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Comparative study of cyclophosphamide, 6-mercaptopurine, azathiopurine and methotrexate

RELATIVE EFFECTS ON THE HUMORAL AND THE CELLULAR IMMUNE RESPONSE IN THE MOUSE

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SUMMARY

The effects of cyclophosphamide, methotrexate, azathioprine and 6-mercaptopurine on the concomitant development of the humoral and the cellular immune responses of mice to a single antigen, the El_4 tumour cell, were investigated. The measurements of cellular and humoral immunity were carried out in the same animal using lymphocyte and antibody mediated lysis of the El_4 cell, a measurement system independent of underlying anti-inflammatory effects. A regimen of daily cyclophosphamide had a more pronounced suppressive effect on the humoral response than on the cellular response, in agreement with other investigators. A single low dose of cyclophosphamide stimulated the cellular response and suppressed the humoral response. Single or multiple high doses of cyclophosphamide maximally suppressed both the cellular and humoral response. Azathioprine and 6-mercaptopurine, in contrast to the results of other investigations, caused equivalent inhibition of both the humoral and cellular responses and thus lacked selectivity. Methotrexate also provided equivalent inhibition of both the humoral and cellular responses at all dose levels investigated.

INTRODUCTION

The ability of the immune suppressants cyclophosphamide (CYP), 6-mercaptopurine (6-MP), azathioprine (AZA) and methotrexate (MTX), to inhibit the immune response to a wide variety of antigens has been investigated extensively. However, few studies have sought to determine the selectivity of their suppressive actions on the humoral and the cell-mediated immune response. There is considerable evidence in support of a selective action of CYP on the humoral response (B-cell dependent). For example, Lerman & Weidanz (1970) reported that CYP depresses the ontogenicity of the humoral immune response in chickens without impairing the development of cellular immunity. CYP was also shown to cause a selective depletion of B lymphocytes from the spleen and lymph nodes (Turk & Poulter, 1972; Stockman *et al.*, 1973) and its effects on B lymphocytes appeared to be longer lasting than on T lymphocytes as measured by the ability of those cells to respond to mitogens (Stockman *et al.*, 1973). Reports describing the relative humoral and cellular effects of the purine nucleotide analogs, 6-MP and AZA, are conflicting. Camiener & Wechter (1971) in their review interpret the literature as implying a selective humoral effect. This would appear to be substantiated by the studies of Abdou, Zweiman & Casella (1973). Lymphocytes taken from multiple sclerosis patients treated with AZA were found to have normal T-cell function as measured by mixed lymphocyte culture and phytohemagglutinin responsiveness, but decreased B-cell function as measured by responsiveness to the mitogenic effects of anti-immunoglobulin

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sera and pokeweed mitogen. However, opposite effects have also been reported. *In vitro*, AZA has been shown to inhibit the development of cell-mediated immune response at concentrations where it failed to inhibit the antibody response (Rollinghoff, Schrader & Wagner, 1973). T-lymphocyte function was also selectively inhibited when T-cell rosetting was used as the criteria of normal function (Bach, 1971). Similarly, Borel & Schwartz (1964) found that 6-MP blocks the acquisition of the cellular response, as measured by the delayed skin test, without inhibiting antibody synthesis. No reports have appeared suggesting the presence or absence of selectivity for methotrexate.

To define accurately the selectivity of these agents for inhibition of cellular and humoral immunity *in vivo*, we undertook to measure their effect on the immune response to a single antigen. Thus, differences in the effective dose of immunosuppressives caused by using antigens of different strength are avoided (Dukor & Dietrich, 1970). To make all measurements internally consistent we chose the El_4 cell as antigen to make possible the determination of both cellular and humoral immunity in the same animal. The system was chosen such that direct measurement of antibody titre and T-lymphocyte-mediated killing of target El_4 cells could be made. Thus, unlike measurements of the immune response based upon skin tests, the presence of anti-inflammatory effects would not interfere with the measurement of immunity.

MATERIALS AND METHODS

Maintenance of El_4 cell line. The El_4 tumour-cell line was a gift from Dr J. Wunderlich, NIH, Bethesda, Maryland. El_4 cells were maintained by passage in syngeneic C57/BL6 mice. The cells (2×10^7) were injected i.p. on day 0. On day 7, approximately $3–5\times10^8$ cells per mouse were harvested from the peritoneum and after washing with Hanks's minimum essential medium (HMEM), they were used either to replant the cell line, for immunization or as target cells in immune assays.

Immunization and drug treatment. On day 0, 1×10^8 El_4 cells were injected i.p. into groups of ten allogeneic BALB/c mice. All mice from a single group were treated orally using one of two schedules: either saline (control group) or drug was administered on day 0 alone or given daily from 3 days prior until 9 days post immunization. In the latter case, dosing was omitted on Sundays, usually day +2 and day +9. Measurements of cell-mediated and humoral immunity were made on day +10.

^{51}Cr labelling of El_4 cells. Both the humoral and cellular assay are based upon lysis of ^{51}Cr-labelled El_4 cells. Labelling was carried out according to the method of Canty & Wunderlich (1970). To 1×10^7 El_4 cells in 20% foetal calf serum and HMEM were added 60 μc of ^{51}Cr. The mixture was incubated for 30 min in 10% CO_2 at 37°C. The labelled cells were washed three times in HMEM with foetal calf serum to remove unbound ^{51}Cr radiolabel and resuspended in the same medium to a concentration of 1×10^5 cells/ml.

Cell-mediated immunity. Measurement of cellular immunity was carried out according to the method of Canty & Wunderlich (1970). Spleen cells were obtained from immunized BALB/c mice treated with either drug or saline. After washing three times in HMEM, the spleen cells were resuspended in HMEM with 20% foetal calf serum to a concentration of 3×10^7 El_4 cells per ml. The spleen cell suspension (0·5 ml) was mixed with 5×10^4 labelled El_4 cells (0·5 ml) in a 35-mm petri dish (Falcon Plastics, Oxnard, California) and incubated for 3 hr at 37°C in 10% CO_2 on a rocker platform making eight reciprocations per min. After incubation, the cells were resuspended, transferred into test tubes and centrifuged. The amount of ^{51}Cr label in the pellet and supernatant was determined. No difference in lysis was found between El_4 cells alone or El_4 cells in the presence of non-sensitized spleen cells. Thus the final percentage lysis from each group of animals was calculated after correction for the spontaneous release (2–3%) of label from El_4 cells. All assays were performed in triplicate. To determine the effect of drug treatment, the percent release of the saline control group was compared with the percent release of the drug-treated group and a percent inhibition of release computed.

Humoral immunity. This was measured by an adaptation of the procedure of Sanderson (1964). The sera from each group of ten immunized and drug treated BALB/c mice were collected, pooled and diluted in HMEM containing foetal calf serum (20%). 0·2 ml, containing $2·5\times10^5$ ^{51}Cr-labelled El_4 cells were mixed with 0·3 ml of a 1/40 dilution of rabbit complement absorbed with 1×10^8 El_4 cells/ml and then added to 0·5 ml of serial dilutions of the mouse sera. The dilution giving 50% lysis of El_4 cells was determined from the van Krogh equation (Kabat & Mayer 1961), to be the titre of the serum. Drug effects were computed as the percent decrease in the titre of sera when drug-treated mice were compared to saline-treated mice.

Drugs. 6-mercaptopurine was purchased from Nutritional Biochemicals, Cleveland, Ohio. Cyclophosphamide, azathioprine and methotrexate were obtained as gifts from Mead-Johnson, Evansville, Illinois, Burroughs-Wellcome, Tuckahoe, New York and Lederle Laboratories Division, American Cyanamid, Pearl River, New York, respectively. The drugs were administered orally in 0·2 ml of a saline suspension except that MTX was a saline dilution of the 50 mg/ml parenteral formulation.

RESULTS

Immunization of the BALB/c mouse with El_4 cells

Three modes of immunization with El_4 cells were tried (Table 1). Cellular and humoral immunity were assayed on day 10. Intravenous immunization with 1×10^7 El_4 cells resulted in no measurable humoral and cellular response. Subcutaneous immunization gave poor cellular immunity (14% lysis of El_4 cells) and sub-optimal humoral immunity (antibody titre of 1/283). Intraperitoneal immunization gave both good cellular (33% lysis of El_4 cells) and humoral immunity (antibody titre of 1/662). Thus, the peritoneal route of immunization was chosen for the subsequent experiments. Further experimentation led to the use of an immunizing dose of 1×10^8 El_4 cells as it gave still higher cellular (circa 40–50% lysis) and humoral (antibody titre, 1/800–1/1000) responses.

The time course of development of cellular immunity

Groups of ten mice were immunized on day 0 with 1×10^8 El_4 cells. At various days thereafter a group of mice was sacrificed and the cellular response determined. The cellular response first became measurable 6 days after immunization and by the 10th day had reached a plateau that did not change significantly through day 20 (Fig. 1). For comparison, three large doses of MTX (8·5 mg/kg) on days −3, 0, and 3, completely suppressed the development of cellular immunity.

TABLE 1. Effect of route of immunization on the elucidation of the humoral and cellular immune response

Route of immunization	Cellular response percentage lysis of El_4 cells	Humoral response serum titre for 50% lysis of El_4 cells
i.v.	2	1/10
Skin Graft C57Bl→BALB/c	31	1/147
i.p.	33	1/662
s.c.	14	1/283

* All mice immunized with El_4 tumour cells received 1×10^7 cells.

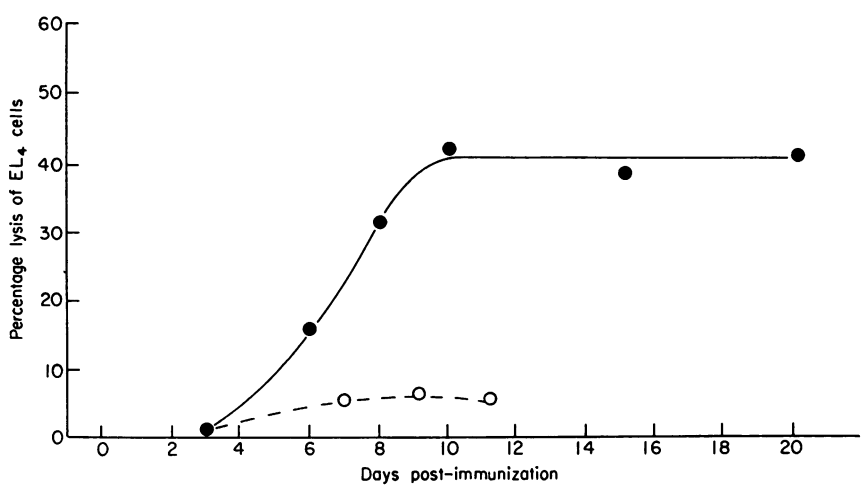

FIG. 1. Kinetics of the appearance of cell-mediated immunity to El_4 tumour cells. Groups of ten BALB/c mice were immunized with 1×10^8 El_4 cells. On the indicated days, cellular immunity was determined as the ability of $1{\cdot}5 \times 10^7$ spleen cells to lyse 5×10^4 ^{51}Cr-labelled El_4 cells. The appearance of cell-mediated immunity is shown for untreated mice, (●) and mice treated with methotrexate, 8.5 mg/kg p.o. on day −3, ○, and +3 (○).

The time course of development of the humoral immune response

A pool of ten mice was immunized with 1×10^8 El_4 cells. On various days thereafter, the mice were bled from the orbital plexus, the sera pooled and the humoral immunity determined. The development of the humoral immune response (Fig. 2) closely paralleled that of the cellular response. Antibody was first detected 5–6 days after immunization. The titre reached a plateau by day 10 and remained constant through day 20. The earliest time both the cellular and humoral immune responses to El_4 cells appeared to reach a constant level was at day 10. Therefore, in subsequent studies with immunosuppressive drugs, all measurements of the immune response were carried out on day 10. Fig. 2 also shows for comparative purposes that MTX administered at 8·5 mg/kg on days −3, 0 and 3 completely suppressed the development of humoral immunity (Fig. 2).

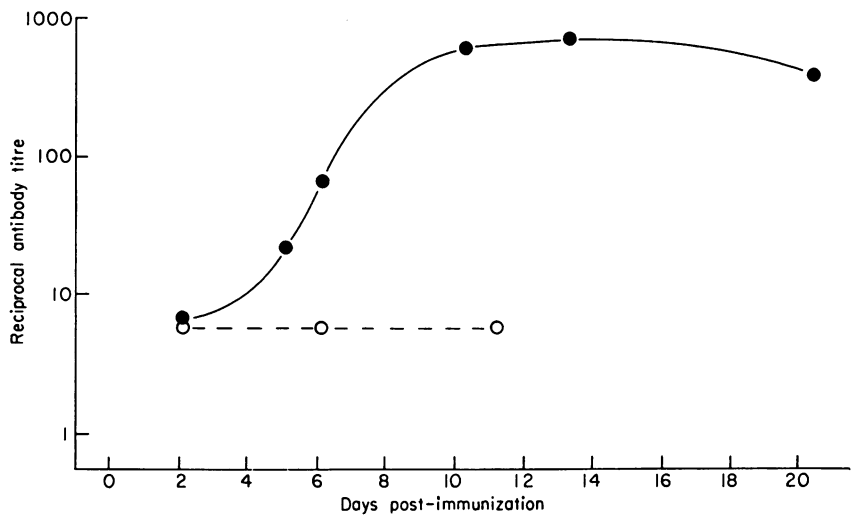

FIG. 2. Kinetics of the appearance of humoral immunity to El_4 tumour cells. A group of ten BALB/c mice was immunized on day 0 with 1×10^8 El_4 cells. They were bled on subsequent days and humoral immunity determined as the serum titre that would lyse 50% of the ^{51}Cr-labelled El_4 cells in the presence of complement. The development of humoral immunity is shown for untreated mice (●) and mice treated with methotrexate, 8·5 mg/kg p.o. on days −3, 0 and +3, (○).

Effects of cyclophosphamide on the development of the humoral and the cell-mediated immune response

The effects of various daily doses of cyclophosphamide on the development of humoral and cell-mediated immune response to El_4 cells in the mouse is shown in Fig. 3. Ten animals in each group received drug daily. At 33 mg/kg and 20 mg/kg, the drug totally inhibited both the humoral and the cell-mediated immune responses. At 10 mg/kg nearly complete inhibition (96%) of the humoral response was obtained while the cellular response was only partially suppressed (52%). At 5 mg/kg no suppresson of the cellular response was found yet the humoral immune response was still suppressed by 63%. No suppression of either humoral or cellular immunity was found at 2·5 mg/kg p.o. A single dose of CYP administered on day 0 at either 330 mg/kg or 100 mg/kg, p.o. appeared to completely inhibit both limbs of the immune response (Table 2). At lower doses (20 mg/kg and 10 mg/kg) the drug stimulated the cell-mediated response while continuing to suppress the humoral response (Table 2).

Effects of 6-mercaptopurine on the development of the humoral and the cell-mediated immune response

The effect of different doses of 6-MP on the development of the humoral and the cell-mediated immune response to El_4 cells is shown in Fig. 4. The drug completely suppressed the development of both the humoral and the cell-mediated immune response at 20 mg/kg and 33 mg/kg. In the range of partial suppression, 10 mg/kg p.o., it suppressed both branches of the immune response equivalently.

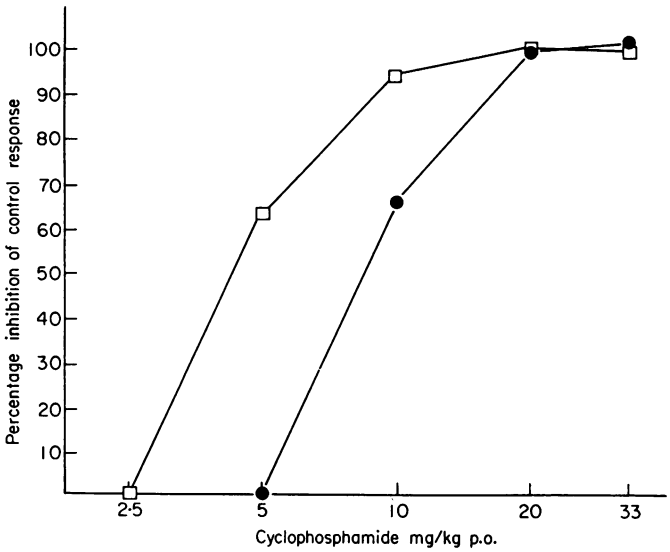

FIG. 3. The effect of cyclophosphamide dose on the humoral and cellular immune response to El_4 cells. Groups of ten BALB/c mice were immunized with 1×10^8 El_4 cells on day 0 and on day 10 their humoral and cellular immune response was measured as described in the text. Each group was treated either with cyclophosphamide or a saline placebo from day -3 to day $+9$. Inhibition of the humoral response (□) and the cellular response (●) by cyclophosphamide is shown.

TABLE 2. Effect of a single dose of cyclophosphamide at the time of immunization with El_4

Dose mg/kg p.o.	Percentage inhibition of control responses	
	Cellular	Humoral
330	>90	>90
100	80	>90
100	88	>90
100	21	>90
33	−46	48
20	−24	52
10	−39	66
10	−43	21

6-MP was inactive at 5 mg/kg. When 6-MP was given as a single dose at the time of immunization, no inhibition of cellular or humoral immunity was found up to the highest dose tested, 200 mg/kg.

Effects of azathioprine on the development of the humoral and cell-mediated immune response

AZA totally suppressed both the humoral and the cell-mediated immune response at 80 mg/kg. In the dose range of partial suppression, both responses were equivalently inhibited, the cellular and humoral response at 40 mg/kg, 62% and 65% respectively, and at 20 mg/kg, 54% and 51% respectively. AZA was inactive at 10 mg/kg (Fig. 4). When given as a single dose, AZA was inactive to the highest dose tested 200 mg/kg.

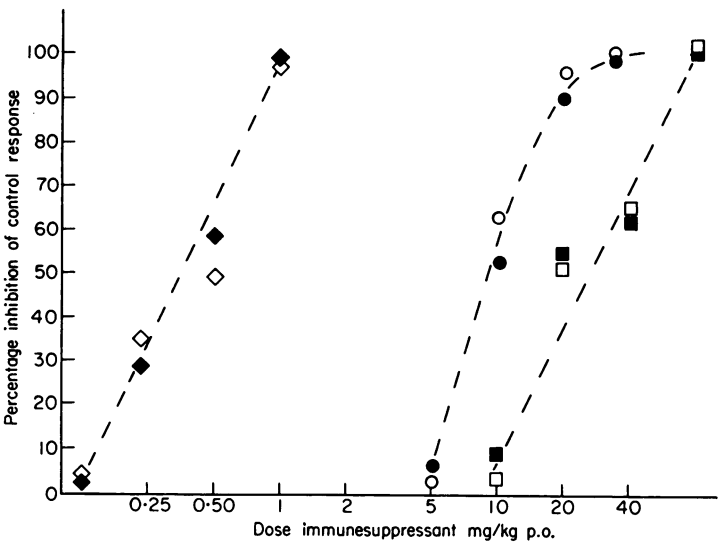

FIG. 4. The effect of immunosuppressant dose on the humoral and cellular immune response to El_4 cells. Groups of ten BALB/c mice were immunized with 1×10^8 El_4 cells on day 0 and on day 10 their cellular and humoral immune response was measured as described in the text. Each group was treated either with an immunosuppressant drug or a saline placebo from day −3 to day +9. The inhibition of the humoral response, (◇, ○ and □) and of the cellular response, (◆, ● and ■) is shown for methotrexate, (◇ and ◆), 6-mercapto- (○ and ●) and azathioprine, (□ and ■).

Effects of methotrexate on the development of the humoral and the cell-mediated immune response

The effect of methotrexate on the development of immune response is shown in Fig. 4. The drug completely suppressed both the humoral and the cell-mediated immune response at 1·0 mg/kg p.o. In the range of partial suppression, both branches of the immune response were inhibited to approximately the same extent, cellular and humoral inhibition at 0·5 mg/kg, 49% and 59% respectively and at 0·25 mg/kg 35% and 28% respectively. No inhibition was seen at 0·125 mg/kg p.o. As a single dose at the time of immunization, MTX was inactive at the highest dose tested, 20 mg/kg.

DISCUSSION

It is well known that the induction of an immune response is dependent on a variety of features. The choice of antigen could affect the selectivity observed; for example, Galanaud, Crevon & Dormont (1975) found *in vitro* that T-cell dependent antibody production is much more sensitive to AZA than T-cell independent antibody production. We chose for our antigen the El_4 cell. It is a leukaemic tumour derived from the C57Bl/6 mouse and the immune responses measured are developed in allogeneic BALB/c mice. Thus the primary antigen involved in the present model of immune response is probably the major histocompatability locus since an allogeneic skin graft from the C57BL mouse sensitizes to El_4 (Table 1) and BALB/c immune sera and immune spleen cells raised against El_4 cells lyse C57Bl spleen cells (data not shown). Some specificity for tumour-associated antigens may also be involved (Kedar & Bonavida, 1975).

The route of antigen administration has a primary effect on whether one obtains a humoral or a cellular immune response or a mixture of both. This is demonstrated by the studies of Lagrange, Mackaness & Miller (1974) who showed that at certain doses of sheep red blood cells, i.v. administration of antigen gives a pure humoral response and s.c. administration of antigen leads to a pure cellular response. We sought a route of immunization that would provide good cellular and humoral responses to El_4 cells and found that the only satisfactory route in the absence of adjuvant was the i.p. one.

Since the object of this study was to determine the selectivity of inhibition of cellular and humoral immunity, it was imperative to have both responses well developed so that drug effects on either limb of the immune response could be assayed accurately in parallel in the same animal. The El_4 cell system meets this requirement.

Immunosuppression achieved with drugs has been shown to be critically dependent on the time of treatment relative to the time of immunization, and on the time chosen for measuring the effects (Santos, 1967; Lagrange *et al.*, 1974). In this study, only two dosing regimens were explored; a daily dosing regimen from day -3 to day $+9$ and a single drug dose at time of antigen administration (day 0).

The present results show that all four drugs, CYP, AZA, 6-MP and MTX, given in a sufficient dose, are capable of completely inhibiting the development of both the humoral and the cellular immune response to El_4 cells when measured by antibody titre and by T-cell mediated destruction of target cells respectively. At lower dose levels, where the immune response is only partially suppressed, it is possible to detect selective effects of these drugs.

Evidence for selectivity of CYP was first reported by Lerman & Weidanz (1970). They showed that CYP depressed selectively the development of the humoral response in newborn chickens as shown by the absence of significant IgM and IgG production with apparently normal cellular immunity as measured in the graft-versus-host reaction. These results are in agreement with histologic observations which demonstrate that CYP causes a selective depletion of B lymphocytes from lymph follicles, germinal centres and the cortico-medullary junctions in lymph nodes and from equivalent thymus-independent areas in the spleen of both mice and guinea-pigs (Turk & Poulter, 1972). The paracortical, thymus-dependent areas retain lymphocytes. Chronic treatment may lead to depletion of thymus-dependent areas also (Winkelstein, 1973). Our results indicate a selective effect of CYP at low doses and a non-selective effect at high doses. They further indicate that to observe a selective therapeutic effect one must work in the lower range of therapeutically effective doses. Attainment of pronounced immunosuppressive effects is tantamount to loss of selectivity.

A dramatically different selectivity was observed when a single dose of CYP was administered at the time of immunization (Table 2). At high doses, both responses were suppressed, but at low doses, CYP suppressed the humoral response, but the cellular response was, in fact, stimulated (Table 2). Both Kerckhaert, van den Berg & Willers (1974), and Askenase, Hayden & Gershon (1975) have reported that a single dose of CYP prior to immunization can enhance the delayed skin test to SRBC in the mouse. Our findings demonstrate that a single dose of CYP can also stimulate a quite different measure of cellular immunity, lymphocyte-mediated target cell lysis.

The phenomenon of selective suppression of the humoral response might be explained by the observation of Stockman *et al.* (1973). They observed that the response of mouse lymphocytes to phytohaemagglutinin (T-cell dependent) recovers 3 days after a single dose of cyclophosphamide (400 mg/kg); responsiveness to pokeweed mitogen (primarily B-cell dependent) does not recover for 10 to 14 days indicating that the effect of the drug on B-lymphocytes but not on T lymphocytes should last through our measurement period of 10 days. Daily dosing of drug would be expected to maintain suppression of both types of response, whereas a single low dose might, after about 10 days, be expected to show only humoral suppression. If these data extrapolate to man, it can be speculated that where selective humoral immunosuppression is desired such as in the treatment of diseases with a predominant immune complex etiology, pulsed doses at intervals sufficient to maintain suppression of humoral, but not cellular immunity, would be a desirable therapeutic approach. Moreover, it is possible that the finding of cellular stimulation may be explained by the residual inhibition of B-cell function. Thus B-cell suppression of cellular immunity (Gorczynski, 1974; Katz *et al.*, 1974) would be absent. Alternatively the stimulation of the cellular response might be due to the relief of the T-cell response from feedback inhibition that normally accompanies the development of the humoral immune response as suggested by Mackaness *et al.* (1974) and Lagrange *et al.* (1974).

The lack of selective effect of 6-MP in our measures of immunity contrasts with the selectivity for the cellular response reported by Borel & Schwartz (1964) using antibody formation and delayed skin tests in the rabbit. The apparent selectivity they observed may be attributed to the fact that 6-MP has a sup-

pressive effect on mononuclear cells (Page, Condie & Good, 1962). The observations that the delayed skin test is inhibited by 6-MP at a dose where no effect is seen on humoral antibody formation might be the result of an anti-inflammatory effect of 6-MP which interferes with measurement of the immune response. This view is in accord with the finding of Zweiman & Phillips (1970) that lymphocytes respond normally to antigen *in vitro* even when their skin test has been abolished by 6-MP treatment and in accord with our results that 6-MP is not a selective immunosuppressive.

Like 6-MP, AZA was found to cause equivalent suppression of both the cellular and humoral response in our system. The exquisite sensitivity of T-lymphocyte rosetting to AZA found *in vitro* by Bach (1971) and the sensitivity of cell-mediated immunity to inhibition *vis-a-vis* the humoral response noted by Rollinghoff *et al.* (1973) and by Galanaud *et al.* (1975) was not observed in our studies. This implies that if a selectivity exists for T cells *in vivo* as well as *in vitro*, the humoral and cellular immune response to El_4 cells must have equivalent T-cell dependency or the primary rate limiting inhibitory step of AZA in the development of the immune response is not the T cell. It is equally possible that *in vitro* results cannot be directly extrapolated to *in vivo* results in view of the multiple control mechanisms for immunity.

The effects of AZA noted by Abdou *et al.* (1973) showed, in contrast to our results and to the *in vitro* results cited above, selective effects on the humoral response. They measured B-cell function as the proliferative response to pokeweed mitogen and to anti-immunoglobulin antisera. Both measures were decreased. They found T-cell function unaltered as measured by the proliferative response to phytohaemagglutinin and by the mixed leucocyte culture technique. They found that the selective effect of AZA was manifest only after 2–4 months of therapy. Thus, it is to be expected that a similar selective effect would not be observed in our 13-day assay.

Like 6-MP and AZA, MTX shows equivalent inhibition of the humoral and cellular response in the intermediate dose range. Thus, in our system where the primary humoral and cell-mediated immune response to the same antigen are determined in the same animals and where the measurement of the cell-mediated immune response is not obscured by underlying antiinflammatory effects, the three antiproliferative agents AZA, 6-MP and MTX suppressed both the humoral and the cellular immune response in parallel at all dose levels.

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