stabilizing" effect of methylprednisolone on intracellular or extracellular lysosomal granules was observed. Failure to find higher intracellular lysosomal enzyme levels in steroid-treated rats was evidence against an in vivo effect of steroids on neutrophil granule secretion or granule losses during phagocytosis.

Lack of increased amounts of granule-associated beta glucuronidase in supernatant fluids from steroid-treated rats was evidence against a stabilizing effect of methylprednisolone on extracellular lysosomal granules. In agreement with our in vivo studies, Persillin and Ku have recently shown failure of steroids to stabilize human lysosomes in vitro (13). The in vitro effects of steroids on the stabilization of liver lysosomes have been questioned (31), and probably will vary with steroid concentration, species, method of preparation, and method of injuring the membranes (32). Goldstein has shown less release of lysosomal enzymes from cytochalasin B-treated neutrophils after steroid treatment in vitro (15). In this system lysis did not occur.

This in vivo model system has permitted multiple simultaneous measurements of neutrophil chemotaxis, supernatant and cellular enzymes, and other parameters. In this system the anti-inflammatory action of methylprednisolone was mediated by a marked inhibition of chemotaxis. Decreased supernatant levels of lysosomal enzymes were secondary to the fewer neutrophils entering the inflammatory area. No effect on lysosomal stabilization was observed after steroid treatment.

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