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EFFECT OF ARABINOSYL-6-MERCAPTOPURINE ON THE BLASTOGENIC RESPONSES *IN VITRO* OF HUMAN LYMPHOCYTES TO MITOGENIC AGENTS*

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Abstract—The effects of arabinosyl-6-mercaptopurine (Ara-6-MP) on lymphocyte blastogenic responses *in vitro* to mitogens were studied. Ara-6-MP was not degraded in the cultures until after 5 days of incubation. Levels of drug of 30 μg/ml or greater significantly inhibited blastogenic responses to phytohemagglutinin, streptolysin O and allogeneic leukocytes as measured by [^{3}H]thymidine incorporation (greater than 50 per cent reduction). Levels over 100–200 μg/ml inhibited thymidine incorporation over 90 per cent, but complete inhibition was not achieved at any dose up to 300 μg/ml. Morphological examination of parallel replicate cultures revealed that blastogenesis characterized by cell enlargement and the development of cytoplasmic basophilia was not significantly inhibited by the drug. Inhibition, at least at higher drug levels, began within 2 hr after exposure of the cells to the drug. Inhibition was completely reversible if the drug was washed from the cultures after 48 hr of continuous exposure and partially reversible when washed from the cultures after up to 96 hr of continuous exposure. In contrast to thymidine incorporation, uridine and leucine incorporation were only slightly inhibited by Ara-6-MP. These studies suggest that Ara-6-MP is a potent reversible and incomplete specific inhibitor of DNA synthesis among mitogen-stimulated, cultured leukocytes *in vitro* and that it might be an inhibitor of the proliferative phase of the specific immune response *in vivo*. In addition, these studies indicate that the morphological events associated with lymphocyte transformation can proceed in the virtual absence of DNA synthesis.

THE LEUKOCYTE culture system *in vitro* and the blastogenic responses *in vitro* of human lymphocytes to specific and nonspecific mitogens[1] have proven useful and important in the evaluation of the biological and immunological activities of many agents. These include corticosteroids,[2] busulfan,[3] antimetabolites such as methotrexate,[4] cytosine arabinoside[5] and azotomycin,[6] enzymes such as L-asparaginase,[7] and miscellaneous agents such as cinanserin,[8] *N*-ethyl-D-galactosamine,[9] isopentyladenosine[10] and amantadine.[11] Studies with these agents have yielded data regarding: (a) the mechanism of action of the agent, (b) the mechanism of the lymphocyte blastogenic phenomenon, and (c) the potential *in vivo* immunological or immunosuppressive activity, or both, of the agent. Indeed, some investigators have proposed this system *in vitro* as a method of choice to predict, identify and characterize clinically useful immunosuppressive agents.[6]

With these considerations in mind, a unique antitumor agent, β-D-arabinofuranosyl-6-mercaptopurine (Ara-6-MP),[12,13] was investigated for its effects on mitogen-stimulated cultures of human lymphocytes. Ara-6-MP is apparently an inhibitor of

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DNA synthesis by reason of its inhibition of ribonucleotide reductase.[12,13] Unlike many nucleoside analogs, it does not appear to require activation by conversion to nucleotide[14] and is not measurably converted to 6-mercaptopurine (6-MP).[12] This agent has been shown to be immunosuppressive *in vivo* in mice,[15,16] where it suppresses the skin homograft reaction, but has no net effect upon the antibody response to sheep red blood cells (RBC). Transient effects of Ara-6-MP upon spleen cell foci responding to sheep RBC were cancelled by enhanced proliferation at a later time.[17]

In the current study, the effects of Ara-6-MP on the blastogenic responses of human lymphocytes to mitogenic agents *in vitro* were explored. Ara-6-MP was found to inhibit partially and reversibly the blastogenic responses to specific and nonspecific mitogens. The effect was mainly on DNA synthesis, and morphologic parameters of blastogenesis were only slightly inhibited.

MATERIALS AND METHODS

Ara-6-MP was prepared by the method of Reist *et al.*[18] Ara-6-MP-^{35}S was prepared from Ara-6-MP by a procedure described for other nucleosides[19] and recrystallized until radiochemically pure (99·7%). This was shown by chromatography on 3MM papers with two systems (5% KH_2PO_4, $R_f = 0{\cdot}65$, and 0·65% boric acid in ethanol–concentrated NH_4OH, 100:1, $R_f = 0{\cdot}66$). The initial radiochemical contamination, crystalline sulfur from the labeling procedure, did not move on either chromatographic system.

In order to determine whether Ara-6-MP levels in culture fluids changed during the incubations, aliquots of the fluids were taken from cultures treated with Ara-6-MP-^{35}S after 1 hr and after 3, 5 and 7 days. These were deproteinized with perchloric acid and chromatographed on 3MM papers with 5% KH_2PO_4. The Ara-6-MP spots were cut from the papers and counted directly in a scintillation counting system as a measure of residual Ara-6-MP concentration.

Peripheral blood lymphocyte cultures were set up as previously described.[20] Venous blood from normal volunteers was defibrinated by swirling with glass beads and the red blood cells were sedimented with one part 4% dextran* (mol. wt., 240,000) to ten parts of blood. The white blood cell count of the resultant leukocyte-rich serum was measured and the cell concentration adjusted to 1×10^6 lymphocytes per ml. Cultures contained 1 ml of this serum and 2 ml of Spinner modified Eagle's minimal essential medium† in 13 × 100 mm screw-cap Pyrex glass tubes. The medium was supplemented with 1 ml penicillin–streptomycin solution† per 100 ml and 2 mM L-glutamine.†

Mitogens included 0·05 ml phytohemagglutinin-M (PHA),‡ 0·10 ml streptolysin-O (SLO)‡ and 0·5 or $1{\cdot}0 \times 10^6$ allogeneic lymphocytes (WBC).

PHA- and SLO-stimulated lymphocyte cultures were harvested after 5 days of culture at 37° in a moist atmosphere of 5% CO_2 in air. WBC-stimulated cultures were harvested after 7 days of culture. Two hr before harvesting, 2 μc [^{3}H]thymidine§ (sp. act., 1·0 c/m-mole) was added to each culture. At the end of the culture period, the cultures were centrifuged at 240 *g* in the cold for 10 min and washed once with

* Pharmachem, Bethlehem, Pa.
† Gibco, Grand Island, N.Y.
‡ Difco Laboratories, Detroit, Mich.
§ Schwarz BioResearch, Orangeburg, N.Y.

isotonic saline; the sediment was treated with 10% ice-cold trichloroacetic acid (TCA) for 10 min, washed twice with 5% TCA, once with absolute methanol, and the precipitate containing the acid-insoluble radioactivity dissolved in Hyamine* was brought to a volume of 10 ml with phosphor–toluene solution and counted in a liquid scintillation counter.* Results were recorded as counts per minute per 10^6 lymphocytes. In some experiments, duplicate cultures were also exposed to 2 μc[^{3}H]uridine (sp. act., 20 c/m-mole) or 0·5 μc [^{14}C]leucine (sp. act., 312 mc/m-mole) in order to determine the drug's effect on RNA and protein synthesis respectively.

Slides for microscopic examination of the cultures were prepared by centrifugation at 240 *g* for 10 min, gradual suspension of the button in 10% glacial acetic acid in ethanol with continuous stirring, centrifugation of the fixed cells at 240 *g* for 5 min, spreading the resultant button on slides, air drying, and staining with Giemsa stain.† On each slide, 500 cells were counted and the per cent lymphoblasts scored.

In the washing experiments, cells were centrifuged at 240 *g* for 10 min, resuspended in 10 ml of Hank's balanced salt solution containing 5% fetal bovine serum, centrifuged as above and resuspended in autochthonous serum and medium at the desired cell concentration.

Viable cell counts were determined in a hemocytometer chamber by the trypan blue dye exclusion method.[7]

RESULTS

Media from three unstimulated, three PHA-stimulated, three SLO-stimulated and three mixed lymphocyte cultures were analyzed at 1 hr and at 3, 5 and 7 days after the addition of various levels of Ara-6-MP-^{35}S. These were found to show no measurable decrease in the drug levels of the media by 5 days. Decreases of 80–85 per cent had occurred by 7 days, however.

Figures 1–3 show dose–response studies of the effects of Ara-6-MP on the lymphocyte blastogenic responses to PHA, SLO and allogeneic tissue antigens as measured by thymidine incorporation. A level of 10 μg/ml in five preliminary experiments reduced the lymphocyte responses an average of 35 per cent. This is not significant in this system[6,21] and therefore the data are not included in the figures. A level of 30 μg/ml, present continuously throughout the culture period, reduced the blastogenic responses 40–74 per cent. Ten times that level (300 μg/ml) reduced the blastogenic responses 89–95 per cent but did not completely abolish them. In no instance were blastogenic responses measured by thymidine incorporation under 1000 counts/min per 10^6 lymphocytes. In most experiments, 90 per cent inhibition of blastogenesis, as measured by thymidine incorporation, was achieved by a level *in vitro* of 100 μg/ml, above which the inhibition tended to plateau.

Figure 4 shows the results of four studies in which the effects of this agent on the morphological changes associated with blastogenesis were studied. It can be seen that there was little effect on the morphological manifestation of blastogenesis even at doses which inhibited thymidine incorporation by 90 per cent or more. This disparity between the biochemical and morphological manifestation of blastogenesis is further clarified by comparison of these morphological studies with simultaneous

* Packard, Bowners Grove, Ill.
† Harleco, London, England.

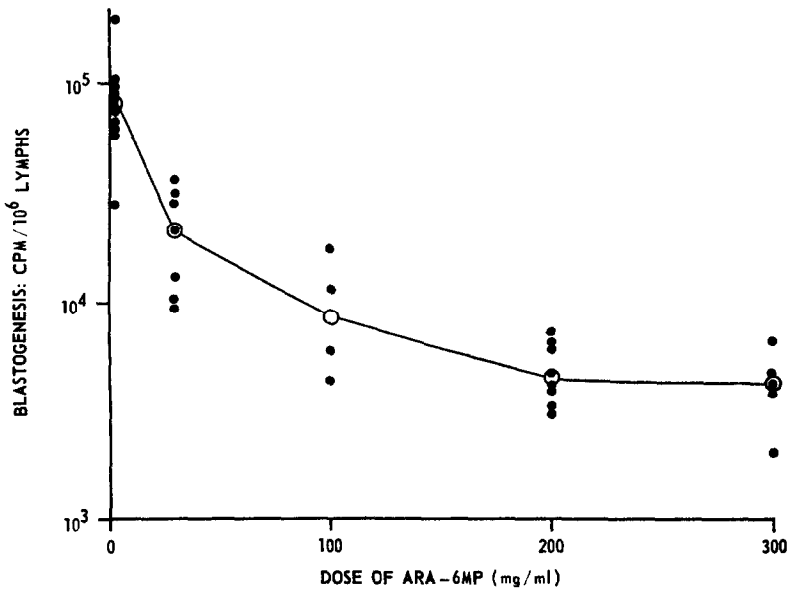

FIG. 1. Ara-6-MP partially inhibited thymidine incorporation into lymphocytes. Closed circles are individual experimental points; open circles are medians. Effect on response to PHA is shown.

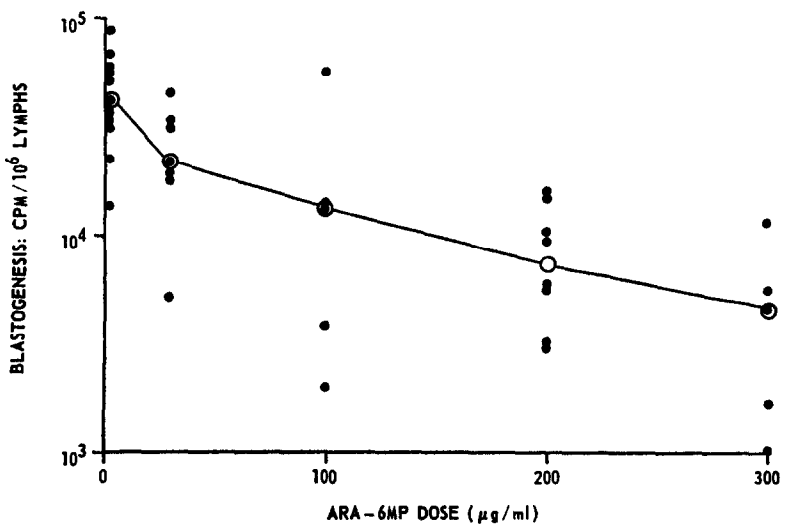

FIG. 2. Ara-6-MP partially inhibited thymidine incorporation into lymphocytes. Closed circles are individual experimental points; open circles are medians. Effect on respone to SLO is shown.

studies of thymidine incorporation in paired cultures (Fig. 5). In all of these experiments there was greater inhibition of thymidine incorporation compared to inhibition of cell enlargement. In four instances blastogenesis was less than 10 per cent of the control biochemically and over 80 per cent of the control morphologically.

Ara-6-MP inhibited lymphocyte responses *in vitro* without marked cytotoxicity. First, serial studies on cultures incubated with Ara-6-MP revealed that the viable

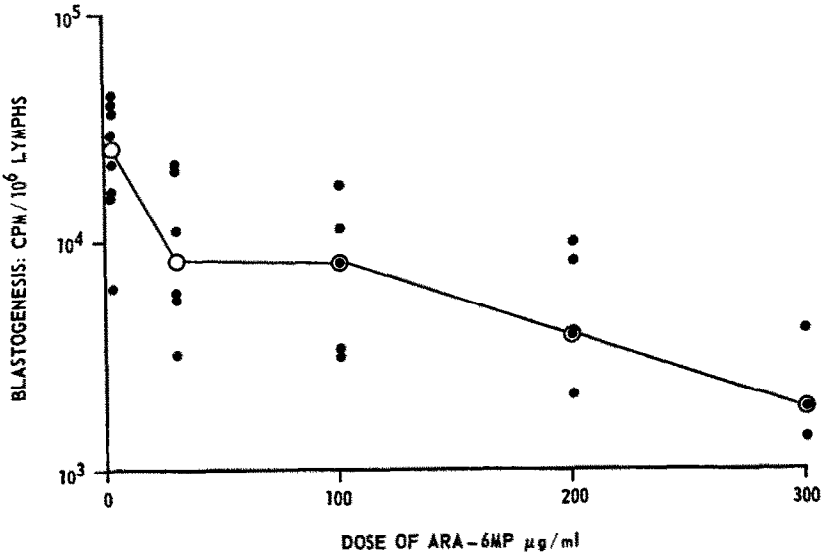

FIG. 3. Ara-6-MP partially inhibited thymidine incorporation into lymphocytes. Closed circles are individual experimental points; open circles are medians. Effect on response to WBC is shown.

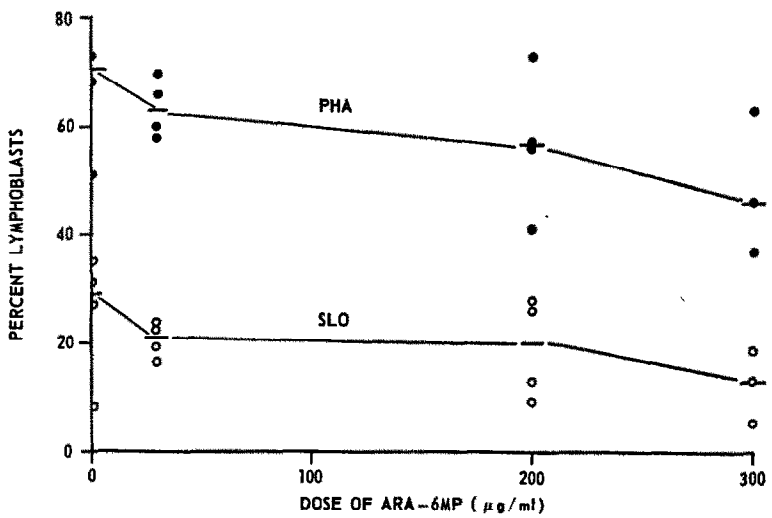

FIG. 4. Ara-6-MP inhibits lymphoblast formation less than 50 per cent. Closed circles, response to phytohemagglutinin; open circles, response to streptolysin-O. Bars are median values.

cell counts were not markedly reduced after up to 7 days of incubation. Second, the drug's effect was reversible when it was washed from the cultures and the mitogen re-added without the drug (Table 1). Even when the mitogens were not re-added to the cultures, the blastogenic responses measured by thymidine incorporation were substantially improved by washing the drug from the cultures, even after up to 96 hr of exposure (Table 2).

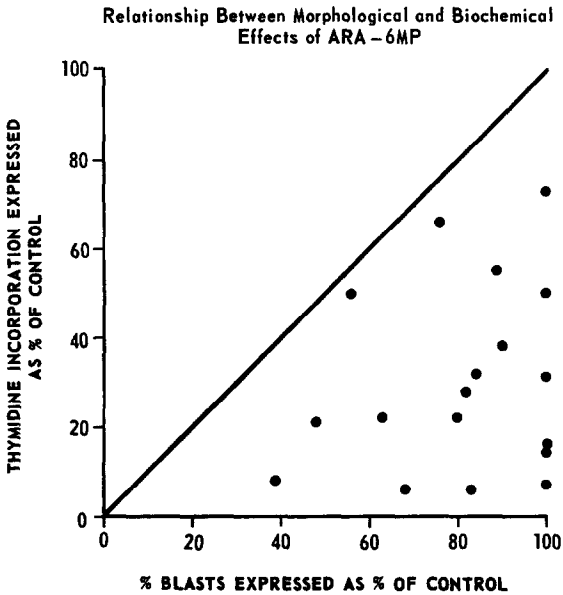

FIG. 5. Ara-6-MP inhibits thymidine incorporation more than morphological blastogenesis. Each point represents one experimental value for thymidine incorporation and blastogenesis.

TABLE 1. CYTOTOXIC EFFECTS OF ARA-6-MP

Time of exposure to drug (hr)*	% Reduction in lymphocyte response					
	PHA		SLO		WBC	
	30†	200	30	200	30	200
0·5	0·0	0·0	0·0	0·0	0·0	0·0
1	0·0	14·0	0·0	0·0	0·0	0·0
4	21·0	18·5	28·2	20·8	35·4	34·5
24	12·8	46·1	0·0	0·0	7·4	56·6‡
48	20·3	53·8‡	0·0	5·5	32·7	65·4‡
Continuous	66·2‡	93·0‡	38·6	65·8‡	52·4‡	90·5‡

* Before removal from culture and readdition of mitogen.
† ARA-6-MP dose, μg/ml.
‡ Significant inhibition.

The lack of major morphological effects on mitogen-stimulated cells while thymidine incorporation was substantially inhibited suggested that DNA synthesis was selectively inhibited by the drug. This was tested by examining sets of cultures in which replicates were simultaneously incubated with [^{3}H]uridine, [^{14}C]leucine or [^{3}H]thymidine (Table 3). In each set of simultaneous cultures, the thymidine incorporation was significantly more inhibited than the uridine incorporation. Incorporation of [^{14}C]leucine was not inhibited at all.

TABLE 2. CYTOTOXIC EFFECTS OF ARA-6-MP

Time of exposure to drug and mitogen (hr)*	% Reduction in lymphocyte response					
	PHA		SLO		WBC	
	30†	200	30	200	30	200
0·5	20·0	0·0	13·5	0·0	46·6	27·7
1	27·0	0·0	0·0	0·0	22·5	18·3
4	0·0	0·0	0·0	13·2	39·5	40·7
24	55·6‡	46·4	28·2	43·2	6·1	0·0
96	72·2‡	59·9‡	5·4	39·5	20·3	44·6
Continuous	50·0‡	78·4‡	11·0	62·6‡	36·8	57·8‡

* Before removal from culture.
† ARA-6-MP dose, μg/ml.
‡ Significant inhibition.

TABLE 3. EFFECTS OF ARA-6-MP ON INCORPORATION OF [^{3}H]THYMIDINE, [^{3}H]URIDINE AND [^{14}C]LEUCINE

ARA-6-MP dose (μg/ml)	% Reduction in lymphocyte response		
	Precursor incorporation		
	Thymidine	Uridine	Leucine
100	89·5* 58·3–100·0†	20·0 0·0–55·0	0·0 0·0–0·0
200	89·0 74·5–96·8	72·0 44·5–93·0	not done

* Median.
† Range.

TABLE 4. ONSET OF ACTION OF ARA-6-MP EFFECT

Hours of drug exposure	ARA-6-MP dose (μg/ml)		
	0	100	300
2	38·6*	33·9	25·2
6	43·6	29·2	11·8
24	46·5	15·1	10·9
48	63·6	28·2	12·4

* Response to phytohemagglutinin in counts/min/10^6 lymphocytes × 10^3. Mitogen added 72 hr before drug. Isotope added during last 2 hr of drug exposure.

Table 4 indicates the timing of onset of the action of Ara-6-MP. PHA-stimulated cultures were incubated for 72 hr at which time drug was added for progressively longer periods of time. Isotope was added during the last 2 hr of the drug exposure period. It can be seen that the DNA synthesis was not significantly inhibited until 24 hr after exposure to a low level of the drug, but was immediately inhibited by a higher drug dose. To further explore late effects of the drug, it was added at various times after addition of PHA (Table 5). It was inhibitory even when added 72 hr after PHA, but at this time the cells were partially resistant to its effect.

TABLE 5. EFFECT OF LATE ADDITION OF ARA-6-MP ON THE RESPONSE TO PHYTOHEMAGGLUTININ

Hour of drug addition	Hours of drug exposure	ARA-6-MP dose (μg/ml)		
		Control	100	300
0	96	60·2*	5·7	3·8
4	92		4·0	2·6
24	72		10·9	4·9
48	48		10·2	5·2
72	24		31·0	21·6

* Counts/min/10^6 lymphocytes harvested at 96 hr with a 3-hr pulse of [^{3}H]thymidine $\times$ 10^3.

DISCUSSION

The results obtained on lymphocyte cultures with Ara-6-MP suggest a highly selective action of Ara-6-MP on the DNA-mediated features of blastogenesis. This is compatible with the earlier observations concerning its biochemical effects on experimental tumors.[12,13] The stability of the drug in the culture media is also compatible with earlier reports of its pharmacological behaviour in mice.[12] The effects were obtained at drug concentrations (100 μg/ml) easily obtained *in vivo* at tolerated doses.[12]

The lymphocyte culture system *in vitro* which has been developed over the last 10 years[1] is a useful tool in the investigation of the human immunological system. It is now generally agreed that the responses to the nonspecific mitogens such as PHA are a partial measure of the individual's general immunological competence, the responses to specific mitogenic antigens a measure of the subjects' population of circulating memory cells (those immunologically committed to antigens), while the response to allogeneic leukocytes is a measure of the individual's ability to mount an allograft reaction against those specific histocompatibility antigens present on the allogeneic leukocytes.[1] The lymphocyte culture system *in vitro* has been used to study many aspects of fundamental and applied human and animal immunology. These studies have included investigations of antibody production *in vitro*,[22] release of mediators of cellular immunity,[23] macrophage lymphocyte interaction,[24] development of immunological commitment after immunization,[25] histocompatibility relationships among potential graft recipients,[26] various immunological deficiency states, [27]and the effects of immunosuppressive therapy.[28]

The kinetics and extent of response *in vitro* to the various classes of mitogens vary, but the basic cellular events of increased RNA and protein synthesis associated with cell enlargement and cytoplasmic basophilia, followed by DNA synthesis and mitosis, are characteristic of responses *in vitro* to all mitogens.[1] These studies with Ara-6-MP show that the functions of cell enlargement and the development of abundant basophilic cytoplasm, in other words, the morphological hallmarks of response, can be completely separated from the most commonly accepted biochemical hallmark ([^{3}H]thymidine incorporation). This supports the observation of Turk and Stone[29] that methotrexate therapy blocked cell division but not the formation of blast-like cells in lymph nodes responding to antigenic stimulation. It shows first that DNA synthesis, although a usual concomitant of blastogenesis, is not necessary for it. Second, when potential states of modified immunity are studied (such as chemical immunosuppression), biochemical studies must be confirmed by morphological examination of the treated cells or the cells from the treated individuals.

The blastogenic responses *in vitro* of lymphocytes to mitogens have proved to be particularly useful in the characterization of chemical and other immunosuppressive agents. The effects of such diverse agents as corticosteroids,[2] antiproliferative antineoplastic agents,[4–6] irradiation,[30] antilymphocyte serum[31] and enzymes[7] have been studied. Other agents investigated include amantadine,[11] cinanserin[8] and immunoregulatory globulin.[32]

The recent studies with asparaginase reveal the usefulness of the system *in vitro*.[7] Asparaginase, when added to lymphocytes *in vitro*, completely inhibited blastogenesis. This inhibition was completely reversible by washing out the enzyme and no cytotoxicity was observed. The enzyme was much less inhibitory if added some time after the mitogen and the effect was reduced as the number of cells per culture was increased. These observations *in vitro* were subsequently found to be closely related to the effects *in vivo* of the enzyme. It was not cytotoxic to the lymphoid system,[33,34] did not inhibit established delayed hypersensitivity,[34] but did inhibit the primary immune response if administration was started before administration of the antigen.[33,34]

On the basis of the current studies, it is possible that Ara-6-MP might partially, but not completely, suppress the cellular, humoral or both components of the primary immune response in man. This partial effect would be anticipated, since Ara-6-MP did not completely inhibit blastogenesis. Also, since it specifically inhibited only lymphocyte DNA synthesis, it would be most effective when administered during the proliferative phase of the response. It should be administered at the beginning of the proliferative phase, since the studies *in vitro* suggest decreased drug sensitivity when blastogenic proliferation is on-going. Since it is not particularly lymphocytotoxic, it would not be expected to inhibit established delayed hypersensitivity. It would have no effect on the uptake or processing of antigen or on the development of immunological commitment, since blastogenesis was not significantly inhibited when both drug and mitogen were washed from the cultures after up to 96 hr of exposure. Ara-6-MP has already been demonstrated to inhibit the immune responses of mice, particularly to skin grafts.[15,16]

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