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USE OF IMMUNOSUPPRESSIVE AGENTS IN CONNECTIVE TISSUE DISORDERS -- DIFFERENTIAL EFFECTS OF AZATHIOPRINE, METHOTREXATE AND CYCLOPHOSPHAMIDE

or

ALL IMMUNOSUPPRESSIVES ARE NOT ALIKE

Use of Immunosuppressive Agents in Connective Tissue Disorders -- Differential Effects of Azathioprine, Methotrexate and Cyclophosphamide

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VI. Summary

I. *Introduction*

Cytotoxic agents are being used increasingly in the treatment of diseases mediated by immune processes such as the rheumatic and connective tissue diseases. Almost every anticancer drug has been shown to have immunosuppressive properties (Table 1).

Table 1

Classes of Commonly Used Agents With Immunosuppressive Activity

Class of Drug	*Examples of Drugs Included in the Class*
1. Alkylating Agents	Cyclophosphamide (Cytoxan) Nitrogen mustard (HN_2) Chlorambucil (Leukeran) Busulfan (Myleran)
2. Antimetabolites	
a) Purine antagonists	6-Mercaptopurine (6-MP, purinethol) Azathioprine (Imuran) 6-Thioguanine (Tabloid)
b) Pyrimidine antagonists	5-Fluorouracil (5-FU), Fluorodeoxyuridine (FUDR) Bromodeoxyuridine (BUDR) Cytosine arabinoside (ara-c, cytarabine)
c) Folate analogs	Methotrexate (amethopterin, MTX)
3. Antibiotics	Antinomycins C and D, Puromycin
4. Urea derivatives	Bis-Chloroethyl-nitroso-urea (BCNU)
5. Steroids	Prednisone
6. Antibody	Antilymphocyte serum (ALS) or antilymphocyte globulin (ALG)
7. Enzymes	Asparaginase
8. Vinca Alkaloids	Vincristine (VCR), Vinblastine (VLB)
9. Methyl hydrazine derivatives	Procarbazine (Matulane)

from Mitchell and Bertino (2)

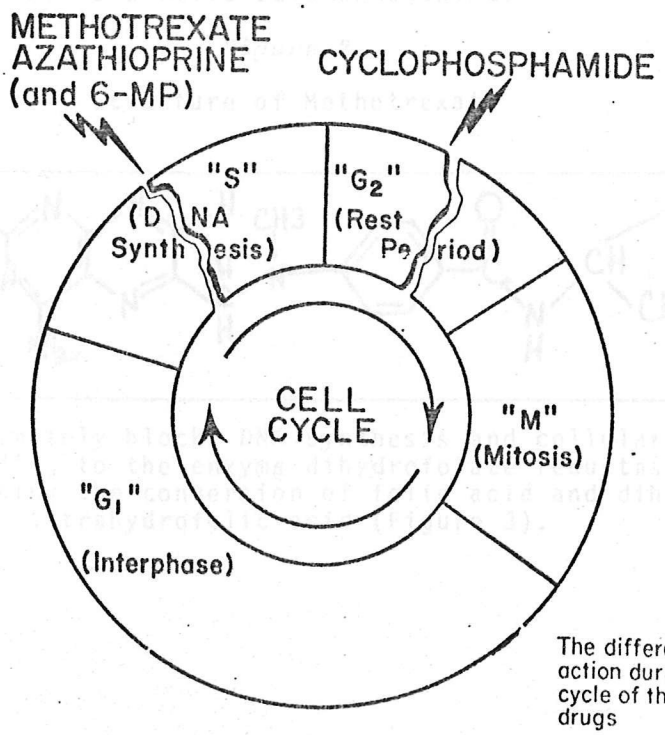
However, the three agents that are currently most actively used in the treatment of nonneoplastic disease are methotrexate, azathioprine and cyclophosphamide. While these drugs are usually all loosely termed "Immunosuppressive," they are actually quite different, not only from the standpoint of pharmacologic differences but also from the standpoint of mechanism of action, toxicity and efficacy for treatment of these diseases. These differential effects will be discussed in some detail.

II. *Pharmacology of Methotrexate, Azathioprine and Cyclophosphamide*

A. General Considerations

A very useful concept which greatly helps in understanding the action of these agents is the concept of *cycle active* and *noncycle active* drugs. Bruce and coworkers (1) demonstrated that certain drugs, primarily alkylating agents (and x-ray therapy), will kill cells whether or not they are *in cycle* -- i.e., in the process of replicating or not, while certain other agents, primarily antimetabolites, will not be able to kill cells if the cell is not in cycle (Figure 1).

Figure 1



The different sites of action during the cell cycle of the cytotoxic drugs

Modified from Starzl et al; TRANSP PROC; Dec.,1972 (3)

The cell cycle may be divided into a G_1 (Gap 1) or pre-synthetic gap, S(DNA synthesizing), G_2 (Gap 2) or pre-mitotic resting phase and M phase (mitosis). While a cycle active agent may kill a cell in any part of the cycle, the antimetabolites such as methotrexate, azathioprine and 6-MP appear to act only on cells going through the S-phase of the cycle. In a non-stimulated lymphocyte population, many of the cells are not in cycle, but are in a so-called *prolonged* G_1 or "G_0" state. These cells are not affected, therefore, by exposure to cycle active agents, but may be killed by alkylating agents such as cyclophosphamide or x-ray, although even these agents seem to kill cells in cycle to a greater degree than cells not in cycle.

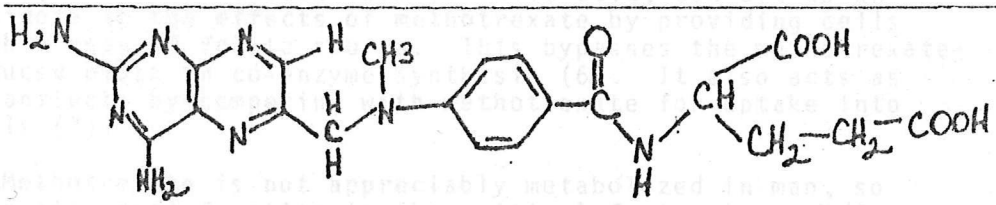
Of the three drugs mentioned, methotrexate and azathioprine (or 6-MP) are *cycle specific* S-phase inhibitors, while cyclophosphamide has properties of both cycle and noncycle specific drugs. Thus, while methotrexate, azathioprine and 6-MP primarily kill rapidly dividing cells, cyclophosphamide kills both dividing and non-dividing (resting) cells.

B. Methotrexate

Methotrexate (4-amino-N^{10}-methylpteroylglutamic acid) (Figure 2) is a folic acid antagonist.

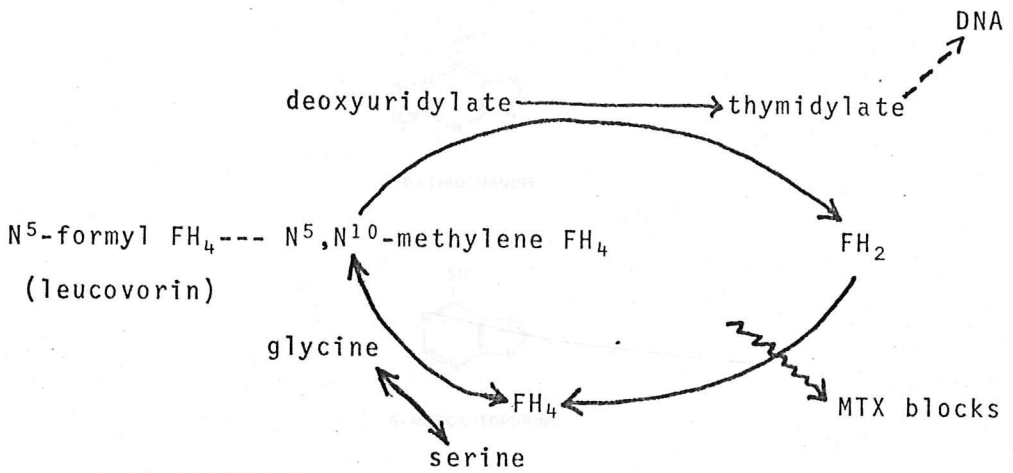
Figure 2

Structure of Methotrexate



It ultimately blocks DNA synthesis and cellular replication by binding to the enzyme dihydrofolate reductase, and thus inhibiting the conversion of folic acid and dihydrofolic acid to tetrahydrofolic acid (Figure 3).

Figure 3. Methotrexate site of action and leucovorin rescue. FH_2, dihydrofolate; FH_4, tetrahydrofolate.



from Bertino (4)

Tetrahydrofolic acid is the active co-enzyme form of folic acid, which is necessary for the formation of thymidylate and the purine ring. Thus, methotrexate blocks thymidylic acid synthesis, which is a pyrimidine found in DNA but not in RNA and thereby ultimately blocks DNA synthesis and cellular replication (5).

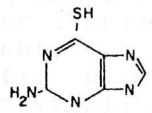
Leucovorin (citrovorum factor, N^5-formylatetrahydrofolic acid), a stable reduced form of folic acid, can act as an antidote to the effects of methotrexate by providing cells with a reduced folate source. This bypasses the methotrexate-induced block in co-enzyme synthesis (6). It also acts as an antidote by competing with methotrexate for uptake into cells (7).

Methotrexate is not appreciably metabolized in man, so adequate renal function is the critical factor in avoiding prolonged, sêvere toxicity. The drug binds to dihydrofolate reductase enzyme in the liver and kidney, and appears to be retained by these organs for several weeks (8).

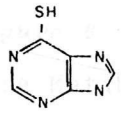
C. Azathioprine

Azathioprine is a purine analogue which is a derivative of 6-mercaptopurine (6-MP) (Figure 4). As shown in Figure 5,

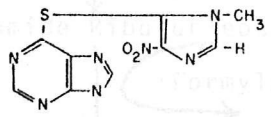
Figure 4. Structure of 6-mercaptopurine, 6-thioguanine and azathioprine.



6-THIOGUANINE

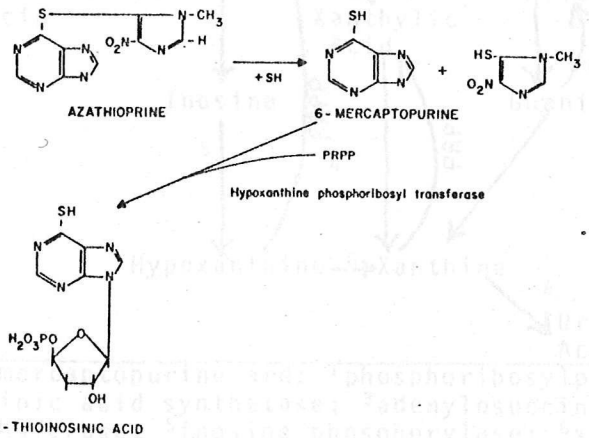


6-MERCAPTOPURINE



AZATHIOPRINE

Figure 5. Conversion of azathioprine to 6-mercaptopurine and 6-thioinosinic acid.



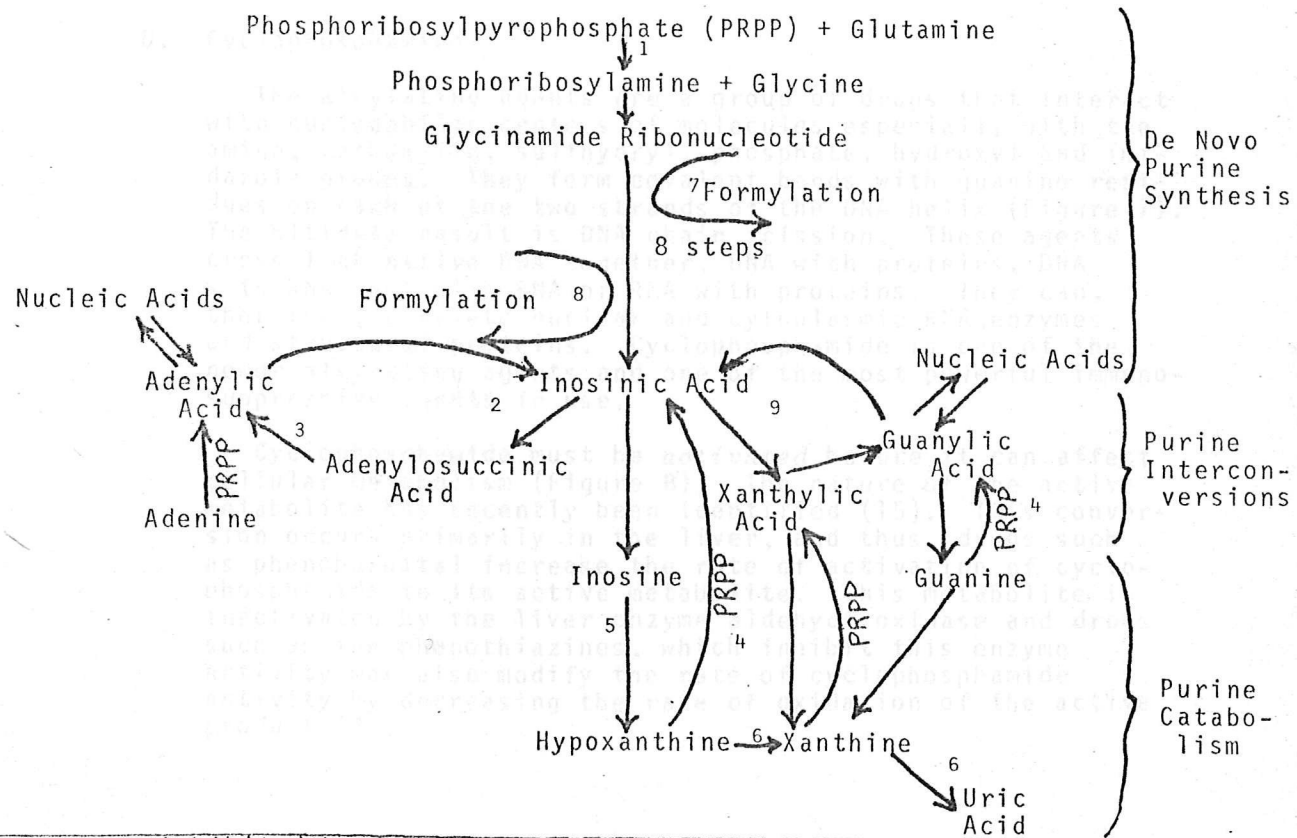
from Bertino (4)

sulfhydryl groups present in plasma and cells (as on glutathione, cysteine or proteins) react rapidly with azathioprine to generate 6-MP. There is some evidence to suggest that azathioprine has a better therapeutic index than 6-MP as an immunosuppressive agent, i.e., azathioprine can be used for a longer period of time than 6-MP with less risk of toxicity such as thrombocytopenia or leukopenia.

The exact mechanism of action of purine analogs in inhibiting cell replication is still not clear, although several sites of actions have been elucidated (9) (Figure 6).

Figure 6

Pathways of Purine Metabolism in Man



Enzymes inhibited by 6-mercaptopurine are: [1]phosphoribosylpyrophosphate amidotransferase; [2]adenylosuccinic acid synthetase; [3]adenylosuccinase; [4]hypoxanthine-uanine phosphoribosyltransferase; [5]inosine phosphorylase; [6]xanthine oxidase; [7]glycinamide ribonucleotide transformylase; [8]5-amino-4-imidazolecarboxamide ribotide transformylase; and [9]inosinic acid dehydrogenase.

from Kelley, Rosenbloom and Seegmiller (13)

As shown in Figure 5, 6-thioinosinic acid, formed by the action of hypoxanthine phosphoribosyl transferase, is the active form of 6-MP.

In addition, a small amount of 6-MP is incorporated into RNA and into DNA in the form of thioguanine which also has potent immunosuppressive effects (10). Both 6-MP and azathioprine are converted to thiouric acid by the enzyme xanthine oxidase. Therefore, the dose of 6-MP and azathioprine should be reduced to one-third or one-fourth of its usual level if allopurinol is given concomitantly since allopurinol is a xanthine oxidase inhibitor. Since some of the drug is excreted unchanged by the kidney, lower doses should be used in patients with renal insufficiency. In the anuric patient, doses may have to be decreased by one-half (11), although this point is disputed by Bach and Dardenne (12), who claim that no reduction in dosage is necessary in patients in renal failure.

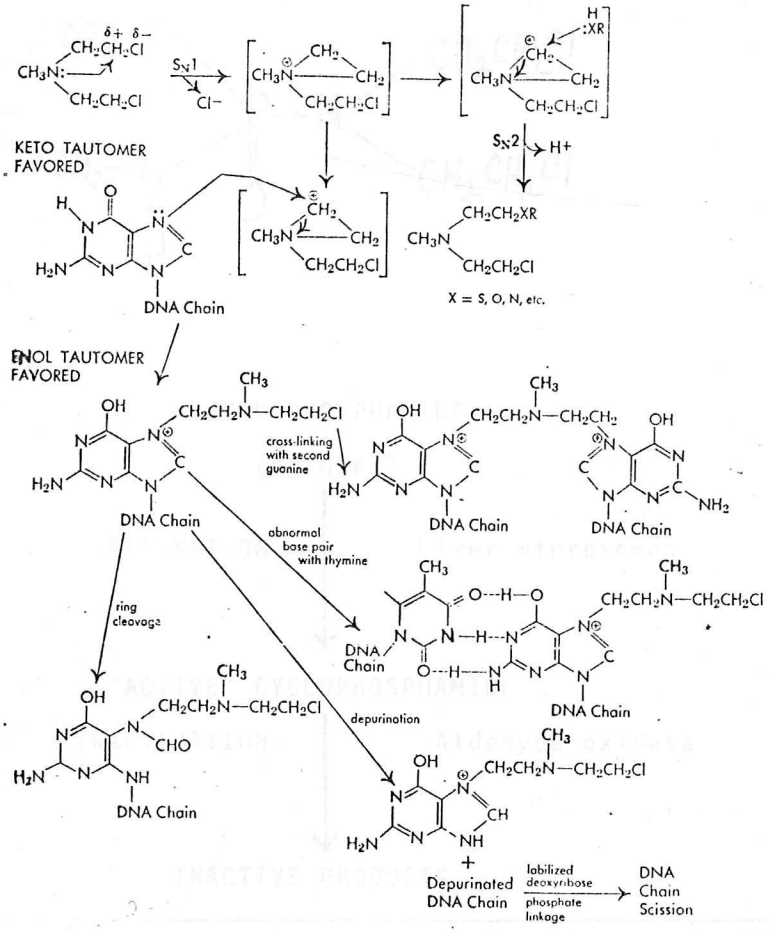
D. Cyclophosphamide

The alkylating agents are a group of drugs that interact with nucleophilic centers of molecules especially with the amino, carboxylic, sulfhydryl, phosphate, hydroxyl and imidazole groups. They form covalent bands with guanine residues on each of the two strands of the DNA helix (Figure 7). The ultimate result is DNA chain scission. These agents cross-link native DNA together, DNA with proteins, DNA with RNA, RNA with RNA or RNA with proteins. They can, therefore, alkylate nuclear and cytoplasmic RNA,enzymes and structural proteins. Cyclophosphamide is one of the newer alkylating agents and one of the most powerful immunosuppressive agents in use.

Cyclophosphamide must be *activated* before it can affect cellular metabolism (Figure 8). The nature of the active metabolite has recently been identified (15). This conversion occurs primarily in the liver, and thus, drugs such as phenobarbital increase the rate of activation of cyclophosphamide to its active metabolite. This metabolite is inactivated by the liver enzyme aldehyde oxidase and drugs such as the phenothiazines, which inhibit this enzyme activity may also modify the rate of cyclophosphamide activity by decreasing the rate of oxidation of the active product (4).

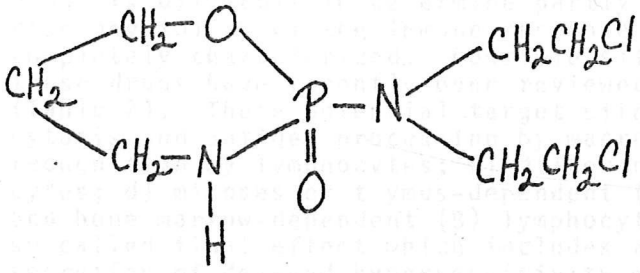
Figure 7

Mechanism of Action of Alkylating Agents



from Calabresi and Parks (14)

Figure 8. Conversion of cyclophosphamide to its active metabolite and its subsequent inactivation.



CYCLOPHOSPHAMIDE

(CYTOXAN)

ACTIVATION — Liver microsomes

"ACTIVE" CYCLOPHOSPHAMIDE

INACTIVATION — Aldehyde oxidase

INACTIVE PRODUCTS

from Bertino (4)

III. Immunosuppressive and Antiinflammatory Properties of Azathioprine, Methotrexate and Cyclophosphamide -- Differential Effects in Mechanism of Action

A. General Considerations

The exact mechanism of action of immunosuppressive drugs is difficult to determine partly because the precise physiology of the immune response has not been completely characterized. Possible sites of action by these drugs have recently been reviewed by Bach (16) (Table 2). These potential target sites are: a) phagocytosis and antigen processing by macrophages; b) antigen recognition by lymphocytes; c) differentiation of lymphocytes; d) mitoses of thymus-dependent (T) lymphocytes and bone marrow-dependent (B) lymphocytes; and e) the so-called final effect which includes antibody production, secretion of delayed hypersensitivity mediators and lymphocyte cytotoxicity of the contact type.

Table 2

Possible Target Sites for Cytotoxic Drugs

1. Phagocytosis and Antigen Processing by Macrophages
2. Antigen Recognition by Lymphocytes
3. Differentiation (induction period)
4. Mitoses of Thymus-dependent (T) and bone marrow-dependent (B) Lymphocytes
5. Final Effect
(a) Antibody Production
(b) Secretion of delayed hypersensitivity mediators
(c) Lymphocyte cytotoxicity

from Bach (16)

The three cytotoxic drugs, cyclophosphamide, azathioprine and methotrexate, are representative of the three major classes of compounds which have been most used for treatment of the rheumatic diseases.

All three of these drugs have been shown to suppress primary and secondary humoral immune responses, delayed hypersensitivity, skin graft rejection and animal diseases of autoimmunity. Unfortunately, there are few studies which have compared the effects of these three drugs under the same experimental conditions. However, some rather striking differences in mechanism of action of the three agents have become apparent, and these are summarized below.

B. Antiinflammatory Properties

Page, Condie and Good (17) showed that, if one injects egg albumin into the skin of a rabbit, polymorphonuclear leukocytes begin to arrive in the lesion during the first four hours and these are followed by mononuclear cells. If, however, the animals are pretreated with 6-MP, the polymorphonuclear leukocytes arrive in the lesion normally, but the numbers of mononuclear cells are markedly decreased. Neither cyclophosphamide nor methotrexate was effective in this respect (18).

We have also studied this phenomenon (19) and were able to demonstrate that relative to control animals, animals treated with 6-MP had significant decreases in the numbers of large lymphocytes and monocytes in the blood without a significant fall in the numbers of polymorphonuclear leukocytes or small and medium lymphocytes. Concurrently, a significant decrease was also seen in the percentage of tissue mononuclear cells in the inflammatory skin lesion. There was a highly significant correlation between the numbers of monocytes in the blood and the percent of mononuclear cells in the lesion. These data suggest that the antiinflammatory effect of 6-MP results from suppression of a bone marrow response to local inflammation, affecting principally proliferating precursors of blood monocytes and the large lymphocytes.

In agreement with these results, Hersh, Wong and Freireich (20), studying experimental inflammatory sites in man, have shown an inhibition of mononuclear cell exudation in patients taking 6-MP or methotrexate.

Stevens and Willoughby (21) have induced two types of acute inflammation in rats, thermal injury and turpentine-induced pleurisy. In these studies, all three drugs were effective in reducing the acute inflammation. They found a rough correlation between improvement and decrease in blood lymphocytes and monocytes, but no correlation with polymorphonuclear leukocytes. A recent study by Currey (22)

compared the effects of all three agents on adjuvant arthritis and found that therapeutic/toxic ratios were most favorable with cyclophosphamide and least favorable with azathioprine. Winkelstein (23) has recently demonstrated that cyclophosphamide treatment brought about a much greater decrease in both lymphocytes and macrophages than either 6-MP or methotrexate in a glycogen-induced peritoneal exudate in the guinea pig.

C. Effects on Antibody-forming System

Studying antityphoid antibody production in mice, Berenbaum and Brown (24) compared the LD_5 dosage of cyclophosphamide, methotrexate and 6-MP with its ID_2 (Table 3).

Table 3

Therapeutic Indices of Agents Inhibiting Antibody Production

Agent	LD_5*	ID_2†	TI††
Cyclophosphamide	300	50	6.0
Methotrexate	6.3	1.25	5.0
6-Mercaptopurine	240	100	2.4

*LD_5, dose (in mg/kg) killing 5 per cent of animals within 1 week.

†ID_2, dose (in mg/kg) lowering antibody titre to $1/2^2$.

††Therapeutic index -- LD/ID.

from Berenbaum and Brown (24)

The LD_5 was the dose which killed 5% of the animals within one week. The ID_2 was the dose required to reduce the antibody response of the mice by a factor of 2. They then calculated a therapeutic index which was defined as the ratio of the two doses (LD_5/ID_2). Cyclophosphamide had the highest therapeutic index of 6.0, followed by metho-

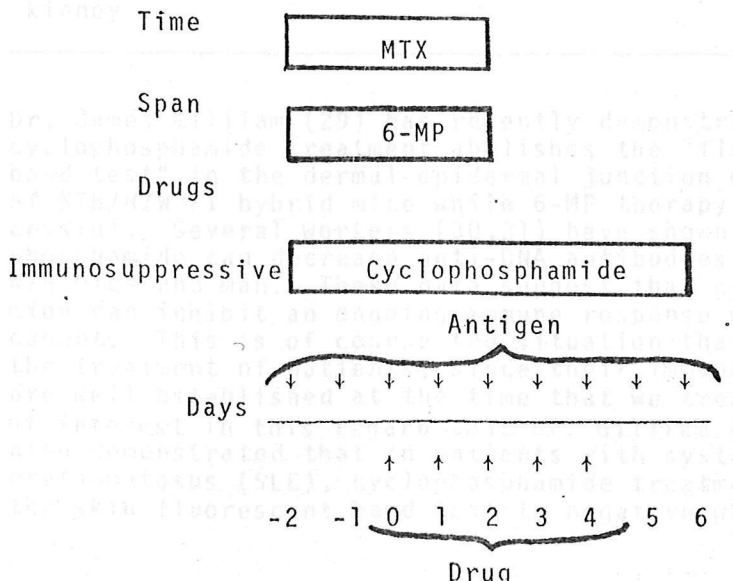
trexate with 5.0 and 6-MP with 2.4. However, these workers emphasize the fact that the toxicity and immunologic effectiveness of these agents are highly dependent on their mode of administration, the nature and quantity of the antigen employed, its mode of administration and the species of animal used. Results of studies by Currey (22) comparing the effects of these three drugs on the primary antibody response to sheep erythrocytes in the rat were very similar, i.e., cyclophosphamide had the best therapeutic/toxic ratio, followed by methotrexate and azathioprine with the least favorable ratio.

Mackay and coworkers (25) assessed the differential effects of azathioprine and cyclophosphamide on humoral antibody production in patients using flagellin as a test antigen. Their results demonstrated a significant suppression of humoral antibody to flagellin in cyclophosphamide-treated patients while the results for azathioprine-treated patients did not differ significantly from those for nontreated control patients.

Santos and Owens (26) made a comparative study of cyclophosphamide, 6-MP and methotrexate, given at maximally tolerated doses, on the primary agglutinin response in rats immunized with sheep erythrocytes (Figure 9).

Figure 9

Primary Agglutinin Response in Rats



from Santos and Owens(26)

Antigen was given before, at the beginning or in the middle of a five-day course of treatment. They found that methotrexate and 6-MP were immunosuppressive only when the antigen was administered from two days before to the second day of the five-day course of drug treatment. Cyclophosphamide was found to be immunosuppressive when the antigen was given from two days before to even two days after the end of the five-day course of therapy.

Thus, the antimetabolites 6-MP and methotrexate appeared to suppress mainly the intermediate stage of the primary response, presumably the cellular proliferative phase, while cyclophosphamide appeared to suppress all stages. Sterzl (27) also has shown that 6-MP works only on the so-called inductive phase. In our laboratory, we have shown (28) that cyclophosphamide decreased both Coombs' antibody titer and amount of γ-globulin deposition in the glomeruli of NZB mice, while 6-MP had neither effect (Table 4). This has recently been confirmed by Hahn and coworkers (151).

Table 4

Differential Effect of Cyclophosphamide and 6-MP on Immunologic Features in NZB Mice (28)

	Cyclophosphamide	6-MP
Coombs Titer	Decrease	No effect
Deposition of γ-globulin in kidney	Decrease	No effect

Dr. James Gilliam (29) has recently demonstrated that cyclophosphamide treatment abolishes the "fluorescent band test" in the dermal-epidermal junction of the skin of NZB/NZW FI hybrid mice while 6-MP therapy is unsuccessful. Several workers (30,31) have shown that cyclophosphamide can decrease anti-DNA antibodies, both in NZB mice and man. These data suggest that cyclophosphamide can inhibit an ongoing immune response whereas 6-MP cannot. This is of course the situation that we face in the treatment of patients, since their immune responses are well established at the time that we treat them. It is of interest in this regard that Dr. Gilliam (32) (Fig. 10) has also demonstrated that in patients with systemic lupus erythematosus (SLE), cyclophosphamide treatment converts the skin fluorescent band test to negative whereas steroid

therapy alone is not effective. This is of importance because he has also demonstrated (33) a good correlation between a positive band test and the presence of clinical or biopsy-proven renal disease and hypocomplementemia, suggesting that the positive band results from deposition of circulating immune complexes in the skin in the same way that they are deposited in the kidney.

Studies have been done by Uyeki (34) in which the effect of the three drugs on the production of spleen hemolysin plaque-forming cells was measured in mice (Table 5).

Table 5

Action of Immunosuppressants on the Primary Immune Response to Sheep RBC in Mice

Treatment	Per cent of control			
	Before Antigen Injection		After Antigen Injection	
	Increase	Decrease	Increase	Decrease
Amethopterin, 100 mg/kg	163	---	---	21
6-Mercaptopurine, 120 mg/kg	161	---	---	15
Cyclophosphamide, 200 mg/kg	---	1	---	1

from Uyeki (34).

When they were administered *prior* to the antigen, the numbers of plaque-forming cells were increased by methotrexate and 6-MP but markedly suppressed by cyclophosphamide. When the drugs were given after antigen injection, there was suppression by all three drugs, but cyclophosphamide produced the greatest decrease. In earlier experiments along the same lines, Chanmougan and Schwartz (35) showed that 6-MP treatment can cause enhancement of antibody production when antigen is administered five days after the last dose of a one-week course of 6-MP. They suggested that this enhancement was due to the adjuvant effect of nucleic acids released from cells killed by the drug. Such an effect could possibly explain the deterioration seen in patients in whom azathioprine has been discontinued (153).

D. Effects on Cellular Immunity

Studies of the effects of these drugs on cellular immunity have shown that all three have in some circumstances depressed cellular immunity. However, comparative studies have tended to show a greater effect on the part of cyclophosphamide (Table 6).

Table 6

Effects on Cellular Immunity

Author	Results		
	CYCLOPH	AZA or 6-MP	MTX
Maguire and Maibach (36)	↓DNCB	No Change	---
Arinoviche and Loewi (37)	↓↓PPD	↓PPD	---
Zweiman and Phillips (38)	---	↓PPD skin test Lymphocytes respond normally to PPD	---
Winkelstein (23)	↓↓PPD	↓PPD	↓↓PPD
	Depletion of Thymic-dependent areas of lymph nodes	No effect	No effect
	↓↓PHA	No effect	No effect
Levy and Coworkers (39)	↓Ig synthesis	↓Ig synthesis	---
	↓Lymphocyte response to mitogen	No effect on lymphocyte response	---
Tripathy and Mackaness (40)	↓passive transfer of cellular immunity	No effect	Partial effect

For example, Maguire and Maibach (36) found that cyclophosphamide, but not 6-MP, delayed dinitrochlorobenzene (DNCB) responses. Arinoviche and Loewi (37) demonstrated a greater depression of the tuberculin reaction with cyclophosphamide than with azathioprine. Winkelstein (23) has shown that while both cyclophosphamide and methotrexate were more effective than 6-MP in suppressing a PPD skin test in guinea pigs, only cyclophosphamide depleted thymic-dependent areas of the lymph nodes. An *in vitro* PHA response was likewise inhibited only by cyclophosphamide. Zweiman and Phillips (38) showed that even when high doses of 6-MP had suppressed the tuberculin skin test, the animals' lymphocytes responded normally to *in vitro* stimulation by tuberculin, suggesting that what they observed was an antiinflammatory effect. Levy and coworkers (39) reported that while both azathioprine and cyclophosphamide suppressed immunoglobulin synthesis in patients, only cyclophosphamide suppressed the response of lymphocytes to mitogen. Tripathy and Mackaness (40) have shown complete suppression of passive transfer of cell-mediated immunity with cyclophosphamide and little effect with methotrexate or azathioprine. The donor lymphoid cell was shown to be a resting cell which was sensitive to cyclophosphamide.

With respect to tolerance production, Kovacs and Steinberg (41) have shown that tolerance is much easier to achieve in mice treated with cyclophosphamide than with azathioprine or methotrexate.

E. Effects on Cell Populations (Table 7)

In our laboratory (28) we have demonstrated that in NZB mice, cyclophosphamide primarily decreases the numbers of recirculating small lymphocytes in the blood, whereas 6-MP preferentially reduces the numbers of polymorphonuclear leukocytes and large mononuclear cells. In more recent studies (42), we have noted eventual total depletion of small lymphocytes in the majority of our patients on cyclophosphamide. Winkelstein (23) has also demonstrated a marked decrease in blood lymphocytes with cyclophosphamide treatment but no effect with either 6-MP or methotrexate. Very recently, we (43) have demonstrated that in patients on cyclophosphamide, both the bone marrow derived or B-lymphocytes and thymus-derived or T-lymphocytes are decreased. These decreases in total numbers of B and T lymphocytes occur in a parallel manner. This data could, of course, explain the fact that cyclophosphamide decreases both humoral and cellular immunity. Others (44,45) have also demonstrated depletion of B cell

compartments in animals on cyclophosphamide. Finally, Petrov and coworkers (46) have also shown that cyclophosphamide has a selective lymphotoxic action, while 6-MP and azathioprine had only mitostatic properties and no effect on non-dividing lymphoid cells.

Table 7

Differential Effect of Cyclophosphamide and 6-MP on WBC

	Cyclophosphamide	6-MP	MTX
Lemmel, Hurd, Ziff (28)	Decrease of Small and Medium Mononuclear Cells	Decrease of PMN and Large Mononuclear Cells	---
Hurd, Ziff (42)	Depletion of Small Lymphocytes	---	---
Winkelstein (23)	↓↓Lymphocytes in Blood and Exudate	No effect	No effect
Petrov and Coworkers (46)	Killing of Lymphoid Cells	Mitostatic Effect No Effect on Lymphoid Cells	---
Hurd, Ziff (43)	Depletion of B and T Lymphocytes	---	---

F. Other Effects

In graft versus host disease in mice, cyclophosphamide suppressed the disease and prevented mortality while neither methotrexate nor 6-MP could suppress the disease (47). However, Storb and coworkers (48) compared methotrexate and cyclophosphamide treatment in canine graft-versus-host disease and found methotrexate to be superior.

In a study in which the effects of each of the three drugs on experimental allergic encephalomyelitis were measured (49), cyclophosphamide was much more effective in

suppression of this disease than either of the other two agents.

Dukor and Dietrich (50) demonstrated that cyclophosphamide but not azathioprine produced potentiation of suppression of anti-sheep-red-cell antibody and of homograft rejection in thymectomized mice. Thus only small doses of cyclophosphamide produced a highly significant immunological defect in thymectomized animals which was not seen in intact animals.

In a study by Sensenbrenner and coworkers (51), it was shown that both cyclophosphamide and methotrexate achieved more antibody suppression with less hematopoietic cell suppression than 6-MP. These authors also found that methotrexate and 6-MP profoundly affected actively proliferating transplanted hematopoietic and antibody-producing cells while cyclophosphamide affected all phases of the cells' proliferative cycle.

Finally, studies by Casey (52, 53) suggest that azathioprine predisposes more to the development of cancer than cyclophosphamide. However, there appears to be no doubt that cyclophosphamide can also increase the incidence of neoplasia in the NZB/NZW mouse (54).

G. Conclusions (Table 8)

Although there are many discrepancies in the literature concerning the mechanism of action of these drugs, Table 8 is an attempt to summarize their known effects. All three drugs apparently suppress the primary immune response, although the effects of azathioprine and methotrexate are highly dependent on timing. Cyclophosphamide appears to be more effective in suppressing a secondary or ongoing immune response. It is for this reason, perhaps, that cyclophosphamide decreases deposition of immune complexes in the kidney of the NZB mouse whereas 6-MP does not. Azathioprine, 6-MP and methotrexate have more profound antiinflammatory and mitostatic effects than cyclophosphamide.

Cyclophosphamide seems to have greater effects on delayed hypersensitivity with depletion of thymic-dependent areas and the passive transfer of cellular immunity than either of the other two drugs.

Finally, cyclophosphamide appears to be able to deplete small lymphocytes more effectively and produce tolerance much more easily than either azathioprine or methotrexate.

On the basis of these effects, certain differences in therapeutic indication become apparent.

For suppression of an ongoing immune response and immune complex formation such as one finds in rheumatoid arthritis and systemic lupus erythematosus, cyclophosphamide would be the drug of choice (Table 9). It would also be the drug of choice for decreasing delayed hypersensitivity mechanisms or for situations in which elimination of clones of small lymphocytes is desired, since it has a lymphopenic effect. For an antiinflammatory effect, azathioprine or 6-MP would be the best choices because these drugs, being antimetabolic agents, have their greatest effect on dividing cells. It is presumably for this reason than they are effective in diseases such as psoriasis, in which there is rapid cellular proliferation. However, they also affect the primary immune response. This may be a factor in their known effectiveness in organ transplantation.

Methotrexate appears to have both immunosuppressive and antiinflammatory properties which, in general, resemble those of azathioprine.

Table 8

Summary of Effects of Immunosuppressive Drugs

	Cyclophosphamide	Azathioprine & 6-MP	Methotrexate
↓Primary Immune Response	++	++	++
↓Secondary Immune Response	++	±	+
↓Immune Complexes in NZB Mice	++	0	0
Antiinflammatory Effect	+	++	+
Mitostatic Effect	+	++	++
↓Delayed Hypersensitivity	++	+ ?Antiinflammatory	+
Suppression of Passive Transfer of Cellular Immunity	++	±	±
Lymphopenia	++	±	±
Tolerance Induction	++	+	+

Table 9

Summary of Differential Effects

Cyclophosphamide	↓Ongoing Immune Response ↓Immune Complexes ↓Delayed Hypersensitivity ↓Small Lymphocytes Production of Tolerance
Azathioprine and 6-MP	Antiinflammatory effect ↓Primary immune response
Methotrexate	Some immunosuppressive properties but primarily antiinflammatory

IV. Toxicity

The most common type of toxicity is, of course, that due to these drugs' effects on the bone marrow and hematopoiesis. All are capable of inducing bone marrow suppression (14). The degree of marrow suppression is usually dose related and can be modulated by dose changes, although they do rarely cause idiosyncratic and irreversible marrow failure. In Table 10, an attempt is made to summarize the differential toxic effects of the three agents.

Table 10

Relative Frequencies of Unwanted Drug Effects

	Cyclophosphamide	Azathioprine	Methotrexate
Oral ulcers	-	-	+
GI intolerance	+	-	+
Hair loss	++	-	+
Cystitis and bladder fibrosis	++	-	-
Infection	+	+	+
Teratogenesis	+	+	+
Hepatic damage	-	+	++
Aspermia	++	-	+
Anovulation	++	-	-
Neoplasia	+	++	+

from Decker (55)

Methotrexate can cause difficulties throughout the gastrointestinal tract, including painful oral ulcerations. With higher doses ulcerative lesions of the esophagus, stomach and small bowel occur and may produce a hemorrhagic enteritis. Nausea, vomiting and occasionally diarrhea may be seen even in the absence of apparent ulcerative lesions. Nausea and, more rarely, vomiting, may complicate oral cyclophosphamide administration but, in controlled trials, the frequency of these results is only slightly greater than in placebo controls (55).

Hair loss occurs often with cyclophosphamide and more rarely after treatment with methotrexate (55,86). Total alopecia is uncommon and regrowth invariably occurs upon drug withdrawal and usually, to some degree, even when the drug is continued (55).

A second and important side effect of cyclophosphamide is

cystitis produced by one of the breakdown products of the drug in the urine (15,56-59). Acute cystitis may occur with or without bleeding and may be associated with abnormal cytology (56,60). With time, the drug may slowly produce bladder fibrosis and telangiectasia of the mucosal wall (61). Uncontrollable bleeding from the bladder may occur months after the drug has been stopped. Bladder fibrosis of this type may prove to be a seriously disabling complication. Transitional cell carcinoma of the bladder has been reported in cyclophosphamide-treated patients (62).

To minimize bladder exposure to the irritant, one should encourage large fluid intakes and have the patient empty his bladder during the early morning hours of the night.

The risk of infection is increased in patients on these agents, presumably because of suppression of the immune mechanism (63).

Ordinary virus infections such as measles (64) and varicella (65,66) may disseminate with fatal outcome but, more likely, the treated patient seems to respond quite normally. The incidence of herpes zoster seems increased but again, the course of the lesion seems much as it is in otherwise normal individuals. An increased susceptibility to virus infections could possibly be related to the fact that these drugs may inhibit interferon production, as has been shown for cyclophosphamide (67).

Tuberculous and fungus infections are much more likely to disseminate on these drugs than in the untreated individual (68,69).

There are many reports of exotic infections such as cytomegalovirus (69) or *Pneumocystis carinii* (70) and fungal infections such as Candida and Aspergillus (68,69).

Teratogenic effects have been observed with each of these agents, but mothers receiving these agents have also borne normal children (71-74). The appropriate delay between stopping drug and permitting conception is of uncertain duration; 4 months is sometimes suggested. Chromosomal changes have been described (75).

Hepatic damage is a serious and limiting factor in the chronic administration of methotrexate, whereas azathioprine occasionally produces changes in liver chemistries which do not seem to persist. Hepatitis and fibrosis result from methotrexate therapy, and these are clearly related to total dose and duration of administration (76), whereas azathioprine or mercaptopurine hepatotoxicity includes features of both intrahepatic cholestasis and parenchymal cell necrosis either of which may

predominate (77-79). Irreversible liver damage has been reported with both azathioprine and 6-MP (77-79).

The effects, particularly of cyclophosphamide and other alkylating agents, upon gonadal tissues must be of major concern in younger patients (80-83). Ovulation stops, perhaps permanently, in a substantial proportion--probably 20 to 30%--of women maintained on effective doses of cyclophosphamide (2 mg/kg/day) (55). Recent evidence from semen analyses, testicular biopsies and serum FSH levels, shows even more profound effects on spermatogenesis, with permanent aspermia in men who receive as little as six months of treatment (81). The mechanism of the destruction of ova is unclear. It is of interest that neonatally thymectomized mice develop identical ovarian pathology (84). This raises the possibility that cyclophosphamide affects the ovary indirectly via its effect on the thymus and cellular immunity.

Probably the major concern with these agents is that of induced malignancy (85). Since one of the roles of the immune system is presumably surveillance for and eradication of neoplastic clones, malignancies are to be expected in patients on immunosuppressive drugs. Results of several studies are shown in Table 11.

Table 11

Incidence of Neoplasms in Patients on Immunosuppressive Drugs

Disease	No. Cases	Incidence (%)			Ref.
		Cyclophosphamide	Azathioprine	Methotrexate	
Rheumatoid Arthritis	2/108	2%			(86)
Psoriatic Arthritis	7/204			3.4%	(87)
Transplant Patients					(88,89
incl. skin tumors			6%		
excl. skin tumors			3.8%		
Non-transplant	3/4000		0.07%		(91)
Normal Incidence			0.06%		(85)

In 108 patients with rheumatoid arthritis treated with cyclophosphamide, two developed malignancies, one with reticulum

cell sarcoma and the other with chronic lymphatic leukemia (86). In 204 patients with psoriatic arthritis treated with methotrexate, seven patients developed neoplasms (87). In the studies by Penn and coworkers of the Denver kidney transplantation experience, the occurrence of neoplasms was 6% if skin tumors were included, and if excluded, the incidence was about 3.8% (88,89). Patients were treated with azathioprine and high dose steroids. The expected tumor occurrence in the general population at a comparable age range is 0.06% (85). A particularly worrisome and peculiar lesion has occurred in the transplant group; in a report by Penn and coworkers, 22 cases of lymphoma, of which 11 were intracerebral, occurred in a group of 5,000 recipients of kidney grafts (90). The foreign tissue in these patients presumably acts as a chronic antigenic stimulus and this, as well as the adrenal corticosteroids given to all of the patients, may be contributing to the development of the tumors. A slightly encouraging survey of 4,000 non-transplant patients treated with azathioprine revealed only three cases of neoplasm for an incidence of 0.07%, or approximately the normal expected incidence (91). It is of interest that in the NZB/NZW FI mouse, 6-MP appears to be more likely to induce tumors than cyclophosphamide (52,53).

A very recent study from the American College of Surgeons Human Renal Transplant Registry (92) reports that among 6,297 kidney-transplant recipients, the risk of developing lymphoma was 35 times higher than normal and was derived almost entirely from a risk of reticulum-cell sarcoma, which was 350 times greater than expected. Skin and lip cancers occurred four times more often than expected and other cancers, mostly soft-tissue sarcoma and hepatobiliary carcinoma, were 2.5 times more common. Other studies (93,94) have also demonstrated the increased incidence of skin cancer in immunosuppressed patients.

Finally, a number of unusual and peculiar side-effects and reactions have occurred in patients taking these agents, e.g., meningitic reactions (95), fever, rash and headache (96), and acute renal insufficiency (97) with azathioprine; pulmonary infiltrates with methotrexate (98,99); and pigmentation of the nails and teeth with cyclophosphamide (100,101).

V. *Efficacy of Immunosuppressive Drugs in Rheumatic Diseases*

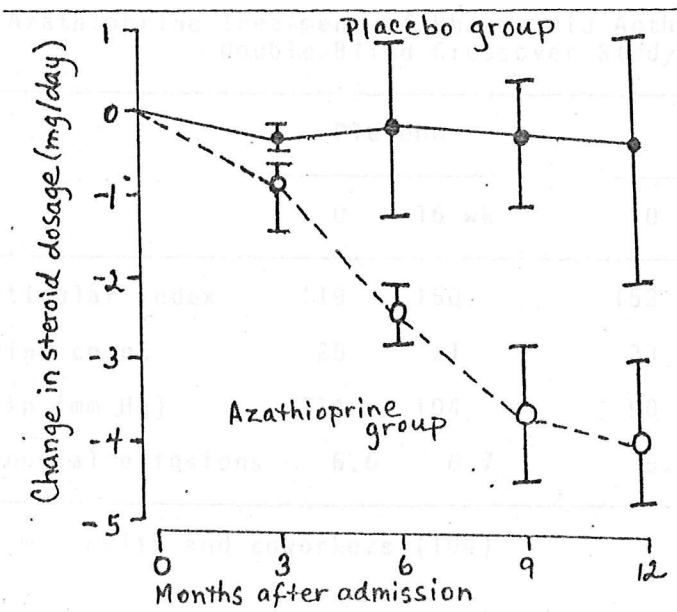
A. General Considerations

The evidence that immunosuppressive drugs may be useful in the therapy of connective tissue diseases will be summarized. Most of the discussion will be confined to two drugs--cyclophosphamide and azathioprine--because these are the two about which there is sufficient controlled information. For the same reason, the discussion will primarily be confined to two diseases: rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). By a controlled trial is meant a study in which patients are admitted to the study on the basis of fixed criteria, randomized into treatment groups and then evaluated by objective methods.

B. Results of Controlled Studies--RA and SLE

The results of some of the controlled trials are summarized in Table 17. In a study by Mason and coworkers (102) in England, patients with severe RA requiring prednisone therapy were randomized to receive either a placebo or azathioprine and they were then followed for 12 months. There was a constant attempt to reduce the steroid dose without increasing disease activity. At the end of the year, it was found that the group who had received azathioprine had a significant reduction in steroid requirement as compared with the placebo group (Figure 11).

Figure 11



from Mason et al (102)

In a study by Levy and coworkers (103), azathioprine given for 6 months was significantly better than placebo in reducing the active joint count, improving grip strength and reducing morning stiffness (Table 12).

Table 12

A Double-Blind Controlled Evaluation of Azathioprine Treatment in Rheumatoid Arthritis and Psoriatic Arthritis

		Placebo (Mean±SE)			Drug (Mean±SE)			
Disorder	Study	Start	6 mos.	Δ	Start	6 mos.	Δ	*p*
Rheumatoid arthritis	Active joint count	11 ± 3	16± 3	+ 5	17± 3	6± 2	- 10	<.01
	Grip strength(mm Hg)	111 ± 13	108±11	- 3	92±12	135±16	+ 44	<.05
	Morning stiffness(min)	106 ± 34	211±52	+105	188±48	69±35	-114	<.00
Psoriatic arthritis	Active joint count	17 ± 6	17± 6	0	18± 5	7± 2	- 11	<.01
	Grip strength(mm Hg)	140 ± 32	134±35	- 6	140±20	159±27	+ 55	<.05
	Morning stiffness(min)	40 ± 34	65±38	+ 25	90±44	10±10	- 80	<.05

from Levy and coworkers (103)

In a similar study by Urowitz and coworkers from Toronto (104), azathioprine was significantly superior to placebo in reducing the articular index, reducing the joint count and improving the grip strength after 16 weeks of treatment. There was also a reduction in synovial effusions in the azathioprine group, but this was not statistically significant (Table 13).

Table 13

Azathioprine Treatment of Rheumatoid Arthritis (RA) -- Double-Blind Crossover Study

	Placebo		Drug	
	0	16 wk	0	16 wk
Articular index	119	150	153	110
Joint count	25	31	33	23
Grip (mm Hg)	114	104	90	132
Synovial effusions	6.6	6.7	6.9	3.2

from Urowitz and coworkers (104)

High doses of cyclophosphamide have also been found effective in reducing the symptoms of RA. In the 32-week study of the Cooperating Clinics Committee of the American Rheumatism Association, high doses (100-150 mg/day) of cyclophosphamide were compared with low or placebo doses (5-15 mg/day) (105). In the high-dose group, there was a significant improvement in grip strength, significant reduction in the number of painful joints, number of swollen joints and a significant reduction in the time to walk 50 feet. There was a one-hour reduction in morning stiffness, but that was not quite statistically significant. There was no change in the erythrocyte sedimentation rate. More of the high-dose patients had reductions of rheumatoid-factor titer and of serum IgG than did the placebo group. Of considerable importance was the finding that x-ray erosions were markedly reduced in the high-dose group as compared with the placebo group (Table 14).

Table 14

Number of Joints with New or Worse Erosions

Group	No. of Joints Evaluated	Joints Worse on Initial Film		Joints Worse on Final Film	
		Observer A	Observer B	Observer A	Observer B
Low-Dose (5-15 mg)	864	2	2	41	25
High-Dose (100-150 mg)	648	0	0	3	2

from Cooperating Clinics Committee of American Rheumatism Association (105)

Townes and coworkers have confirmed the findings of the CCC study (106). They found that cyclophosphamide, in high dose, when compared with placebo, significantly decreased morning stiffness, increased grip strength and decreased the number of painful and the number of swollen joints. They also found a significant reduction in time to walk 50 feet (Table 15).

Table 15

Controlled Trial of Cyclophosphamide in Rheumatoid Arthritis (RA): An 18-month Double-Blind Cross-over Study

Treatment	No. of patients	Morning stiffness	Grip strength R	Grip strength L	Painful joints (No.)	Swollen joints (No.)	50 Ft. Walk
Drug	11	-65.9	43.9	39.6	-14	-13.4	-5.5
Placebo	11	6.4	6.6	6.6	1	0.36	1.2

from Townes, Sowa and Shulman (106)

However, it appears that the efficacy of cyclophosphamide in RA is dose-dependent. A study by Lidsky and coworkers demonstrated that when lower doses of cyclophosphamide, 50 mg/day, or about 0.7 mg/kg/day, were given for one year, there was no difference when compared with placebo with regard to grip strength, fist formation, ring size, joint swelling or x-ray progression (107). No difference was detected in immune reactivity of the two groups. A similar result was found by the Cooperating Clinics Committee in a second study which was done to compare high and low dose regimens (108). With low doses of cyclophosphamide (up to 75 mg/day) they found no significant improvement as compared with placebo (Table 16).

Table 16

A Controlled Trial of High and Low Doses of Cyclophosphamide in 82 Patients with Rheumatoid Arthritis

Median improvement	Previous trial dose	This trial dose		Previous trial
	≤ 150	≤ 150	≤75	Controls *
Morning stiffness(hr)	1	1	.5	0
Grip strength(mmHg)	21.5	16.35	4.4	-6.7
Painful joints (No.)	10	11	5	-1.5
Swollen joints (no.)	8.0	7.5	3	-1.0
50-Foot walk(sec)	1.4	2.5	1	-0.7

*deterioration

from The Cooperating Clinics Committee of The American Rheumatism Association (108)

This contrasted with their previous good results with high doses of cyclophosphamide, between 100 and 150 mg/day (105). The toxic side effects of the drug were almost as great at the low dose as they were with the high.

In summary, the controlled trials of azathioprine and cyclophosphamide in the therapy of severe RA suggest that both drugs, when given in sufficient quantity, are capable of suppressing disease activity. However, toxic effects of these drugs have also been observed.

Table 17 also summarizes results from controlled studies of systemic lupus erythematosus. A study by Sztejnbok and coworkers from Brooklyn compared azathioprine with control, untreated patients in the therapy of SLE (109). The creatinine clearance increased in the azathioprine-treated group but not in the control group. Proteinuria was decreased on the average of 0.75 g/day in the azathioprine group. There was little change in the control group. Patients receiving azathioprine required statistically less prednisone (5.8 mg/day) as compared with controls (14.7 mg/day). Furthermore, rehospitalizations were markedly reduced in the azathioprine group. Finally, there was no increased tendency to develop infection and there was a decreased mortality in the azathioprine-treated group. Discontinuation of the azathioprine was associated with severe exacerbations of disease. Toxicity was minimal. A study of SLE nephritis by Donadio and coworkers from the Mayo Clinic (110) compared an average dose of prednisone (49 mg/day) with that dose of prednisone plus azathioprine (2 to 3 mg/kg/day). They studied the changes after six months of therapy. Creatinine clearances were either stable or improved in both groups. Proteinuria improved in two of three prednisone-treated patients. Complement improved in three of five in each group. There was one significant infection and that occurred in a patient receiving prednisone plus azathioprine. Their conclusion was that no benefit was obtained by the addition of azathioprine to therapy with prednisone. In a very recent report, Cade and coworkers (111) demonstrated that azathioprine was very effective in treatment of SLE proliferative nephritis as compared with prednisone therapy. However, if azathioprine was given with either prednisone or heparin, it was even more effective. Complications with prednisone therapy were higher than those with azathioprine. Steinberg and coworkers have compared the use of cyclophosphamide versus azathioprine versus placebo in the therapy of lupus nephritis (112) (Table 18). There was little difference in creatinine clearance among the three groups. Proteinuria was reduced by both cyclophosphamide and azathioprine but the urine sediment improved primarily in the cyclophosphamide-treated group. Serum complement was increased more by cyclophosphamide than by azathioprine. Anti-DNA antibodies were decreased equally by cyclophosphamide and azathioprine. Patients

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Table 18

Controlled Trial of Azathioprine, Cyclophosphamide or Placebo in Lupus Nephritis

Measure	Results*					
	Azathioprine (N=6)		Cyclophosphamide (N=8)		Placebo (N=4)	
	+	-	+	-	+	-
Creatinine clearance	3	2	3	1	4	3
Urine sediment	2	2	6	0	3	4
Proteinuria	1	0	2	1	0	5
Serum complement	1	1	3	0	2	1
Anti-DNA antibodies	4	0	4	0	2	0
Extrarenal disease	0	1	0	0	0	6
Net change	+5/36		+16/48		-8/66	

from Steinberg and coworkers (112) * + = improvement
- = deterioration

receiving cyclophosphamide did significantly better than the placebo-treated group. Azathioprine, however, was not statistically better than placebo. Toxicity appeared to be equal with cyclophosphamide and azathioprine. In the first NIH study, cyclophosphamide was compared with placebo in a double-blind trial in a 10-week, in-hospital program (113). There was no difference between cyclophosphamide and placebo with regard to creatinine clearance changes. However, patients with relatively stable creatinine clearances were selected for study. Proteinuria was reduced in the cyclophosphamide group as were red cells in the urine sediment. Anti-DNA antibodies decreased and the serum complement increased in patients receiving cyclophosphamide. This study suggested that cyclophosphamide was useful in the therapy of lupus nephritis. However, the study was short-term with a relatively small number of patients.

Finally, in a low-dose cyclophosphamide study, Fries and coworkers (114) observed no improvement in treated SLE patients. Thus, both azathioprine and cyclophosphamide appear to be effective in RA if doses are high enough. In SLE, cyclophosphamide appears to be more consistently effective than azathioprine. Thus, the controlled studies completed to date suggest that both azathioprine and cyclophosphamide

are potentially effective adjuncts to therapy, but that more long-term information is necessary to determine whether the beneficial effects outweigh the toxic ones.

A number of additional *uncontrolled* studies also generally claim a beneficial effect with both azathioprine and cyclophosphamide in both RA (115-117) and SLE (118-127). An excellent recent review of the use of these agents in RA is that by Currey (128).

C. Efficacy in Other Rheumatic Diseases

There appears to be little doubt that these agents (particularly azathioprine, 6-MP and methotrexate) are very effective in treatment of the classical type of psoriatic arthritis as defined by Wright and Moll (129). Two controlled studies demonstrating improvement have been published, one using methotrexate (130) and the other azathioprine (103). We have also observed striking improvement in all of 11 patients with classical psoriatic arthritis who were treated with 6-MP (131).

Although no controlled studies have been reported, the beneficial effects of these agents have been published for polymyositis (132,133,152), juvenile rheumatoid arthritis (134), Behcet's syndrome (135), Reiter's syndrome (136-139), Wegener's granulomatosis (140-144) and scleroderma (145).

D. Guidelines for Use of Cytotoxic Drugs in Rheumatic Disease

Guidelines for the use of cytotoxic drugs in the rheumatic diseases set forth by Schwartz and Gowans (146) are abstracted in Table 19.

Table 19

Guidelines for Use of Cytotoxic Drugs in Rheumatic Diseases

1. Life-threatening or seriously crippling disease
2. Reversible lesions must be present
3. Failure to respond to conventional therapy or intolerable side effects
4. No active infection
5. No hematologic contraindication
6. Meticulous follow-up
7. Objective evaluation must be used
8. Informed consent
9. Protocol must be submitted for peer review

from Schwartz and Gowans (146)

It is apparent that more controlled trials and long-term follow-up are necessary before a final decision can be made concerning the use of immunosuppressive drugs in the treatmetn of patients with connective tissue diseases. Future studies may demonstrate combined therapy to be superior to use of single agents without increased toxicity (111,147,148, 150).

VI. Summary

The use of immunosuppressive drugs for the treatment of various types of arthritis and the connective tissue diseases has increased over the past several years. Three major types of antimetabolites have been the primary agents used. They are (1) alkylating agents, particularly cyclophosphamide (Cytoxan); (2) purine analogues such as 6-mercaptopurine or 6-MP (Purinethol) and azathioprine (Imuran) which is a derivative of 6-MP and (3) folic acid antagonists, e.g., methotrexate. The differential mechanisms of action of these three groups of drugs have been discussed. In general, they all exert antiinflammatory effects and decrease both humoral and cell-mediated immunity. However, cyclophosphamide appears to be the best agent for killing small lymphocytes, decreasing circulating immune complexes, decreasing a secondary,or ongoing, immune response, decreasing cellular immunity and producing tolerance. The remaining drugs, 6-MP, azathioprine, and methotrexate, have more potent antiproliferative and antiinflammatory effects and, while able to decrease a primary immune response, have little effect on a secondary immune response. These agents kill predominantly monocytes and the large lymphocytes.

For the above reasons, cyclophosphamide should be the drug of choice for treatment of diseases with circulating immune complexes or deposition of complexes such as one finds in systemic lupus erythematosus or rheumatoid arthritis.

The other agents are most indicated for their antiproliferative effects, as in treatment of psoriasis and psoriatic arthritis.

Although the clinical results have, in general, been encouraging, the incidence of harmful drugs side effects have mitigated against widespread usage of these agents.

The differential toxicity of these agents has been discussed. In general, the major side effects include bone marrow toxicity, increased risk of infection of all types, possible increased risk of teratogenesis, increased incidence of sterility (particularly with cyclophosphamide), liver damage (with 6-MP and methotrexate) and hemorrhagic cystitis and urinary bladder fibrosis (with cyclophosphamide) and finally, the possibility of increased incidence of malignancy.

The use of these agents should be restricted for those patients with life-threatening disease such as systemic lupus erythematosus with renal disease or life-crippling disease such as a patient with severe chronic disabling rheumatoid arthritis who has failed to respond with conventional means of therapy.

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