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SHORT COMMUNICATION

DIFFERENTIAL EFFECTS OF 6-MERCAPTOPURINE AND CYCLOPHOSPHAMIDE ON AUTOIMMUNE PHENOMENA IN NZB MICE

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SUMMARY

The effects of 6-mercaptopurine (7·5 mg/kg) and cyclophosphamide (15 mg/kg and 30 mg/kg) on various aspects of the immune response have been compared in New Zealand Black (NZB) mice. Injections were given daily for 5–8 weeks. Serologic and morphologic studies during and after treatment revealed the following: (1) 6-MP decreased the levels of circulating polymorphonuclear leucocytes, monocytes, and large lymphocytes; small and medium lymphocyte counts remained unchanged. In contrast, cyclophosphamide decreased mainly the small and medium lymphocytes, leaving the other cell types unchanged. (2) In older animals, 6-MP produced a marked fall in haematocrit, while cyclophosphamide did not do so. (3) Cyclophosphamide delayed the onset of Coombs positivity and decreased the Coombs antibody titre. It also decreased immunofluorescent staining of γ-globulin deposits in the kidney. 6-MP had no such effects.

The results suggest that 6-MP exerted an anti-proliferative action on the bone marrow, as shown by a decrease of short-lived cells in the blood. Cyclophosphamide, on the other hand, appeared to have mainly a cytotoxic effect on circulating cells, resulting in depletion of the long-lived small lymphocytes of the blood and leading to inhibition of autoantibody formation and immune complex deposition in the kidney. Cyclophosphamide would, therefore, appear to be the preferable drug where suppression of ongoing cellular and humoral immune reactions is desirable.

INTRODUCTION

Immunosuppression appears to be a logical therapeutic approach to diseases which are initiated or sustained by immunological reactions. However, the clinical use of immuno-

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suppressive agents is still in an experimental state because of insufficient knowledge of their mechanism of action. In this study, experiments were undertaken to clarify the mode of action of two commonly used immunosuppressive agents: 6-mercaptopurine (6-MP) and cyclophosphamide.

The differential effects of these drugs were determined in the New Zealand Black (NZB) mouse on the Coombs antibody titre, cell counts of the individual circulating leucocytes, and the pattern of fluorescent antibody staining of the immunoglobulin deposits in the renal glomeruli. NZB mice develop a well-described entity of spontaneously occurring autoimmune phenomena (Bielschowsky, Helyer & Howie, 1959; Helyer & Howie, 1963; Holmes & Burnet, 1963; Mellors, 1966) with immunohaemolytic anaemia, renal disease, and frequent development of lymphomas. The cause of this disease is not known. Functional thymus deficiency (Burnet & Holmes, 1962; de Vries & Hijmans, 1966) and virus infection (Mellors & Huang, 1966) have been claimed to play an important aetiological role.

MATERIALS AND METHODS

NZB mice were obtained from the University of Otago Medical School, Dunedin, New Zealand. They were bred by strict litter mating through two to three generations until used. Daily intraperitoneal injections of the sodium salt of 6-MP (Burroughs Wellcome & Co., Inc., Tuckahoe, New York) or cyclophosphamide (Cytoxan, Mead Johnson & Co., Evansville, Indiana) were given over a 6–8-week period. Both drugs were dissolved (6-MP in distilled water, cyclophosphamide in normal saline) not longer than 15 min prior to injection. 6-MP was given to all experimental groups in an initial daily dosage of 7·5 mg/kg which was the highest dose tolerated in long-term treatment. Cyclophosphamide was used in two dosage schedules, 15 and 30 mg/kg daily. The higher dosage proved to be toxic after 3–4 weeks, as shown by weight loss, diarrhoea and a high mortality rate. Groups of eight to twelve mice, composed of equal numbers of both sexes, and falling in a series of specified age groups between 3 and 12 months, were bled at weekly intervals from the retroorbital plexus. White blood cell counts and differential counts of polymorphonuclear cells, large mononuclear cells, and medium and small mononuclear cells were performed. For the determination of the titre of anti-erythrocyte antibodies (Coombs titre), mouse erythrocytes were carefully washed and resuspended in 2% suspension in phosphate-buffered saline. Commercial rabbit anti-mouse γ-globulin (Hyland, Los Angeles, California) was absorbed three times with equal volumes of washed packed erythrocytes from 8–10-week-old C57Bl/6 mice and diluted 1:25 with buffered phosphate saline. From this stock solution, serial dilutions (up to 1:2560) of antiserum were prepared. Equal amounts of the 2% erythrocyte suspension and the diluted antisera were incubated in microtitre plates (Cooke Engineering Co., Alexandria, Virginia) at room temperature for 1 hr and at 4°C overnight. Agglutination, easily determined by the trailing pattern of the erythrocytes in the wells, was read after setting the plates at a 60° angle for a few minutes. Coombs titres were expressed as the maximal dilution of antiserum capable of producing erythrocyte agglutination.

The kidneys of treated animals and of matched controls were evaluated for glomerular depositions of γ-globulin. Sections, approximately 4 μ thick, were incubated with fluoresceinated rabbit anti-mouse γ-globulin. Histologic evaluation was carried out in a blind fashion. The observer did not know the treatment group from which the renal tissue of a particular animal was obtained and it was evaluated by using a Leitz-Orthomat microscope

fitted with appropriate dark-field condenser and filters for fluorescence. The staining pattern was classified either as negative to minimal or as moderate to severe.

RESULTS

Fig. 1 shows the effect of 6-MP on the circulating white blood cells and on the Coombs titre in a group of 3-month-old NZB mice. The initial dosage of 6-MP was 7·5 mg/kg/day, although it was necessary to decrease the dosage during the treatment period because of a fall in haematocrit. It is seen that with 6-MP treatment the polymorphonuclear cells and the

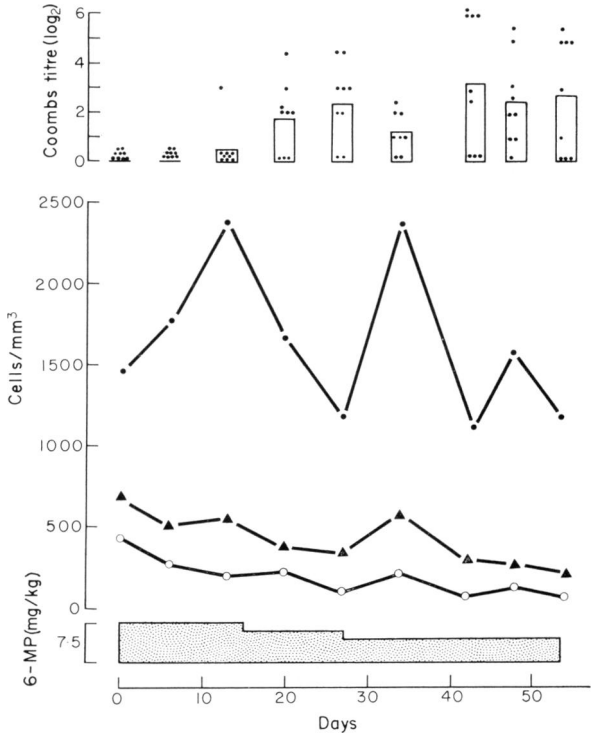

FIG. 1. Effect of 6-MP on Coombs titre and circulating white blood cells in 3-month-old NZB mice. Cells are differentiated into polymorphonuclear cells, ▲; large mononuclear cells, ○; small and medium mononuclear cells, ●. Mean values of counts of nine animals treated are given. Coombs titres are expressed as the maximal dilution of a standard anti-mouse γ-globulin serum capable of producing erythrocyte agglutination; the bars represent the geometric mean titres of the groups.

large mononuclear cells fall off gradually while the counts of small and medium mononuclear cells are not appreciably depressed. In addition, the development of Coombs positivity in this age group was not prevented. The titres given are similar to titres in untreated control animals of the same age. In older age groups (6, 8, 10, and 12 months old), no decrease of existing Coombs titres was observed on 6-MP administration.

The effect of cyclophosphamide (15 mg/kg) on circulating white blood cells and Coombs

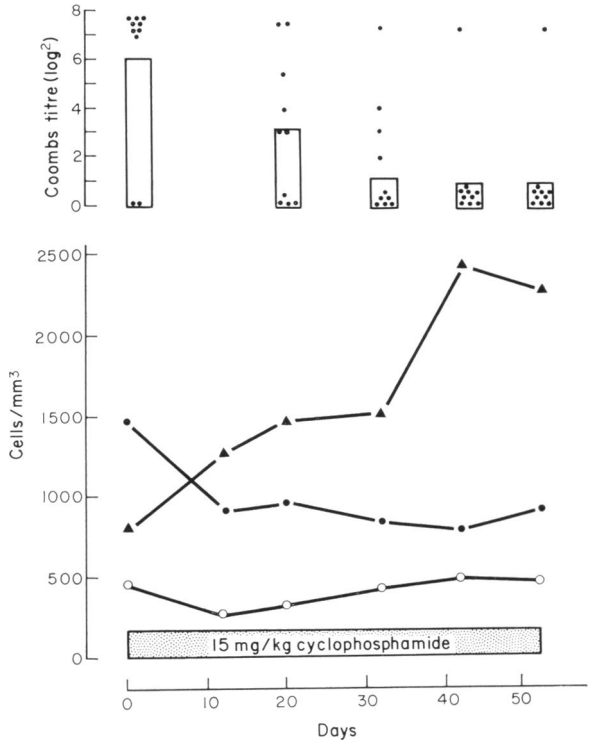

FIG. 2. Effect of cyclophosphamide on Coombs titre and circulating white blood cells in 6-month-old NZB mice. Differentiation of cells and Coombs titres same as in Fig. 1. Data represent the mean values of ten animals treated.

titre in 6-month-old mice is seen in Fig. 2. On this treatment, in contrast to 6-MP, the polymorphonuclear cells and large mononuclear cells remain unchanged or even increase in number, while the small and medium mononuclear cells decrease. In addition, in this and in the other age groups (3, 8, 10, and 12 months old) tested, administration of cyclophosphamide led to a fall in Coombs titre. In Coombs negative animals, the development of erythrocyte antibodies was prevented. There appeared to be a correlation between the fall in medium and small lymphocyte counts and the fall in the Coombs titres.

Fig. 3 summarizes the differential effect of cyclophosphamide and 6-MP on the circulating white blood cells in three groups of 8-month-old mice; eight to twelve animals were present in each group. The bars represent the average percentage change from initial values of the cell counts of the individual leucocytes at the end of 6-8 weeks of treatment. 6-MP produced a predominant decrease of polymorphonuclear and large mononuclear cells. In contrast, cyclophosphamide, at the high dosage level (30 mg/kg), effected a marked decrease of small and medium mononuclear cells, while the numbers of polymorphonuclear and large mononuclear cells remained essentially unchanged. On the lower dosage of this agent (15 mg/kg), there was in fact a rise in polymorphonuclear cell count when the total of small and medium lymphocytes fell. These data suggest that cyclophosphamide acts predominantly on the small and medium mononuclear cell populations.

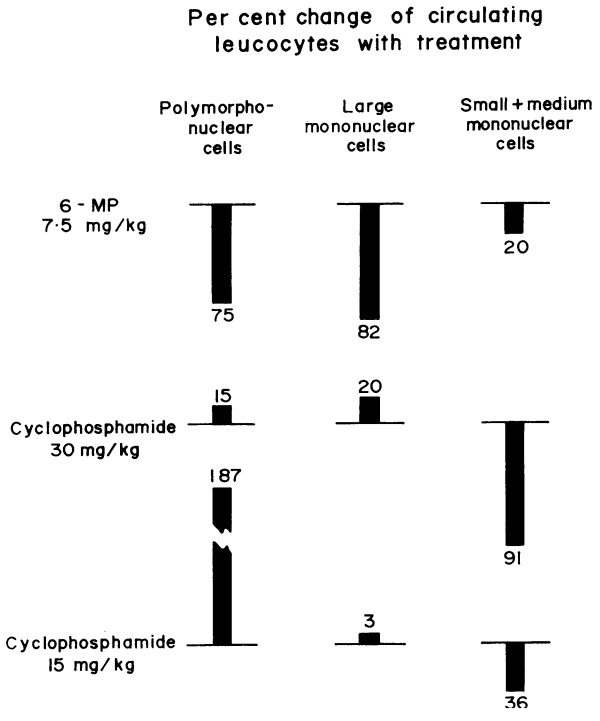

FIG. 3. Differential effect of 6-MP and cyclophosphamide on the circulating white blood cell populations of 8-month-old NZB mice. The bars represent the average per cent change (eight to twelve animals per group) of cell counts from initial values at the end of 6–8 weeks of therapy.

In view of the differing effects of 6-MP and cyclophosphamide on the circulating leucocytes, a kinetic study was carried out in 8-month-old mice to measure the rates of formation of the three cell populations at the end of 6 weeks of treatment. The 6-MP-treated animals and the group receiving cyclophosphamide at the 15 mg/kg dosage level were injected intraperitoneally with [^{3}H]thymidine (specific activity 6·7 Ci/mM, New England Nuclear Corp., Boston, Massachusetts) daily (1 μCi/g body weight) during the last 7 days of treatment. An untreated control group was also injected with [^{3}H]thymidine. The percentage of labelled cells in each cell population was determined autoradiographically by the following method: blood smears were dipped in Kodak NTB-3 nuclear track emulsion, stored 1 week in the cold, and developed. The slides were then stained with Leishman's stain and differential cell counts made. A minimum of 5 grains per nucleus was required to record a cell as labelled. As shown in Table 1, the percentage of labelled cells in the cyclophosphamide treated group was very similar to that of the controls, indicating a normal and uninhibited rate of formation of all cell types in these animals. In the 6-MP treated group, there were too few polymorphonuclear cells and large mononuclear cells to count as a result of the severe depletion of these populations. The small and medium mononuclear cells, however, showed a significant decrease in labelling, indicating that the rate of formation of this population was also depressed by 6-MP.

TABLE 1. Labelling of leucocytes of 8-month-old NZB mice after treatment with cyclophosphamide and 6-MP

Leucocyte type	Per cent of cells labelled		
	Untreated	Cyclophos-phamide*	6-MP
Polymorphonuclear	70 (66–82)	66 (55–82)	—†
Large mononuclear	61·5 (53–70)	86·7 (73–100)	—†
Small & medium mononuclear	14·5 (13–19)	17·5 (17–18)	6·6 (5–10·3)

* 15 mg/kg.
† Too few cells to count.

Examination of renal glomeruli for γ-globulin deposits by the immunofluorescent technique revealed that the majority of the kidneys of the untreated mice (fourteen out of twenty) and all of the 6-MP treated mice showed moderate to severe staining, predominantly in the mesangial region, while only a small fraction (four out of fourteen) of the cyclophosphamide treated animals showed this degree of staining. The majority of the cyclophosphamide treated animals (ten out of fourteen) had negative to minimal staining. These results indicate that, in contrast to 6-MP, treatment with cyclophosphamide prevented the deposition of γ-globulin in the renal glomeruli.

DISCUSSION

These findings are, in part, a confirmation of earlier reports. Casey (1968a) has shown that 6-MP, given in a dosage of approximately 10 mg/kg/day, did not delay or modify the development of anti-erythrocyte autoantibodies in NZB mice. The same author (Casey, 1968b), however, studying the effect of cyclophosphamide on the auto-immune disease of NZB/NZW F_1 hybrids, was not able to demonstrate a beneficial effect of cyclophosphamide on Coombs titre when this drug was given in a single injection of 2 mg per mouse once a week. No reports on the effect of cyclophosphamide on Coombs titres in NZB mice are available. A beneficial action of cyclophosphamide on the renal disease of NZB/NZW mice has been demonstrated (Casey, 1968b; Russell, Hicks & Burnett, 1966; Horowitz *et al.*, 1969), while azathioprine, an analogue of 6-MP, had no demonstrable effect on the renal changes of NZB mice (Casey, 1968c).

From the data presented here, it would appear that the difference in action of 6-MP and cyclophosphamide may be a reflection of their differing effects on the individual population of circulating leucocytes. In keeping with its known anti-proliferative effect, 6-MP appeared to affect predominantly the formation and consequently the circulating levels of the short-lived, i.e. the polymorphonuclear and large mononuclear cell populations. In association with this effect, an ongoing immune reaction such as erythrocyte autoantibody formation was

not inhibited. Cyclophosphamide, on the other hand, appeared to act on the circulating leucocytes, thus decreasing predominantly those cell types with a slow rate of formation, i.e. the small and medium mononuclear cells. These cells are known to be associated with the immune response, both of the delayed hypersensitivity (Gowans *et al.*, 1962) as well as the humoral antibody (Gowans & Uhr, 1966) type. A decrease of these cell populations would result in suppression of both of these types of immune response.

It is not surprising that the circulating levels of small lymphocytes did not fall in the 6-MP treated animals in spite of the marked decrease in rate of formation of these cells noted in these animals (Table 1). It would be expected that the level of this cell type would not fall after only 7 days of treatment since the majority of these small lymphocytes are long-lived cells with a slow turnover time and a potential life span of many months (Everett, Caffrey & Rieke, 1964). It is also not surprising that cyclophosphamide, a cytotoxic agent, effectively decreased the small lymphocyte count without affecting the rate of formation of these cells, since the small lymphocyte, in contrast to the larger mononuclear cells, is very slowly replaced (Everett, Caffrey & Rieke, 1962).

From the data presented, it would appear that the clinical use of 6-MP would be indicated under circumstances where suppression of infiltration with large mononuclear cells would be desirable. 6-MP would appear to be less effective where an ongoing immune response is well established. Cyclophosphamide, on the other hand, because of its predominant effect on small and medium lymphocytes would appear to be the preferable drug where suppression of established cellular and humoral immune reactions is desirable.

It should be pointed out that the present observations pertain to mice receiving 6-MP and cyclophosphamide in relatively high dosage. The possibility exists that undetected infection of the treated mice might have stimulated a polymorphonuclear and large mononuclear response, tending at least in the cyclophosphamide treated animals, to maintain these cell types at higher levels than would otherwise occur. The extrapolation of these results to man may not be warranted.

ACKNOWLEDGMENTS

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ANNOUNCEMENT

Immunological Research and Diagnosis Course

The Center for Immunology of the State University of New York at Buffalo will again present a three-week course on current methods of immunological research and diagnosis from July 12 to July 30, 1971. It will consist of practical laboratory exercises supplemented by demonstrations, lectures and discussions, designed to provide the participant with a survey of presently available methodology and insight into the underlying immunological principles. The topics will include: antigen preparation methods, gel diffusion precipitation, active and passive agglutination, mixed agglutination, immunofluorescence, complement determination, haemolysis in gel, blood group determination and compatibility testing, immediate hypersensitivity, delayed hypersensitivity, and tissue typing. Attendance will be limited to twenty participants in order to give maximum opportunity for individual instruction. Limited fellowship support can be provided to applicants from abroad.

The course will be held on the campus of the State University of New York at Buffalo. Tuition has been set at $300. Suitable living accomodations and opportunities for recreation are available. The faculty will consist of staff members of the State University at Buffalo, Roswell Park Memorial Institute, and affiliated hospitals. The course is supported in part by a grant from the World Health Organizatian.

Potential applicants should contact:

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