Effect of 6-Mercaptopurine on Enzymes of the Polymorphonuclear Leuocyte* (33449)

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Page and Good (1) showed in 1958 that migration of mononuclear cells into an area of inflammation was dependent upon the prior presence of neutrophils at the site and was delayed in the neutropenic animal. Page, Condie, and Good (2) subsequently demonstrated that administration of 6-mercaptopurine (6-MP) virtually eliminated the appearance of mononuclear cells at the local inflammatory site. This effect of 6-MP developed only after a period of treatment with the drug, and the duration of this period was inversely related to the dose. These observations raised the possibility that the appearance of mononuclear cells at an inflammatory site results from the local release of an active agent from polymorphonuclear cells, and that this agent is not released from these cells in the animal that has received 6-MP for a sufficient period of time. The possibility was therefore considered that a lysosomal enzyme might be the mediator released by the polymorphonuclear cell that initiated the mononuclear cell response and that the synthesis of this enzyme in polymorphonuclear cells might be inhibited in 6-MP–treated animals. Three lysosomal enzymes of the polymorphonuclear leukocytes of rabbits, i.e., acid phosphatase, β-glucuronidase, and ribonuclease, were, therefore, assayed in 6-MP-treated and control animals to determine whether there was a reduction in the lysosomal enzyme content of the cells of the treated animals.

Materials and Methods. Rabbits. Albino rabbits of both sexes, weighing 2.0–3.0 kg, were obtained from a single local breeder in groups of eight from the same litter and randomly separated into two equal groups. One group was treated with 6-MP and the other served as a control group. Both groups were pair-fed a standard Purina pellet diet in order to keep weight gain or loss equal in the two groups.

6-Mercaptopurine. 6-MP was obtained in the form of the sodium salt in 0.5-g sterile vials (kindly supplied by Dr. Donald S. Searle of Burroughs Wellcome and Co., Tuckahoe, New York) and water added to a concentration of 37.5 mg/ml. A fresh vial was used daily, and the dissolved drug injected immediately after addition of water. Injections were given intravenously into the marginal ear vein daily for 8 days in a dosage of 18 mg/kg. Injected solutions had a pH of 10.5 and were only mildly irritating to the vein. This dosage has been shown by Page *et al.* (2) to produce a good anti-inflammatory effect. Control animals received equivalent volumes of phosphate-buffered saline, pH 10.5

Inflammatory response. The studies of Page and co-workers (2) were repeated using 4 6-MP–treated animals and 4 controls in each group. A solution of egg white and India ink (0.1 ml) was injected subcutaneously into several sites along the rabbit's shaved back. Biopsy specimens were obtained at 4–6 hr after injection when Page and co-workers observed the first significant effect of 6-MP on inflammation. The loose subcutaneous connective tissue was spread on clear glass slides, the tissue fixed by air-drying, and then stained with Wright-Giemsa stain as described by Page and co-workers (2) and by Kolouch (3).

Peritoneal exudates. Peritoneal exudates were prepared according to the method of Cohn and Hirsch (4). After 8 days of treatment with 6-MP, 6-MP–treated and control rabbits were injected intraperitoneally with 200 ml of 0.1% glycogen in sterile saline.

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Four hours later, 100 ml of heparinized saline were injected into the peritoneal cavity and the exudate withdrawn immediately after gentle kneading of the abdomen. For this purpose, a no. 13 short-bevel needle with multiple holes in the tip was used. This was attached to a McGaw intravenous administration set and introduced in the linea alba below the umbilicus. The fluid was then collected by gravity drainage. Total fluid yield was 150–250 ml. Exudates contaminated with large numbers of erythrocytes were discarded. Smears and wet mounts revealed 95% or more of polymorphonuclear leukocytes. The peritoneal fluid obtained was centrifuged at 1000 rpm for 10 min. A pellet of white cells was usually obtained. If considerable erythrocyte contamination was present, the pellet was discarded. The cells were then washed twice with 0.85% saline, counted, and resuspended in the appropriate volume of saline so that all suspensions contained the same number of cells per unit volume. Smears of these centrifuged suspensions revealed 5–10% contamination with erythrocytes. The cell suspensions were then rapidly frozen and thawed 5 times in a dry ice-alcohol solution, and then centrifuged at 27,000*g* for 10 min. The resulting clear supernatant fractions were then assayed for enzyme activity. The only variation from this method occurred in the assay of β-glucuronidase. In this assay, the cells were suspended in 1% gelatin in saline for counting and then washed and suspended in saline as previously described.

Enzyme analysis. Acid phosphatase was determined using as substrate, *p*-nitrophenyl phosphate (5–7), obtained from Sigma Chemical Company, St. Louis, Missouri. The yellow color of *p*-nitrophenol, which is liberated from this substrate, was read in alkaline solution (8) at 410 mμ. Results were expressed as micrograms of phosphorus per 1 $\times 10^6$ cells.

Beta glucuronidase activity was assayed by a modification of the method of Fishman, Springer, and Brunetti (9), using 0.01 *M* phenolphthalein glucuronide as substrate. The enzyme extract and substrate were incubated in 0.1 *M* acetate buffer, pH 4.5, at 38° for 3 hr, and the reaction stopped using 10% trichloracetic acid. The solutions were centrifuged at 27,000*g* for 10 min. The supernatant fraction was placed in a graduated conical centrifuge tube, 2.5 ml of an alkaline reagent mixture added, and then distilled H_2O up to a volume of 6 ml. This final solution was read at 550 mμ.

Ribonuclease was determined by the following modification of the method of Kalnitsky and co-workers (10). The RNA substrate, prepared from baker's yeast, was obtained from Worthington Biochemical Corporation. A 1% RNA solution in 0.1 *M* acetate buffer (0.2 ml), pH 5.0, was incubated 4 min at 37° with 0.2 ml cell extract and was precipitated with 0.2 ml uranyl acetate (0.75% in 25% perchloric acid). The resulting cloudy solution was cooled in an ice bath and centrifuged at 3°. A portion of the clear supernatant fraction (0.2 ml) was promptly diluted to 0.6 ml with water and read at 260 mμ.

Results. In rabbits treated with 6-MP in a dosage of 18 mg/kg for 8 days, white blood cell and differential counts of the peritoneal exudates revealed slightly decreased total numbers of leukocytes in the peritoneal exudates of the treated animals. Differential counts in 6-MP–treated animals and controls were comparable. A peripheral leukopenia was noted in most of the 6-MP–treated animals with moderate decreases in total neutrophils and lymphocytes. Examination of the polymorphonuclear leukocytes in the exudates by phase-contrast microscopy revealed no obvious differences between 6-MP–treated and control animals in the appearance of the lysosomal granules.

When egg white and India ink were injected subcutaneously on day 9, and biopsy specimens taken 4–6 hr later, the results of Page and co-workers (2) were confirmed, i.e., a definite decrease in the mononuclear cell concentration in the areas of induced inflammation was noted in the sections of the 6-MP–treated animals as compared with control animals. The average percentage of mononuclear cells in the tissue lesion in the control animals was 32.1% (3.2–51.0) while in the treated animals the average was 9.6% (0.8–45.8). The numbers of polymorphonu-

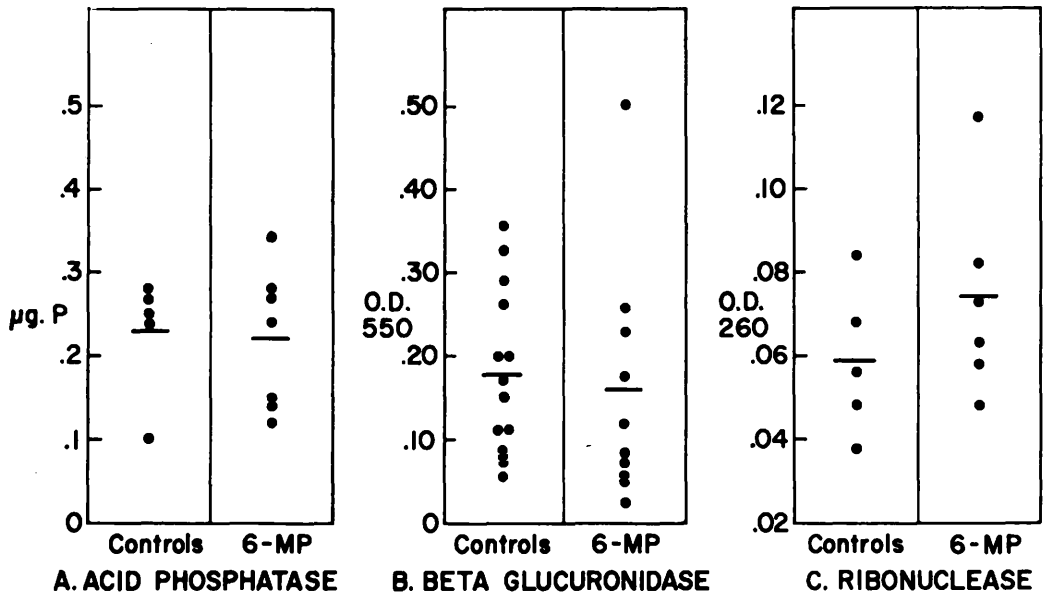

FIG. 1. (A) Acid phosphatase activity of extracts of peritoneal leukocytes from control and 6-MP–treated animals expressed as micrograms P liberated per 1 × 10^6 cells. (B) Beta glucuronidase activity of extracts of peritoneal leukocytes from control and 6-MP–treated animals expressed as OD 550. Each sample contained an extract of 8.75 × 10^6 peritoneal cells. (C) Ribonuclease activity of extracts of peritoneal leukocytes from control and 6-MP–treated animals expressed as OD 260. Each sample contained an extract of 4 × 10^6 peritoneal leukocytes.

clear leukocytes that appeared initially were essentially the same in both treated and control animals.

Results of the assays of the three lysosomal enzymes, acid phosphatase, β-glucuronidase, and ribonuclease in the extracts of the peritoneal leukocytes of the 6-MP–treated and control animals are shown in Fig. 1. It is evident from these data that there was essentially no difference in the enzyme levels per cell of the 6-MP–treated animals as compared with the control animals in the case of the three enzymes examined. Thus, although the same degree of delay and reduction in the mononuclear cell response in an area of inflammation was induced in the 6-MP–treated animals as reported by Page and co-workers (2), it was not possible to demonstrate a decrease in the lysosomal enzyme levels of the polymorphonuclear leukocytes of the animals in spite of the fact that they had received 6-MP in the relatively high dosage of 18 mg/kg for an 8-day period.

Discussion. A number of studies have attempted to localize the site of action of 6-MP in the biosynthetic pathways of the purines and pyrimidines. It has been well demonstrated that this purine analog inhibits a variety of metabolic processes that normally utilize adenine and hypoxanthine (11–15). In some contrast to these observations of interference with metabolic reactions involved in the synthesis of nucleic acids, the studies by Page and co-workers (2) have led to 3 different hypotheses to explain the decrease in mononuclear cell infiltration observed in 6-MP–treated animals: (1) that neutrophils are affected by 6-MP so that substances chemotatic for lymphocytes are not produced at the inflammatory site; (2) that lymphocytes and monocytes are injured by 6-MP and rendered unresponsive to chemotactic substances; and (3) that circulating mononuclear cells that are active in acute inflammation are eliminated. The present data would be most compatible with one of the latter two hypotheses.

Available evidence indicates that the mononuclear cells that are active in acute

inflammation are short-lived cells that constitute a minority of the mononuclear cells of the blood, and which are derived from rapidly proliferating precursors (16–20). Recently, it has been observed in this laboratory (21) that rabbits receiving 6-MP in a dosage sufficient to reduce the mononuclear infiltration of egg albumin–induced skin lesions do, in fact, concomitantly reduce the concentration of large mononuclear cells in the blood. Accordingly, the absence of an effect on lysosomal enzyme synthesis noted in the present report would be in accord with this observation.

Summary. Mononuclear cell infiltration of an egg albumin–induced skin lesion is preceded by infiltration with polymorphonuclear cells. In rabbits in which mononuclear cell infiltration of this lesion was suppressed by 6-MP, the possible effects of immunosuppression on the lysosomal enzyme content of the polymorphonuclear cells was investigated. The acid phosphatase, beta glucuronidase, and ribonuclease content of extracts of the polymorphonuclear cells of these animals were determined. In spite of adequate suppression of mononuclear infiltration of the induced skin lesion, no difference in the concentration of the three enzymes in the polymorphonuclear cells was noted. The results indicate that the failure of mononuclear cell infiltration in 6-MP–treated animals is not due to an effect on lysosomal enzyme synthesis in cells of the neutrophil series.

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