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Immunosuppression in the treatment of inflammatory bowel disease

II. THE EFFECTS OF AZATHIOPRINE ON LYMPHOID CELL POPULATIONS IN A DOUBLE BLIND TRIAL IN ULCERATIVE COLITIS

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SUMMARY

Blood lymphocytes and rectal plasma cells have been studied in patients with ulcerative colitis taking part in a double-blind trial of treatment with azathioprine.

Treatment for 1 year resulted in a modest fall in blood lymphocyte count, with little change in neutrophils or platelets. There was no major change in the proportions of circulating T and B lymphocytes, suggesting that the number of such cells per millilitre of blood fell in proportion to the change in lymphocyte count. The number of plasma cells in the rectal lamina propria was reduced to a mean less than half that of the control patient group. Blood K-cell cytotoxic activity fell at least 25-fold after 1 year's treatment. PHA-induced cytotoxicity was also reduced, but less consistently. Reduced K-cell activity is interpreted as reflecting depletion of effector cells from the circulation. The fall in lymphocyte count, K-cell activity and gut plasma cells was slow, indicating continuous inhibition of lymphopoiesis or differentiation throughout the trial period. Thus, azathioprine has some immunosuppressive effects which develop only after prolonged treatment. The clinical results of the trial did not show a major beneficial effect of azathioprine in the treatment of ulcerative colitis, nor were there clear correlations between the results of lymphocyte assays and clinical response in individual patients.

INTRODUCTION

Since the demonstration by Schwartz and Dameshek that 6-mercaptopurine in appropriate dosage and timing can suppress primary antibody responses and induce specific tolerance (Schwartz & Dameshek, 1959), the thiopurines have been widely used as immunosuppressants. Despite the fact that antiproliferative agents are much more effective in preventing primary immune responses than in suppressing secondary or ongoing responses to antigens experienced before the onset of treatment (Levin, Landy & Frei, 1964; Swanson & Schwartz, 1967), such agents have been applied in the treatment of many diseases whose pathogenesis may be mediated by immunological mechanisms (Parker & Vavra, 1969). In a few cases, such as autoimmune haemolytic anaemia, idiopathic thrombocytopenia (Bouroncle & Doan, 1969; Dacie & Worlledge, 1969) and severe rheumatoid arthritis (Currey, 1974), thiopurines appear to have an established, if limited, usefulness in therapy. There are reports suggesting that they may be useful in the treatment of ulcerative colitis (Mackay, Wall & Goldstein, 1966; Korelitz, Glass & Wisch, 1973). A large-scale controlled trial has been reported by Jewell & Truelove (1974), and the studies described here were made in association with that trial.

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In order to quantify the effects of azathioprine on some components of the immune system we have studied several lymphoid subpopulations and functions. Blood lymphocytes were characterized as T cells by rosette formation with sheep erythrocytes (Jondal, Holm & Wigzell, 1972), and B cells by staining for surface immunoglobulin (Fröland, Natvig & Berdal, 1971). Functional assays included mitotic response to phytohaemagglutinin (PHA), which appears to be predominantly a T-cell characteristic (Greaves, Janossy & Doenhoff, 1974). Lymphocyte cytotoxic activity against homologous target cells was assayed in both antibody-dependent (MacLennan, 1972; Perlmann, Perlmann & Wigzell, 1972) and PHA-induced (Perlmann & Holm, 1969) systems. Plasma cells were quantified in rectal biopsies.

In a previous report we have described some changes in the circulating lymphocytes and rectal plasma cells of a small group of patients after prolonged treatment with azathioprine (Campbell *et al.*, 1974a). The most striking effects were depression of antibody-dependent lymphocyte-mediated cytotoxicity (K-cell activity) among peripheral blood cells, and depletion of plasma cells from the rectal lamina propria. Following cessation of azathioprine treatment, these cells recovered to near-normal levels within 8–12 weeks. This paper describes a fuller study of lymphoid cells in patients with ulcerative colitis who took part in a trial of azathioprine treatment.

PATIENTS AND TRIAL DESIGN

The patients studied were participants in a controlled therapeutic trial of azathioprine (Jewell & Truelove, 1974). All had a diagnosis of ulcerative colitis, and were in a frank attack at the time of entry to the trial. On entry, they were randomly allocated to receive either azathioprine or dummy tablets of identical appearance.

The initial attack was treated with corticosteroids. Patients who were managed as outpatients received oral prednisolone (20 mg/day). The more severe cases, treated as inpatients, began with a 5-day intravenous regime including prednisolone (40 mg/day). If the clinical response was good, this was followed by resumption of oral prednisolone. Steroid treatment continued for 1 month, and was tailed off over the next 2 weeks.

Treatment with the 'special tablets' (azathioprine or dummy) was begun on admission to the trial. The dose of azathioprine was approximately 2·5 mg/kg body weight/day, and was maintained throughout the 1-year trial period.

During the trial patients were seen each month for clinical, sigmoidoscopic and laboratory assessment. Relapses were treated with steroids as described above. Any patient who suffered three relapses was taken out of the trial. Apart from the treatment of such attacks, no maintenance therapy other than the 'special tablets' was given. A few patients who failed to go into satisfactory remission during initial steroid treatment were withdrawn as 'failures'.

MATERIALS AND METHODS

Blood-lymphocyte tests. Lymphocyte studies were made on a total of thirty-four patients, but some of these were withdrawn as early 'failures', or on suffering their third relapse. Serial studies were carried out on twenty-six patients who remained in the trial for longer than 9 months. Of these, eleven were receiving azathioprine, and fifteen were on the dummy tablets. Since this was a double-blind trial, none of the investigators was aware of which patients were on the drug until the trial was completed.

Blood counts. White cell, differential and platelet counts were performed in the routine haematology laboratory.

Lymphocyte preparation. The lymphocyte harvesting and assay procedures have been described previously (Campbell *et al.*, 1974a, b), and are briefly outlined here. Leucocytes were obtained from defibrinated blood samples (15–25 ml) by gelatine sedimentation. The washed cells were resuspended in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), glutamine, non-essential amino acids and antibiotics. The mononuclear cells were counted, and this preparation was used for assays of mitotic response to PHA and cytotoxic activity. This separation procedure yields 50–80% of the mononuclear cells present in whole blood, but only partially eliminates granulocytes.

For the assays of cells forming rosettes or bearing surface immunoglobulin-mononuclear cells were further purified on Ficoll–Triosil, and were washed twice in phosphate-buffered saline (PBS), pH 7·2.

Spontaneous rosettes with sheep red blood cells (E rosettes). Rosettes were prepared by a technique similar to that of Jondal *et al.* (1972), but with cell suspensions mixed in 100% heat-inactivated FBS. The percentage of cells forming rosettes (three or more adherent SRBC) was estimated on stained smears from a count of 200–300 lymphocytes (Campbell *et al.*, 1974b).

Staining for surface immunoglobulin (SIg). Ficoll–Triosil purified mononuclear cells were fixed in 4% paraformaldehyde, before staining with FITC-conjugated sheep-anti-human immunoglobulin (Wellcome) (Campbell *et al.*, 1974b).

Phytohaemagglutinin stimulation. Triplicate cultures containing 5×10^5 mononuclear cells were set up in 1 ml RPMI 1640 plus 10% FBS with and without PHA (Wellcome dried reagent, lot no. K4416) at a concentration of $1:10^3$, a submaximal stimulating dose. After 72 hr culture, DNA synthesis was estimated by uptake of [^{3}H]thymidine which was added (1 μCi, 150 mCi/mmol) for the last 4 hr. PHA response was expressed as counts per min (ct/min) in the trichloracetic acid-precipitated residues of stimulated cultures *minus* ct/min in cultures without added PHA (Campbell *et al.*, 1974a).

Cell-mediated cytotoxicity. The assays of antibody-dependent and PHA-induced lymphocyte-mediated cytotoxicity have been fully described elsewhere (Campbell *et al.*, 1974b). Target cells were [^{51}Cr]chromate-labelled Chang human liver cells. Triplicate cultures were set up, containing 10^4 Chang cells, plus medium alone, 10^5 and 3×10^5 mononuclear cells. Three separate sets of tubes were set up: (1) with no other reagents; (2) with rabbit anti-Chang antiserum at a concentration of $1{:}10^4$; (3) with PHA at a concentration of $3{:}10^3$. These are concentrations which induce maximal killing of Chang cells by normal mononuclear cells.

After 20 hr incubation the percentage of ^{51}Cr release into the cell-free supernatant was estimated by gamma counting. Specific isotope release was calculated by correcting for baseline release from Chang cells with medium alone, and the maximum attainable on addition of excess effector cells (MacLennan, 1972), and was plotted against the $\log_{10}$ of the number of mononuclear cells in culture. The relative cytotoxic activity of each sample was measured or extrapolated as the $\log_{10}$ number of cells required to produce 50% specific cytotoxicity (SC_{50}).

Proportional and absolute results. The assays as described give proportional estimates of the various subpopulations (or their functional activity) among harvested lymphocytes. Estimates of 'absolute' levels per unit volume of blood have been made by taking into account the blood lymphocyte count at the time the sample was taken.

From the PHA response,

$$\textit{mitogenic capacity} = \log_{10}(\text{ct/min} \times \text{lymphocyte count per millilitre of blood}).$$

From cytotoxicity assays,

$$\textit{cytotoxic capacity} = \log_{10}(\text{lymphocyte count per millilitre blood}) - SC_{50}.$$

Because, in a large series of results obtained for healthy subjects, it has been found that most of these assays approach a Gaussian frequency distribution on a logarithmic scale, all results are plotted and expressed in logarithmic form. Statistical comparisons have been made using the non-parametric Wilcoxon rank sum test, which involves no assumption of a Gaussian distribution.

Plasma cells in the lamina propria of the rectal mucosa. Rectal biopsies were taken 15 cm from the anal–rectal margin, fixed in 4% formalin buffered to pH 7·0) and processed to paraffin wax. Serial sections were taken at 5 μm intervals and every third section was placed on a slide.

The immunoglobulins within plasma cells were identified using the indirect immunoperoxidase method (Burns, 1975). Sections were treated with rabbit antisera against light chains κ and λ (obtained from Dakopatts), which were diluted in PBS (pH 7·4) to a working concentration of 1:40. After three washes in buffer lasting 1 hr, swine anti-rabbit immunoglobulin conjugated to horseradish peroxidase was added at a concentration of 1:40. After washing, the sites of peroxidase were stained dark brown using diamino-benzidine as a substrate. The sections were counter-stained with haematoxylin and mounted.

A modification of the point counting method of Weibel (1963), was used to quantify plasma cells. The slides were projected onto a grid consisting of points, 2 cm apart at the apices of equilateral triangles. The number of points falling on the lamina propria of the mucosa was counted in each section, and also the number of positive staining plasma cells in the same fields. The number of cells per 1000 points falling on lamina propria was calculated. This represents the number of plasma cells in a fixed volume of lamina propria (Skinner & Whitehead, 1974). The total number of plasma cells was calculated by adding the scores for κ and λ staining cells.

RESULTS

The results obtained on admission to the trial will be reported in the third paper in these series. At presentation the mitotic response to PHA was significantly lower in the patients than in healthy controls and the number of plasma cells in the rectal lamina propria was significantly raised (Skinner *et al.*, 1976). In none of the other assays was there a significant difference between the patients and controls at presentation.

Effects of azathioprine

The effects of azathioprine treatment have been analysed by comparing the results obtained in the two patient groups: azathioprine-treated and dummy tablet-treated, at the end of the trial. The last available result has been taken for each patient who remained in the trial for more than 39 weeks. In most cases these tests were performed on completion of the full year's treatment, but final tests were included for a few patients who left the trial on suffering their third relapse in the last 3 months. Statistical analysis of the difference between the groups for the various assays is shown in Table 1. In the assays where a significant effect is attributable to azathioprine, the rate of appearance of the effect is discussed.

Blood counts

The final lymphocyte, neutrophil and platelet counts are shown in Fig. 1. Only the lymphocyte counts were significantly lower in the azathioprine-treated group ($P<0{\cdot}01$). The means for the two groups (2170

and 1390/mm^3) indicate a fall of 36%. The neutrophil counts suggest a slight azathioprine-induced depression, though the difference between the groups is not statistically significant. There was no indication that azathioprine had any effect on platelet counts. The serial results showed that at no point in the trial period was there a significant difference in anything but the lymphocyte counts. The fall was gradual and the difference between the two groups became significant only after 9 months.

TABLE 1. 'End of trial' comparison between azathioprine- (AZA) and 'dummy'-treated patient groups. The results used are the last ones available for patients who remained in the trial for more than 39 weeks

Assay	AZA	Dummy	χ^2, P*
Blood-lymphocyte count/mm^3	3·143±0·179 (13) (1391)	3·337±0·153 (16) (2171)	6·696 $P<0·01$
Blood-neutrophil count/mm^3	3·560±0·126 (13) (3634)	3·668±0·177 (16) (4764)	3·557 $0·1>P>0·05$
Blood-platelet count/mm^3	5·539±0·152 (11) (346×10^3)	5·576±0·153 (16) (377×10^3)	0·351 n.s.
E rosettes (%)	1·845±0·046 (7) (70·0%)	1·778±0·130 (8) (60·0%)	0·568 n.s.
Number/ml blood	5·870±0·217 (7) ($7·4\times10^5$)	6·090±0·174 (8) ($1·23\times10^6$)	2·816 $0·1>P>0·05$
SIg-bearing lymphocytes (%)	1·341±0·112 (11) (21·9%)	1·385±0·169 (14) (24·3%)	0·012 n.s.
Number/ml blood	5·427±0·237 (11) ($2·7\times10^5$)	5·700+0·188 (14) ($5·0\times10^5$)	6·623 $P=0·01$
PHA response (ct/min)	3·280±0·928 (12) (1905)	3·519±0·513 (15) (3304)	0·215 n.s.
Mitogenic capacity	9·406±0·803 (12)	9·833±0·563 (15)	1·736 n.s.
K-cell activity SC_{50}(Ab)	6·127±0·334 (12) ($1·34\times10^6$)	4·919±0·507 (15) ($8·30\times10^4$)	19·072 $P<0·0005$
Cytotoxic capacity (Ab)	1·999±0·360 (12) (1·0)	1·395±0·529 (15) (24·8)	18·648 $P<0·0005$
PHA-induced cytotoxicity SC_{50} (PHA)	5·715±0·724 (11) ($5·20\times10^5$)	5·214±0·298 (11) ($1·64\times10^5$)	4·144 $P<0·05$
Cytotoxic capacity (PHA)	0·426±0·697 (11) (2·7)	1·106±0·337 (11) (12·8)	5·903 $P<0·025$
Plasma cells number in rectal lamina propria/1000 points	3·283±0·333 (15) (1920)	3·613±0·180 (18) (4106)	7·451 $P<0·01$

Results are expressed as logarithmic mean±s.d. (number of individuals tested). Where relevant the antilog mean is shown in parentheses.

n.s. = Not significant.

* χ^2 obtained by Wilcoxon sum of ranks test.

E rosettes (*Fig. 2a*)

This assay was introduced at a late stage in the trial and only a few patients were tested. Although the small number of results does not allow firm conclusions to be drawn, the proportions of rosetting cells in the azathioprine-treated patients were mostly in the high–normal range, indicating that there was no selective loss of this population of lymphocytes. The calculated numbers of rosetting cells per ml blood (Table 1) show that azathioprine caused a fall in these cells approximately in proportion to the change in total lymphocyte count. There is no evidence that these patients with ulcerative colitis had abnormal numbers of T cells in the blood.

SIg staining (*Fig. 2b*)

At the end of the trial there was no significant difference between the two groups in the percentage of cells staining for SIg. The number of such cells per ml of blood was significantly lower in the azathioprine-treated group ($P = 0{\cdot}01$) This fall in SIg-positive cell numbers was to 55% of that seen in the group receiving dummy tablets.

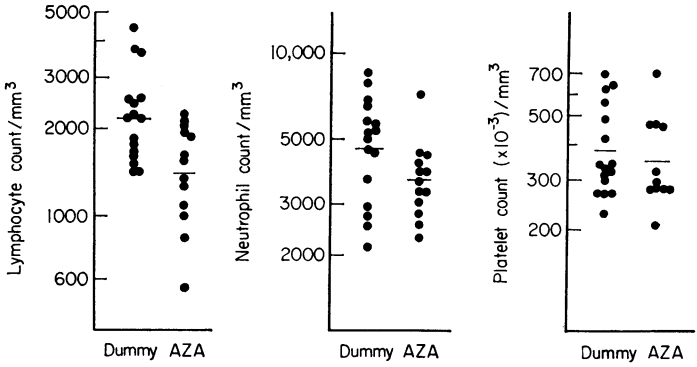

FIG. 1. 'End of trial' lymphocyte, neutrophil and platelet counts. The points shown are the final results obtained for patients who remained in the trial for longer than 39 weeks. 'Dummy' patients treated with dummy tablets, 'AZA' patients treated with azathioprine.

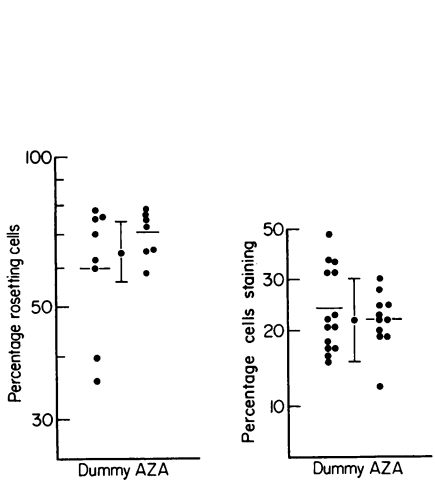

FIG. 2

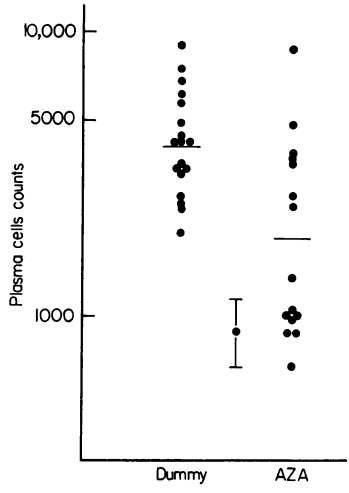

FIG. 3

FIG. 2. End of trial. (a) percentage of lymphocyte forming spontaneous rosettes with sheep red blood cells, (b) percentage of lymphocytes staining for surface immunoglobulin. The central bars indicate the mean ± s.d. established in a large series of tests on healthy adults.

FIG. 3. End of trial (after 12 months), plasma cell counts, expressed as cells per 1000 pts, for the azathioprine (AZA) and 'dummy' treated groups.

Plasma cells in the rectal lamina propria (*Fig. 3*)

There was a fall in the number of plasma cells in the lamina propria of the rectal mucosa. Although several patients showed a 3- to 5-fold fall during the year, the mean reduction was only a little greater than that for the circulating immunoglobulin-staining cells. In a previous study of a group of patients who had been on azathioprine for a much longer period, plasma cell numbers had fallen to considerably

lower levels while the SIg-staining cells in the blood remained in the low normal range (Campbell *et al.*, 1974a). Fig. 4 indicates the slow rate of change in the plasma cells in the azathioprine-treated group. Taking the results of this study with our previous investigation it seems that there is a selective loss of antibody-producing cells compared with their precursors, but that this differential effect is not apparent until after several months treatment with azathioprine.

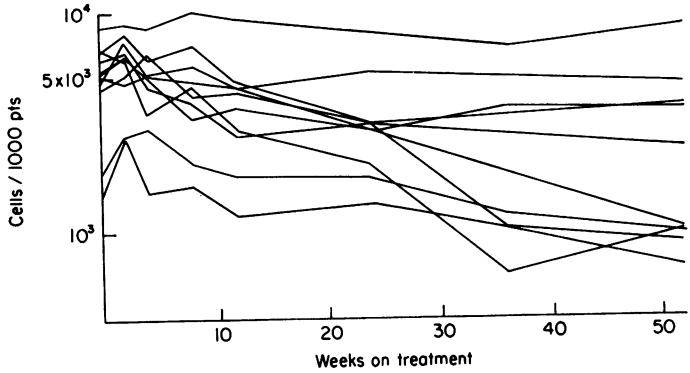

FIG. 4. Serial plasma cell counts at intervals throughout the trial on the azathioprine-treated patients for whom complete data is available. In the other five cases sufficient intermediate biopsies were either not available or of too poor quality to quantify.

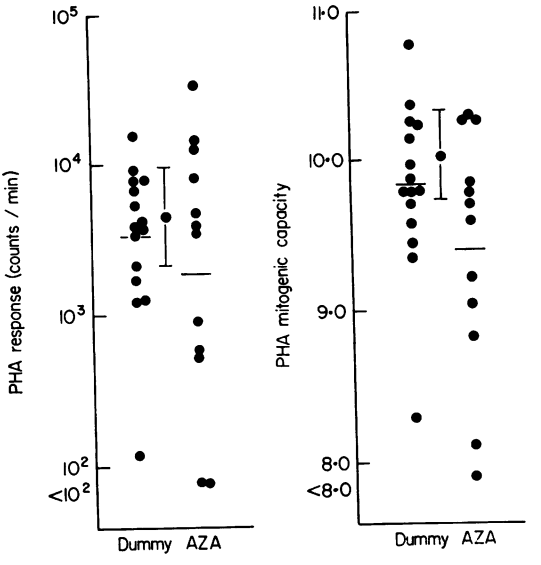

FIG. 5

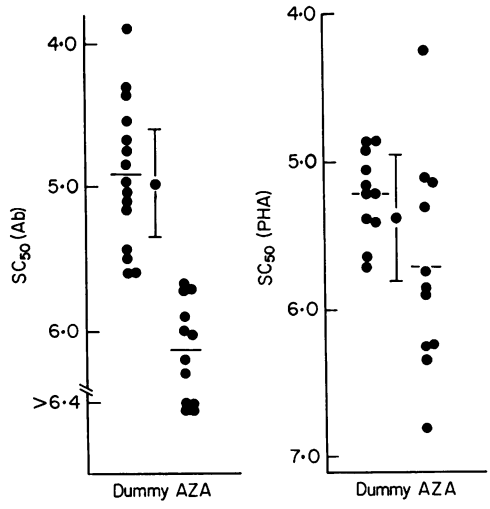

FIG. 6

FIG. 5. End of trial, PHA response (ct/min) and calculated mitogenic capacity of blood. Description as for Fig. 2.

FIG. 6. End of trial, lymphocyte cytotoxic activity expressed as $\log_{10}$ of the number of mononuclear cells required to induce 50% target-cell lysis (SC_{50}), (a) antibody-dependent (K cell), (b) PHA-induced.

Mitotic response to PHA (Fig. 5)

There was no significant difference between the groups, and the overall distribution of results was similar to that found at the beginning of the trial. Although there were more low responses among the azathioprine-treated group, examination of the serial results suggests that this was a chance occurrence. It is a characteristic of this disease that mitotic response to PHA is extremely erratic and that this

variability in responsiveness is not clearly related to clinical activity or treatment (Campbell *et al.*, 1974a; Skinner *et al.*, 1976).

K-cell activity (Fig. 6a)

At the end of the trial the lymphocytes of all the patients who had received the drug had very low cytotoxic activity. The SC_{50} was in fact below the level of accurate quantitation in four of the patients. Results for the control group were distributed in the normal range. Serial results in the azathioprine

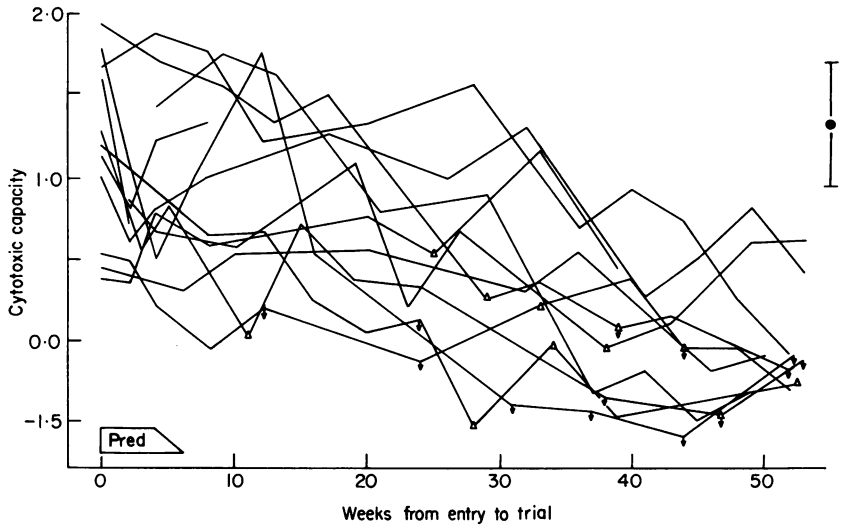

FIG. 7. Serial K-cell activity (cytotoxic capacity) in azathioprine-treated patients. Each downward arrow indicates that cytotoxic capacity was actually lower than that shown, since the SC_{50} was beyond the limit at which it could be accurately quantified (> 6·4). Open triangles represent occasions on which patients were receiving oral prednisolone treatment.

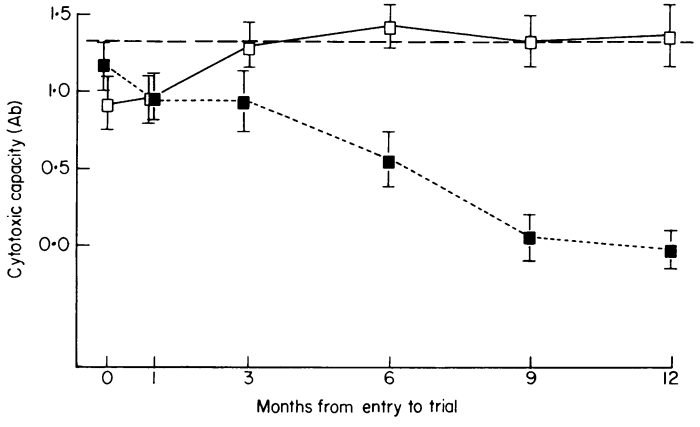

FIG. 8. Serial K-cell activity (cytotoxic capacity) plotted as mean ± standard error at intervals throughout the trial. □———□ dummy treated; ■ - - - - ■ azathioprine treated. The broken horizontal line indicates the established mean for healthy adults. Results at 3, 6, 9 and 12 months have been obtained by collecting the counts made closest to those times (within 3 weeks). With the exception of the '1-month' points, when all patients were receiving oral prednisolone, results have been excluded for patients who were on steroids at the time.

group (Fig. 7) show that K-cell activity in the blood fell in all but one of the patients. The serial results in both groups are summarized in Fig. 8. The azathioprine-induced fall in K-cell activity was continuous over the 12-month period.

PHA-induced cytotoxic activity (Fig. 6b)

The patients on azathioprine showed significant depression of PHA-induced cytotoxicity, though it was less consistent than that found for antibody-dependent cytotoxic activity. Relatively few patients were tested repeatedly throughout the trial by this assay, as it was introduced after the trial had started. These patients showed rather large variations from assay to assay, so the time course for the azathioprine-induced fall in this type of cytotoxicity could not be determined.

Clinical results of the trial, and correlation with assay results

The clinical results of this trial have been described in full elsewhere (Jewell & Truelove, 1974). The therapeutic benefit of azathioprine was found to be undramatic. The addition of azathioprine to the initial treatment of acute attacks was not superior to steroids alone. The effects of maintenance therapy were assessed by the number of relapses occurring during the trial period. There were fewer relapses in the azathioprine-treated than in the dummy-treated group, but the difference was not statistically significant ($P = 0{\cdot}18$). When the patients were divided into those admitted in their first attack, and those who were suffering relapse of established disease, the difference in number of relapses was confined to the established disease group. Even in these, the difference between the treated and control groups did not quite reach statistical significance.

The data reported here have been examined for any evidence of relationships between the patients' clinical course and the effects of azathioprine on lymphoid cell populations. Since the blood-lymphocyte count, K-cell activity and rectal plasma cells were clearly depressed by azathioprine, the relapses occurring during the trial period were examined in relation to these factors, but no correlations were detected. There was no significant difference in the number of relapses during the first 6 months of the trial compared to the second 6 months, in which the depressive effects of azathioprine had become obvious. With regard to K cells, whose function was most markedly reduced by azathioprine, several patients suffered relapses during the last 3 months of the trial, when their lymphocyte cytotoxicity was very low. This can be seen in Fig. 7 in which the occasions on which patients were receiving steroid therapy are identified. Each such occasion indicates that the patient had had an exacerbation of symptoms within the previous 4 weeks.

DISCUSSION

After 1 year of treatment with moderate doses of azathioprine, blood-lymphocyte counts were reduced, though no patient had severe lymphopenia. There was less depression of neutrophil and platelet counts. Staining for SIg and assays of E rosette-forming cells did not indicate any great change in proportions of T and B lymphocytes, suggesting that both circulating populations were diminished approximately in proportion to the fall in total lymphocyte count. Yu *et al.* (1974) have similarly reported finding no selective loss of T or B cells after azathioprine treatment. No change in PHA responses could be attributed to the effects of azathioprine, which is in agreement with the observations of other authors (Denman *et al.*, 1970; Yu *et al.*, 1974).

In the treated patients there was a gross fall in circulating K-cell activity. At the end of the trial the mean cytotoxic capacity was reduced at least 25-fold in comparison to the control patient group. PHA-induced cytoxicity was also clearly reduced, but less consistently than K-cell cytoxicity. Some patients showed a dissociation between the two types of cytotoxic activity, an indication that they are mediated, at least partially, by different cell populations, as has previously been reported (Perlmann, Perlmann & Wigzell, 1972; Campbell *et al.*, 1974b). The K-cell depression and modest lymphopenia induced by azathioprine confirm previous observations on a small group of patients treated for several years with this agent (Campbell *et al.*, 1974a).

The marked loss of plasma cells from the lamina propria provides good evidence that azathioprine can

bring about specific immunosuppression by inhibiting the development of antibody-producing cells from their precursors. Whether this is a direct effect on B-cell maturation or an indirect inhibition resulting from interference with antigen presentation processes remains a matter for speculation.

Through their interaction with pathways of nucleotide synthesis the thiopurines may have multiple effects on cell metabolism (Elion, 1967; Hitchings & Elion, 1969). Inhibition of nucleic acid synthesis is probably a major mechanism by which such agents are anti-proliferative. The cytotoxic effect of azathioprine, or its active derivative 6-mercaptopurine (6MP), is probably largely restricted to cells which are in active cell cycle, though it may not be strictly S-phase specific (Bruce, Meeker & Valeriote, 1966). Thus, azathioprine-induced depletion of circulating leucocytes, which are not themselves proliferating populations, may be due to effects on precursor populations in bone marrow and lymphoid tissue. Numerous factors may be involved in determining the relative depression of different precursor-cell types. For example: (1) pharmacological properties of the agent affect its tissue distribution and metabolism; (2) intrinsic biochemical differences between different cell types may influence their susceptibility to a particular metabolic inhibitor; and (3) kinetic parameters of the cell populations determine their capacity for proliferative recovery in the intervals between repeated drug doses (Skipper, 1968; Berenbaum, 1971). There is no reason to suppose that azathioprine or 6MP are selectively distributed to sites of lymphopoiesis rather than granulopoiesis. It seems likely therefore that granulocytes are either intrinsically more resistant to thiopurines or that the relatively greater depletion of blood lymphocytes than granulocytes was due to more rapid and complete proliferative recovery of the granulocyte precursors in the intervals between daily doses of azathioprine.

A further factor which will affect the rate of depletion of circulating leucocytes is their life-span. Recirculating lymphocytes include both long-lived and short-lived populations. In rodents, there is evidence that a major proportion of recirculating B and T lymphocytes are long-lived cells (Bianco, Patrick & Nussenzweig, 1970; Sprent & Miller, 1972). In man, too, many blood lymphocytes may be of extreme longevity, capable of remaining in the recirculating pool even for several years without undergoing division (Buckton, Court-Brown & Smith, 1967). Thus, the slow reduction in lymphocyte count, resulting in only modest depletion after 1 year of azathioprine, may be a consequence either of the selective loss of relatively short-lived cells or of the slow loss of long-lived cells.

The loss of blood K-cell activity was much more profound than the reduction of B- or T-lymphocyte numbers. This suggests either that the K-cell-precursor population is particularly sensitive to azathioprine as administered, or that K cells are of shorter life-span than most B and T cells. These possibilities involve the assumption that the measured antibody-dependent cytotoxicity is related mainly to the number of effector cells present. Since there are at present no definitive markers by which K cells can be identified directly, it has not been possible to verify this assumption. An alternative explanation of the results might be that azathioprine can depress the function of circulating K cells, without actually eliminating them. Azathioprine, in low concentrations, has been reported to inhibit *in vitro* the cytotoxic activity of immune lymphocytes (Wilson, 1965). However, the slow and continuous decline in K-cell activity detected during this trial does not seem consistent with a direct effect of the drug on effector-cell activity. Azathioprine is rapidly converted *in vivo* to 6MP, which has a short biological half-life (Elion, 1967). Thus any direct inhibitory effect on cytotoxicity would be expected to appear within hours of beginning treatment. The rate of fall of K-cell activity in these patients contrasts with the more rapid recovery (8–12 weeks) in a previous study of patients who stopped treatment after several years (Campbell *et al.*, 1974a). These kinetics may be explained by a 'cell depletion' interpretation of the results: the rate of depletion being the consequence of a fractional reduction of the precursor population by each dose of azathioprine, while rapid recovery is achieved by increased proliferation among precursor cells. In principle, a homeostatically maintained cell population, after depletion by any cytotoxic agent, enters a phase of recuperative expansion (approximating to logarithmic growth) until it approaches its homeostatic norm once more (Berenbaum, 1971).

It remains to ask what may be the clinical implications of these results. The trial did not indicate a major beneficial effect of azathioprine in ulcerative colitis (Jewell & Truelove, 1974), and no obvious correlations were found between the results of lymphocyte assays and the clinical course of individual

patients. In particular, there was nothing to suggest a role of K-cell cytotoxicity in the disease, since several patients suffered acute attacks when their circulating K-cell activity was very low. However, there are a number of other clinical situations in which this agent has more obvious beneficial effects for the patient. The findings which we have described here may correlate more closely with clinical response in these situations, where they might prove a sensitive measure for the individual dose requirement of patients for the drug.

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