

SUPPRESSION OF MITOGENIC LECTIN INDUCED BLAST
TRANSFORMATION OF HUMAN PERIPHERAL BLOOD
MONONUCLEAR CELLS BY
PYRIMETHAMINE

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OBJECTIVE : To investigate the effect of the antimalarial drugs on human lymphocytes response to plant mitogens and allogeneic cell surface antigens.

BACKGROUND : In recent years a combination of the drugs sulfadoxine and pyrimethamine (S-Py), has been widely used both for malarial chemotherapy in areas where *Plasmodium falciparum* is resistant to chloroquine, and for long term prophylaxis of individuals working in highly endemic malarious area.

Although other anti-malarial compounds such as primaquine (Thong, et al, 1978), chloroquine (Forsdyke, 1975), quinine (Gold and Ben-Efraim, 1978) and mefloquine (Thong, et al., 1979) have been shown to have immunosuppressive properties, little is known about the consequences of sulfadoxine and pyrimethamine on human immune response.

It has been reported that pyrimethamine adversely effects hematopoiesis in humans when the drug is taken daily for extended periods of time (Myatt, et al., 1953). Thus, the possibility exists that S-Py taken for prolonged prophylactic purposes may adversely effect an individual's immune competence. Immunosuppression due to S-Py would be undersirable both in the clinical treatment of malaria, since malaria chemotherapy requires a minimal immune response for the elimination of malaria parasites (Cohen, 1974; Hamburger & Kreier, 1975), and also in the chemoprophylaxis against malaria since individuals on S-Py may be at greater risk to other infectious diseases. Recently, however, pyrimethamine has been reported to have a stimulatory effect on both B and T cell responses in mice (Thong, et al., 1980) and on human lymphocytes response to the purified protein derivate of tuberculin (PPD) and pokeweed mitogen (PWM) (Bygbjerg, 1981). These results are unexpected since pyrimethamine is a known folic acid antagonist which blocks the reduction of dehydrofolate to tetrahydrofolate resulting in the inhibition of nucleic acid synthesis of parasites, and to a lesser degree of human cells

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(Hitchings, 1978). However, there is the possibility that pyrimethamine effects folic acid reduction differently in parasites than in the host lymphoid tissue. We have therefore examined the effect of sulfadoxine and pyrimethamine, and of sera from individuals on long-term S-Py chemoprophylaxis on the responsiveness of peripheral blood mononuclear cells (MNC) from normal individuals in an effort to determine if the drugs have any detectable immunosuppressive or immunoenhancing properties. This study used both the mitogenic-lectin induced human lymphocyte blast transformation assay and the one way mixed lymphocyte reaction (MLR) which are considered *in vitro* correlates of cellular immune responsiveness.

MATERIALS AND METHODS :

Lymphocytes : Peripheral blood MNC obtained from 13 healthy volunteers were isolated from heparinized blood as previously described (Wells, et al., 1979). Briefly, whole blood was diluted 1:2 in Hanks balanced salt solution (HBSS) (GIBCO Laboratories, Grand Island, N.Y.) and the MNC isolated by Ficoll-Hypaque centrifugation (Boyum, 1968).

After three washes in HBSS the isolated peripheral blood MNC were adjusted to a concentration of 5×10^6 cells per ml in assay medium RPMI 1640 (GIBCO), containing 2 mM glutamine, 50 U penicillin, 50 ug/ml streptomycin, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer (HEPES) and 20% heat-inactivated fetal bovine serum (FBS). Cell viability was determined using the eosin dye inclusion technique. Viability was greater than 95% in all specimens.

Serum : Sera from individuals on S-Py chemoprophylaxis were kindly provided by Dr. David Johnson, Epidemiology Department, AFRIMS. Individuals serum samples were obtained from adult Thai volunteers in Ban Tablan, Prachinburi Province, Thailand, who were administered 2 tablets of S-Py (a tablet includes sulfadoxine 500 mg and pyrimethamine 25 mg) per week for 26 weeks. Three different serum samples were obtained from each volunteer; the first immediately before S-Py chemoprophylaxis began, the second and third after the volunteers had been on chemoprophylaxis for 5 and 25 weeks, respectively. The samples were obtained from a study of S-Py prophylaxis started by Elliot Pearlman and a description of the study population has been reported previously (Pearlman, et al., 1977). Quantitation of drug levels in volunteers was not done but drug ingestion during the study was individually supervised.

Pyrimethamine and sulfadoxine solutions : Pyrimethamine and sulfadoxine were kindly supplied by Drs. Scholar and Rickle (F. Hoffman-La Roche & Co.), with the following instructions for preparation of stock solutions :

A 10^{-4} M stock solution of pyrimethamine (2, 4-diamino-5-chlorophenyl-6-ethylpyrimidine), MW 248.7, was prepared by suspending 6.23 mg of the pyrimethamine in 0.5 ml H₂O, then forming the salt by adding, under constant stirring, approximately 1.03 ml 0.1 N HCl to reach a final pH of 4. Distilled water was added to give a final volume of 250 ml and the solution

was filtered through a 0.2 μ m membrane filter (Millipore, GS) and then autoclaved for 30 min at 120°C.

A 10^{-3} M stock solution of sulfadoxine, MW 310.3, was prepared by suspending 77.56 mg of sulfadoxine in 0.5 ml H₂O before the salt was formed by adding, under constant stirring, approximately 2.1 ml 0.1 N NaOH to reach a final pH of 7. Distilled water was added to make a final volume of 250 ml and the solution was filtered and then autoclaved as described above.

Lectin-induced mitogenesis : The method used for analysis of lectin-induced blast transformation has been reported (MacDermott, et al., 1978). In brief, MNC were adjusted to 1.0×10^6 cells per ml in assay medium, and 1.0×10^5 cells (0.1 ml) were added to each well of flat-bottomed microtiter plates (Costar, Cambridge, Mass). All dilutions were made in assay medium containing 20% heat-inactivated FBS. The optimal stimulatory concentration for each lectin was determined in separate experiments and used throughout the study: mitogens were added at a final concen of 5 μ g of phytohemagglutinin (PHA; Calbiochem, La Jolla, Calif.) per ml, 2 μ g of concanavalin A (Con A; Calbiochem) per ml, or 25 μ g of pokeweed mitogen (PWM; GIBCO) per ml in a .025 ml volume (the volume of medium in which the lectins were added to the test wells was increased to 0.1 ml in the prophylaxis serum experiments, in order to maintain a final 20% (v/v) test serum concentration in the respective wells). The test wells then received 0.05 ml of the respective concentration of pyrimethamine or sulfadoxine while the control cultures received 0.05 ml assay medium. To assess the effect of serum from individuals on S-Py chemoprophylaxis on *in vivo* MNC responsiveness 0.05 ml of the individuals' serum, obtained after 5 and 25 weeks of S-Py chemoprophylaxis, was added to the test wells. Serum obtained before prophylaxis was started was used for comparison. MNC were incubated at 37°C in a 5% CO₂ - 95% O₂, humid environment. The cultures were then pulsed by addition of 0.4 μ Ci of tritiated thymidine ((methyl-³H) sp. act. 20 ci/mmol., New England Nuclear, Boston, Mass.) after 72 h (for PHA and Con A) or 120 h (for PWM) incubation and harvested 24 h later. All tests were performed in triplicate. The (³H) thymidine incorporation by the cells was determined by using a multiple automated sample harvester (Microbiological Associates, Bethesda, Md.) to impregnate filter disks with the MNC cultures and then counting the radioactivity on each filter in a Hewlett-Packard liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Recognition of allogeneic cell surface antigens : The methods used for the allogeneic mixed leukocyte reaction (MLR) have also been described (MacDermott et al., 1980). In brief, stimulator MNC were prepared by treating cells from one individual at a concentration of 1.5×10^6 per ml with 50 μ g of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) for 45 min at 37°C. The mitomycin C-treated MNC were then carefully washed three times in HBSS containing 5% heat-inactivated FBS and were then suspended in assay medium and adjusted to a concentration of 2×10^6 MNC per ml. Responder cells were diluted in assay medium to a final concentration of 1×10^6 cells per ml. In flat-bottomed microtiter test plates, 0.1 ml (1×10^5 cells) of responder cells and/or 0.1 ml (2×10^5 cells) of stimulator cells were added to the wells; each cell type was cultured alone

(nonspecific blastogenesis control) and with PHA (mitomycin C control). Each of the individual healthy volunteer's cells were tested against another healthy control. Lymphocytes were cultured in the one way MLR in the absence or presence of different concentrations of pyrimethamine or sulfadoxine by adding 0.05 ml of the respective drug concentration, medium and PHA (5 ug/ml) to the test cultures.

The effect of serum from the individuals on long-term S-Py chemoprophylaxis on the responsiveness of peripheral blood MNC₀ in the allogeneic MLR was tested by adding 0.05 ml of pretreatment serum or serum obtained after 5 and 25 weeks of S-Py chemoprophylaxis to the culture wells.

All tests were done in triplicate and the cells were cultured at 37°C in a 5% CO₂ - 95% air, humid environment for 120 h before being pulsed with (³H) thymidine (0.4 uCi) for 24 h. The cultures were processed for estimation of (³H) thymidine incorporation as described above.

Statistics : A log transformation of CPM was used to help stabilize variance and correct skewness. If a set of three triplicates had extremely divergent values due, presumably to laboratory error, the entire set was discarded (Grove and Gilbreath, in preparation) without attempting to identify which of the triplicates were "good" tests and which were "bad". This was to prevent bias in the mean values. If the triplicate with 10⁻⁴ M drug concentration (the highest) was bad, the entire sample was discarded regardless of the other values.

The *in vitro* effect of pyrimethamine and sulfadoxine was assessed by regressing the log CPM of isotope in the respective test wells on drug concentration; drug concentrations used were 0, 10⁻⁹ up to 10⁻⁴ M by steps of ten fold increases in molar concentrations. The effect of S-Py chemoprophylaxis on the immune responsiveness of MNC from healthy individuals was assessed by calculating within subjects paired t-tested using the log CPM for the culture wells to which previously was added serum from individuals on S-Py chemoprophylaxis. The serum was obtained before chemoprophylaxis started or after 5 or 25 weeks of S-Py chemoprophylaxis (2 tablets per week).

If a drug has no effect on the immune response being tested, then the slope of the regression of log CPM on drug concentration is as likely to be positive as negative. If the drug depresses the immune response the slope should be negative, aside from experimental variation. If the effect is strong, then even with small sample sizes one can hope to find regression coefficients (slopes) that differ significantly from zero.

RESULT :

Effect of pyrimethamine and sulfadoxine on the viability of mononuclear cells (MNC) : The effect of pyrimethamine and sulfadoxine on human peripheral blood mononuclear cells was tested by incubating the cell suspensions with the respective drug at the highest concentration used throughout the study. As shown in Table 1, the viability of the MNC in the drug treated cultures

was similar to that found in control untreated cultures. Thus, neither drug was cytotoxic to human MNC.

In vitro drug effect on blast transformation : The effect of pyrimethamine and sulfadoxine on lectin induced blast transformation and the MLR are summarized in Table 2. The only statistically significant regression coefficients are negative which is striking evidence that suppression has occurred. Out of 57 regressions, 13 or 22.8% are significantly different from zero. One would expect by chance alone $57 \times (.05)$ or 2.85 significant regressions. From the poisson distribution the probability of 13 significant test occurring when only 2.85 are expected is less than .0001.

Pyrimethamine had 22 negative regressions out of 25 tests, while sulfadoxine had only 11 negative regressions out of 32. The difference between pyrimethamine and sulfadoxine in proportion of regression coefficients that are negative is very highly significant ($\chi^2 = 16.56$, $p < .001$) when compared with an expected proportion of one half (which is expected if the sign of the slope is determined by chance). The excess number of positive slopes seen in the sulfadoxine test was not significant, which gives no reason to believe that sulfadoxine actually enhances immune reaction. On the other hand, pyrimethamine had 80% of its regression coefficients negative, which is significantly greater ($p < .001$) than the expected proportion of one half of the regression coefficients being negative.

All but two of the regressions significant at the 0.05 level occurred in the Pokeweed mitogen, PHA, and Con A tests with pyrimethamine. This group also had all eight regressions that were significant at the .01 level. Since none of the 18 regressions were positive in the PWM, PHA and Con A transformation assays when pyrimethamine was added to the culture wells, *in vitro* suppression of the immune response appears fairly consistent in this group. Since only 2 significant regressions occurred outside of this group, and since one would expect 2 or 3 significant regressions by chance alone, these data give evidence of immune suppression for pyrimethamine but not sulfadoxine, in the lectin induced blast transformation (but not MLR).

Effect of serum from individuals receiving S-Py on in vitro response of human mononuclear cell : The difference in log CPM before and after taking S-Py combination (2 tablets per week) in a group of 11 subjects was computed. Table 3 gives the mean difference and standard errors for PHA, Con A, PWM, and MLC tests. Cases with "bad" replicates were deleted, so that degrees of freedom vary. Blood was drawn before chemoprophylaxis was started and after 5 and 25 weeks of taking S-Py. In the PHA, Con A and PWM transformation assays, 5 out of the six mean differences were negative, as expected if there had been suppression. However, no test approached statistical significance, so these data provide little support for the hypothesis of serum immune suppression induced by prolonged S-Py chemoprophylaxis (as assessed by lectin-induced blast transformation and the MLR assays).

DISCUSSION : Lectin-induced blast transformation and proliferation in response to stimulation by alloantigens are generally regarded as *in vitro* correlates of immune responsiveness. In the present study pyrimethamine,

but not sulfadoxine, caused a potent suppressive effect on lectin induced blast transformation.

The mode and nature of pyrimethamine suppression *in vitro* is unclear. The viability experiments demonstrated that pyrimethamine and sulfadoxine, at the concentration used, are not toxic to the MNC when the drugs and cells are incubated together for 96 hours which is similar to that found by Bygbjerg (1981). Furthermore pyrimethamine and sulfadoxine were not acting as cell mitogens, since no increase in (³H) thymidine incorporation was observed in cells exposed to the drugs for 48 to 96 hours (data not shown).

The lack of suppression induced by pyrimethamine in the MLR would suggest that the functional capabilities of T cells, which are the principal responding cell in the MLR, are essentially intact. Although the major cell which responds to Con A and PHA is the T cell (MacDermott, et al., 1979), both B cells and Null cells are also capable of responding to PHA and Con A as long as 5 to 20% T cells are also present, while in the MLR Null cells, B cells and macrophages do not function as responder (MacDermott, 1975). On the other hand, failure to detect any suppressive action by pyrimethamine in the MLR experiments could be due to the fact that the normal degree of stimulation in the MLR is much less than that observed in lectin-induced blast transformation, because a smaller percentage of cells proliferate in response to allogeneic cell surface antigens than proliferate in response to non-specific mitogenic lectin stimulation; 60 to 70% of the cells become blast when cultured with mitogenic lectins, whereas only 2 to 5% of the cells become blast in MLR.

The unweighted mean regression of log CPM on pyrimethamine concentrations used in the PHA-stimulated cultures is $-2750 + 540$. By using this value to compare the difference between the means before and after 25 weeks of S-Py chemoprophylaxis, $-0.084 + 0.052$; the observed depressing effect of S-Py is $(-0.084)/(-2750) = 3.05 \times 10^{-5}$ M (with a standard error of 1.98×10^{-5} M). The ninety-five percent confidence limits are -1.63×10^{-5} M to 7.73×10^{-5} M. This means that although the observed effect of 25 weeks use of 2 tablets per week of S-Py is not significantly different from zero, the data also do not allow us to rule out an effect equivalent to an *in vitro* pyrimethamine concentration of 7.73×10^{-5} M (which is almost equal to the highest concentration used in the experiments). By using this statistical method of analysis, we can make similar statements concerning the effect of S-Py chemoprophylaxis on Con A and Pokeweed mitogen stimulated cultures. Admittedly, our experimental error is obviously too large to obtain a precise estimate with only eight subjects. However, the suppressive effect of pyrimethamine on cellular immune responses may be clinically significant since serum concentrations after oral administration of a single dose of 100 mg of pyrimethamine have been found to range from 0.57 to 0.12 mg/l ($2.29 - 0.48$ umol/l) (Smith and Ihrig, 1959), while in studies of Khmer refugees on anti-malaria drug therapy the range of the effective dose (ED)_{99.9} in moles of pyrimethamine per liter of blood (based on the percentage maturation of schizonts against the mean of the control) was found to be 2.0×10^{-4} to 5.1×10^{-5} M (personal communications: Dr. D. Johnson and CDC study team, Sa Kaeo, Thailand).

We are presently unable to explain the differences of our results in respect to the *in vitro* immunopotentiating properties recently reported for pyrimethamine by Bygbjerg (1981). However, the methods of preparation of stocks solutions of pyrimethamine along with the different methods of statistical analysis must be considered. In addition, the influence of varying concentrations of mitogens may alter the effect of pyrimethamine, however, we cannot assess the relative importance of mitogen concentration at this point since the mitogen concentrations used by Bygbjerg were not reported.

The mechanism by which pyrimethamine influences lymphocyte functions remains to be shown. However, the present studies demonstrate that pyrimethamine, a component of many widely used anti-malarial drugs adversely affects *in vitro* lectin-induced blast transformation by human mononuclear cells. The drug concentration at which suppression occurs is equivalent to the blood drug level range found in patients on malaria chemotherapy. Although the extrapolation of the *in vitro* findings to *in vivo* systems awaits careful clinical follow-up studies the combination of suspected immunosuppressive effects, antifolate properties, and other hematological complications associated with pyrimethamine use are important considerations for individuals on long term S-Py chemoprophylaxis. In addition, it is important to note that any immunosuppressive effect of pyrimethamine may be of greater significance in situations in which an intact host immune response is desired, i.e. vaccination. Therefore, further studies are needed to elucidate the specific mode of action by pyrimethamine on human MNC and to clarify whether the abnormalities in immune function result in alteration of immunoregulating or immune effector functions.

Table 1. Effect of Pyrimethamine and Sulfadoxine on Mononuclear Cell Viability^a

	# Moles/Culture ^b	% Dead Cells ^c
Pyrimethamine	0	21
	10 ⁻⁴ M	22
	10 ⁻⁵ M	18
	10 ⁻⁶ M	16
Sulfadoxine	0	21
	10 ⁻⁴ M	19
	10 ⁻⁵ M	18
	10 ⁻⁶ M	17

^a Ficoll-Hypaque purified mononuclear cells were used and incubated in assay medium as described in Materials and Methods.

^b 1 x 10⁵ cells/culture.

^c Viability was assessed after 96 hours incubation using the eosin dye inclusion technique; no difference between drug treated and untreated cultures was observed. Death of mononuclear cells in culture was >85% in presence of 0.05% Triton X-100 detergent.

Table 2. Summary of Regressions of Log CPM on Drug Concentration^a

Test ^b	No. of Positive Slopes	No. of Negative Slopes	No. of Regressions Sig. at .05 Level	No. of Regressions Sig. at .01 level
1. Sulfadoxine				
a) MLC	4	3	1	0
b) Pokeweed Mitogen	6	3	0	0
c) PHA	5	3	0	0
d) Con A	<u>6</u>	<u>2</u>	<u>0</u>	<u>0</u>
Total	21	11	1	0
2. Pyrimethamine				
a) MLC	3	4	1	0
b) Pokeweed Mitogen	0	5	5	4
c) PHA	0	8	3	2
d) Con A	<u>0</u>	<u>5</u>	<u>3</u>	<u>2</u>
Total	3	22	12	8

^a *In vitro* effect of sulfadoxine and pyrimethamine on immune responsiveness of human mononuclear cells was assessed by regressing the log CPM of isotope in the respective test well on drug concentration.

^b Respective drug concentration used ranged from 10^{-4} M to 10^{-9} M.

Table 3. Difference Between Mean Log CPM of Blood Taken Before Drug Administration and After Five or Twenty-five Weeks of Fansidar.

Duration of Drug Use ^a	Immune Test System			
	PHA	Con A	PWM	MLC
5 weeks	\bar{d}^b - .021 ± .046 ^c	- .035 ± .051	.123 ± .067	.110 ± .083
	t^d - .457 (11 df) ^e	- .684 (11 df)	1.884 (9 df)	1.326 (9 df)
25 weeks	\bar{d} - .084 ± .052	- .081 ± .114	- .002 ± .090	.012 ± .121
	t - 1.605 (9 df)	- .709 (9 df)	- .026 (8 df)	0.101 (8 df)

^a Volunteers received 2 tablets S-Py per week; each tablet included 500 mg sulfadoxine and 25 mg pyrimethamine.

^b \bar{d} is the mean difference in log CPM.

^c Negative values are preceded by a minus sign.

^d t is the t -value.

^e df is degrees of freedom in parentheses.

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