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EFFECTS OF SEVERAL ANTITUMOUR AGENTS ON SPLEEN HEMOLYSIN PLAQUE-FORMING CELLS*

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Abstract—The effect of several antitumor agents—amethopterin, cyclophosphamide, 6-mercaptopurine, and vinblastine—on the production of spleen hemolysin plaque-forming cells (HPFC) was investigated in C57L × A/He mice receiving a primary injection of sheep red cells. The production of HPFC in spleen tissues is markedly dependent on the timing of drug injection relative to antigenic stimulation. The administration of the antitumor agents 3 days after antigen resulted in a consistent and marked decrease in HPFC, accompanied by a decrease in splenic cellularity. The numbers of HPFC resulting from drug injections prior to antigen injection showed no consistent pattern of response; 6-mercaptopurine and amethopterin enhanced, vinblastine did not significantly alter, and cyclophosphamide markedly depressed the production of HPFC.

ALTHOUGH many agents have been shown to suppress antibody response, precise mechanisms by which various drugs exert their effects to alter antibody formation are not well understood. Immunosuppressants have been the subject of a recent comprehensive review.[1] A major problem in the past has been quantitatively to assess the antibody-forming cells in lymphoid organs with mixed cell populations. As a result, most of the studies of drug effects have been confined to alterations of serum antibody titers. The use of the Jerne hemolysin plaque technique[2] has largely overcome this difficulty of cell heterogeneity, and the present communication deals with the effect of selected drugs on hemolysin plaque-forming cells.

Our earlier observations of radiation effects on hemolysin plaque-forming cells (HPFC)[3] have indicated a dependence on the timing of X-ray administrations relative to antigen injection. At sublethal doses of 250 R total-body X-irradiation, the numbers of antibody-forming cells are clearly influenced by this time interval; a biphasic response (i.e. a relatively insensitive period between two sensitive periods) to X-rays in the production of antibody-forming cells was demonstrated.

Antibody formation consists of an inductive (or latent) phase and a proliferative (or log) phase. The peak response in terms of HPFC occurs 4–5 days after a single injection of sheep red cell antigen to mice.[2] Studies of drug effects on antibody formation have dealt, for the most part, with multiple injections given over a period of several days; these multiple injections may obscure the effects of a single injection at a specific phase of antibody formation. Recent experiments have indicated that HPFC which were analyzed within a week after a primary antigen injection were producing 19s antibody.[4, 5] This report deals with the effect of several drugs on

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hemolysin plaque-forming cells, injected with a large single dose *in vivo* into mice at various times before and after sheep red cell antigen injection. The immunosuppressants used for the present studies were amethopterin (a folic acid antagonist), cyclophosphamide (an alkylating agent), 6-mercaptopurine (a purine antagonist), and vinblastine. The number of plaque-forming cells of the spleen was correlated with relative spleen weights, total nucleated spleen cells, and serum hemolysin titers at the time of sacrifice.

MATERIALS AND METHODS

Hybrid male LAF (C57L × A/He) mice, approximately 3–5 months of age and weighing between 20 and 27 g, were given a single i.p. injection of 8×10^8 sheep red cells. Animals were sacrificed 5 days after antigen injection. Spleens were removed and immersed in Eagle's medium without serum. Cells were separated from connective tissue stroma by compressing the tissue against the sides of a Dounce homogenizer with the loose-fitting pestle. The cell suspension was then passed through nylon mesh bolting and centrifuged at 800 *g* for 5 min in an International refrigerated centrifuge, model PR-2. The supernatant fluid was withdrawn by aspiration, and the cell density was adjusted so that 0·1 ml of suspension contained approximately one million cells. Sheep red cells in Alsever's solution were washed three times with 0·9% sodium chloride, and the final adjusted volume was brought up with Eagle's medium.[3] The plating technique was done according to Jerne *et al.*[2]

Preliminary studies of drug toxicity were done on LAF mice. The doses used in the current studies were the largest single intraperitoneal injections the animals could tolerate without losing more than 3 g during the 7-day period of observation. Sodium amethopterin was obtained from Lederle Laboratories, cyclophosphamide from Mead Johnson Laboratories, 6-mercaptopurine from Burroughs Wellcome & Co., and vinblastine from Eli Lilly & Co. The dosages used in the experiments were the following: sodium amethopterin, 100 mg/kg; cyclophosphamide, 200 mg/kg; 6-mercaptopurine, 120 mg/kg; vinblastine, 3 mg/kg. 6-Mercaptopurine was dissolved in 4:1 dimethylsulfoxide:water prior to injection. Other abbreviations used in this report are: r.s.w. (relative spleen weight), 6MP (6-mercaptopurine), MTX (sodium amethopterin), VBL (vinblastine), CY (cyclophosphamide).

RESULTS

1. *Effect of sodium amethopterin* (100 *mg/kg*) *on* HPFC

Five groups of LAF mice were given an i.p. injection of 8×10^8 sheep cells and amethopterin on the day of injection (group C), 1 day after (group D), and 3 days after antigen injection (group E). Group B was given amethopterin 2 days prior to sheep red cell injection. All groups, including antigen-injected controls (group A), were sacrificed 5 days after antigen injection, blood was collected for serum antibody titration, and the spleens were weighed and analyzed for HPFC. The drug-treated animals lost little or no weight during the period of treatment (7 days). The results of this experiment (shown in Table 1) indicated that the numbers of HPFC were markedly dependent on the timing of amethopterin injection relative to sheep red cell injection. The HPFC per spleen were significantly higher than for control animals (level of significance used for these studies was 0·05) when MTX was administered 2 days prior to antigen injection, although the serum hemolysin titers indicated a

slight drop from controls. The other measured parameters were not significantly different statistically from controls.

The overall trend in the measured parameters of MTX-injected animals from the day of antigen injection to 3 days post-treatment was one of a decrease from control values. At this dose level of MTX, maximal effects of the drug were observed at 3

TABLE 1. SPLEEN HPFC OF LAF MICE RECEIVING AMETHOPTERIN (100 mg/kg) BEFORE AND AFTER SHEEP RED CELL ANTIGEN INJECTION

Treatment	HPFC per spleen	HPFC per 10^6 nucleated spleen cells	r.s.w. $\left(\frac{\text{mg spleen} \times 10}{\text{g body wt.}}\right)$	Total nucleated spleen cells ($\times 10^7$)	Serum hemolysin $1{:}\log_2$ titer
Group A, controls, antigen given	129,100 ± 12,700 (12)	462 ± 48 (12)	50·7 ± 2·1 (12)	29·4 ± 1·7 (12)	9·6 ± 0·2 (8)
Group B, MTX, 2 days before antigen	210,000 ± 23,000* (9)	601 ± 63 (9)	55·0 ± 2·8 (9)	37·0 ± 7·9 (9)	9·0 ± 0·3 (5)
Group C, MTX, day of antigen	116,900 ± 30,900 (2)	679 ± 135 (2)	49·9 ± 1·3 (2)	35·6 ± 3·6 (2)	8·0 ± 0* (2)
Group D, MTX, 1 day after antigen	51,000 ± 17,200* (4)	184 ± 76* (4)	44·4 ± 0·9 (4)	28·0 ± 3·8 (4)	6·0 ± 0·4* (4)
Group E, MTX, 3 days after antigen	26,900 ± 6,200* (4)	117 ± 41* (4)	36·9 ± 2·2* (4)	26·4 ± 3·6 (4)	7·3 ± 0·2* (4)

Values represent the mean ± standard error of the mean. The number of animals used for each value is in parentheses.

* Asterisks represent values which are significantly different ($P < 0{\cdot}05$) from control values according to Student's *t*-test distribution.

days post-treatment, showing significantly reduced values for relative spleen weights, HPFC, and serum hemolysin titers.

2. *Effect of* 6MP, VBL, *and* CY *on hemolysin plaque-forming cells*

Since maximal differences were observed 2 days before and 3 days after antigen injection, these two time intervals were chosen to analyze the effects of several other compounds which have demonstrable immunosuppressant action.

6-Mercaptopurine at a dose of 120 mg/kg was administered intraperitoneally to male LAF mice 2 days before and 3 days after sheep red cell injections. The animals were sacrificed, and analyses similar to those for MTX were performed. The results of these studies are shown in Table 2. When 6MP was administered to animals 2 days prior to antigen, there was a marked increase in the HPFC per spleen, although little or no change was observable in the r.s.w., total nucleated spleen cells, and in serum hemolysin titers. On the other hand, the injection of 6MP 3 days after antigen injection resulted in a marked decrease in all the measured parameters except for serum hemolysin titers.

The injection of vinblastine at a dose of 3 mg/kg 2 days before antigen did not result in statistically significant differences in the measured parameters when compared with control values. The administration of the drug 3 days after antigen injection

resulted in a marked decrease in the measured parameters except for serum hemolysin titers.

Table 2 also lists observations on the injection of cyclophosphamide at a dose level of 200 mg/kg. Some notable differences were observed. When CY was administered 2 days prior to antigen a marked decrease in HPFC was observed, concomitant

TABLE 2. SPLEEN HPFC OF LAF MICE RECEIVING 6-MERCAPTOPURINE (120 mg/kg), VINBLASTINE (3 mg/kg) AND CYCLOPHOSPHAMIDE (200 mg/kg) BEFORE AND AFTER SHEEP RED CELL INJECTION

Treatment	HPFC per spleen	HPFC per 10^6 nucleated spleen cells	r.s.w. $\left(\frac{\text{mg spleen} \times 10}{\text{g body wt.}}\right)$	Total nucleated spleen cells ($\times 10^7$)	Serum hemolysin 1:1 $\log_2$ titer
Control, antigen given	129,000 ± 12,700 (12)	462 ± 48 (12)	50·7 ± 2·1 (12)	29·4 ± 1·7 (12)	9·6 ± 0·2 (8)
6MP, 2 days before antigen	208,000 ± 20,000 (8)	605 ± 46 (8)	48·2 ± 1·4 (8)	33·8 ± 1·9 (8)	9 (pooled serum)
6MP, 3 days after antigen	19,350 ± 1,500* (4)	115 ± 16* (4)	37·2 ± 1·7* (4)	17·8 ± 2·9* (4)	9·5 ± 0·3 (4)
VBL, 2 days before antigen	162,100 ± 17,800 (8)	505 ± 42 (8)	63·4 ± 5·4 (8)	33·4 ± 2·3 (8)	10·0 ± 0 (4)
VBL, 3 days after antigen	18,700 ± 3,400* (4)	119 ± 35* (4)	35·8 ± 1·6* (4)	16·4 ± 1·4* (4)	9·0 ± 0 (4)
CY, 2 days before antigen	1,386 ± 574* (4)	4 ± 2* (4)	50·3 ± 5·7 (4)	27·8 ± 4·7 (4)	6 (pooled serum)
CY, 3 days after antigen	803 ± 254* (4)	11 ± 3* (4)	21·4 ± 1·6* (4)	7·3 ± 0·5* (4)	9·0 ± 0 (4)

Values represent the mean ± standard error of the mean. The number of animals used for each value is in parentheses.

* As in Table 1.

with a decrease in serum hemolysin titers. At the same time no values significantly different from control animals were obtained in the r.s.w. and total nucleated spleen cells. The serum hemolysin titer was surprisingly high ($\log_2$ 9) in view of the marked reduction in spleen cellularity and HPFC.

DISCUSSION

Clearly, the production of HPFC is affected by the timing of drug injection relative to antigenic stimulation. In view of the current studies, in which several agents either stimulate or do not affect the production of antibody-forming cells, some revision or extension of current thoughts on mechanisms of immunosuppression by the anticancer agents appears to be in order. All of the currently used agents in this study are potent inhibitors of cell division, and this has been the basis of their use as antitumor agents Antibody production is preceded by intense mitotic activity in lymphoid tissues;[6–8] furthermore, the maintenance of serum antibody titers is accompanied by continued cell proliferation of lymphoid tissues. The use of antitumor agents as immunosuppressants is based on the premise that they can inhibit antibody production by inhibiting cell proliferation in lymphoid tissues.

Since spleen tissue showed considerable variation in response to the various drugs administered, a summary of drug effects on HPFC is shown on Table 3. As noted

before, the administration of these agents 3 days after antigen resulted in a consistent and marked decrease in HPFC of spleens. This decrease in HPFC was correlated with a significant decrease in splenic cellularity (as shown by the relative spleen weights and total nucleated spleen cells of Tables 1 and 2). These results were interpreted as indicating that in the later proliferative stages of the primary antibody response these agents exert their action by inhibiting cell division.

TABLE 3. ACTION OF IMMUNOSUPPRESSANTS ON THE NUMBERS OF HEMOLYSIN PLAQUE-FORMING CELLS OF MICE SPLEENS ANALYZED 5 DAYS AFTER A PRIMARY INJECTION OF 8×10^8 SHEEP RED CELLS

	Per cent of control					
	Before antigen injection			After antigen injection		
Treatment	Increase	No effect	Decrease	Increase	No effect	Decrease
Amethopterin, 100 mg/kg	163					21
6-Mercaptopurine, 120 mg/kg	161					15
Vinblastine, 3 mg/kg		113*				15
Cyclophosphamide, 200 mg/kg			1			1
Total body X-ray 250 R†			15			8

* Not significant. Level of significance used for these studies was 0·05.
† From Ref. 3.

The finding that serum antibody levels of several drug-treated groups did not correspond closely to the decrease in the numbers of HPFC and splenic cellularity was somewhat surprising. Injection of VBL and 6MP showed a decrease of less than one $\log_2$ titer dilution when compared to control values. On the other hand, MTX- and CY-treated animals showed a significant drop in serum hemolysin titers 3 days after antigen injection.

One explanation for the lack of correspondence may be that drug effects after the administration of antigen may be obscured by the relatively slower decrease in antibody levels already present in serum as distinguished from more rapid alterations in the cell population of HPFC. Berenbaum[9] in his recent studies on radiosensitivity of HPFC suggested that "marked fluctuations in radiosensitivity found when certain end results of the immune process are investigated may, to some degree, be experimental artefacts due to the delay with which changes in cell population are reflected in serum antibody levels and to the impossibility of following changes in serum antibody below the threshold of detection." This viewpoint is applicable to the present studies of antitumor agents on HPFC and serum hemolysin.

The interpretation of results on animals treated with drugs *prior* to antigen injection is more difficult. Table 3 summarizes the data from Tables 1 and 2 and from an earlier study.[3] 6MP and MTX enhanced, VBL did not significantly alter, and CY and X-rays markedly suppressed the production of HPFC. The reasons for the varied responses to different antitumor agents are not at present understood. Since the enhancement effect on HPFC by MTX and 6MP cannot be explained on the basis of their cytotoxic effect, I suggest that the drugs may interfere with the normal pattern of

cyto-differentiation of "precursor cells" (or less differentiated cells). The response of these cells to chemotherapeutic intervention before the cells have become "committed" to produce HPFC may depend on the drugs' own particular biochemical mechanisms of action. Their effects on differentiating cells may be unique; some may stimulate, whereas others may inhibit production of HPFC.

The above discussion assumes the attainment of drug levels adequate to exert their action. One possibility to explain the divergent results would be a differential uptake by the sensitive cells for different drugs. It may be noted that X-irradiation, not dependent on the vagaries of drug distribution, does markedly inhibit the production of HPFC when it is administered prior to antigen. Recently Kassel *et al.*[10] showed that the uptake of methotrexate (tritiated) may be a major determinant of the antitumor activity of MTX against transplantable mouse leukemias; resistant strains did not incorporate MTX as did sensitive strains. Similarly, Goldstein *et al.*[11] observed that when actinomycin D-sensitive and resistant strains were treated with the tritiated antibiotics, only the nuclei of drug-sensitive cells incorporated the tritiated compound.

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