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ANTI-TUMOUR AGENTS ON ANTIBODY-FORMING CELLS *IN VITRO**

EDWIN M. UYEKI and VICENTE LLACER

Department of Pharmacology, University of Kansas Medical Center, Kansas City, Kans. 66103, U.S.A.

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Abstract—Studies were conducted on the effective concentrations of five anti-tumor agents to alter the production of antibody-producing cells *in vitro*. The Mishell–Dutton technique was used to obtain hemolysin plaque-forming cells arising from dissociated spleen cells of normal LAF_1 mice immunized to sheep erythrocytes *in vitro*. The doses required to obtain 50 per cent inhibition of antibody-producing cells were the following: amethopterin, $2{\cdot}5 \times 10^{-6}$ M; 6-mercaptopurine, $0{\cdot}06 \times 10^{-6}$ M; 5-fluorouracil, $0{\cdot}1 \times 10^{-6}$ M; vinblastine, $0{\cdot}007 \times 10^{-6}$ M; and mechlorethamine, $0{\cdot}2 \times 10^{-6}$ M. The drugs were classified into two groups on the basis of drug concentration ranges required to inhibit the formation of hemolysin plaque-forming cells from 50 per cent to less than 10 per cent of control values. Vinblastine and mechlorethamine required a narrow range (2- to 3-fold), whereas amethopterin, 6-mercaptopurine and 5-fluorouracil required a wide dose range (100- to 400-fold).

PREVIOUS studies *in vivo* on the effect of anti-tumor agents on hemolysin plaque-forming cells of mouse spleens have indicated a marked dependence on timing of antigen and drug injections for their immunosuppressant effects.[1, 2] It was found that amethopterin and 6-mercaptopurine stimulated and that cyclophosphamide, vinblastine and radiation inhibited the production of direct hemolysin plaque-forming cells when given before antigen. However, all the agents suppressed direct hemolysin plaque-forming cells when given 3 days after antigen. A second injection of amethopterin and 6-mercaptopurine administered after a second dose of antigen had no inhibitory effect on the production of hemolysin plaque-forming cells within a 14-day period of observation; cyclophosphamide and vinblastine, however, effectively suppressed hemolysin plaque-forming cells in a similar injection sequence. We speculated that this "unresponsiveness" of antibody-forming cells to these agents was due, in part, to the formation of precursor "memory" cells.[3]

Interpretation of the results *in vivo* is hindered by the possibility of interchange of cells from one lymphoid organ to another. Thus, for example, cells from other lymphoid organs may contribute to the measured effect on hemolysin plaque-forming cells of spleen tissue. The interchange of cells from tissues other than spleen was eliminated in our present studies *in vitro*. A more suitable biological test system to study drug effects on antibody formation would be a system *in vitro* in which cultured cells would respond to antigen, undergo cell proliferation, and mature into cells that are immunologically competent. Mishell and Dutton[4] have recently introduced a

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technique whereby dispersed cell suspensions obtained from spleens of normal mice were immunized *in vitro* to heterologous red cells to produce hemolysin plaque-forming cells. Utilization of this technique introduces the possibility of investigating drug effects on various phases of antibody formation. This communication deals with effective concentrations of anti-tumor agents which inhibit the production of antibody-forming cells *in vitro*.

MATERIALS AND METHODS

Male LAF_1 (C57L $\times$ A/He) mice purchased from Jackson Memorial Laboratory, Bar Harbor, Me., between the ages of 8 and 16 weeks, were used for these experiments. Fetal calf serum, lyophilized guinea pig complement and sheep red blood cells were obtained from Colorado Serum Company, Denver, Colo. Concentrates of vitamins, essential amino acids, nonessential amino acids, glutamine and sodium pyruvate were obtained from Gibco, Grand Island, N.Y.

Cell culture conditions for the induction *in vitro* of hemolysin plaque-forming cells were described by Mishell and Dutton.[4] Procedures for the determination of hemolysin plaque-forming cells by the assay of Jerne *et al.*[5] are given in an earlier study.[2] As a routine procedure, dispersed spleen cells were planted, drugs were added on the second day, and cells were harvested and assayed for hemolysin plaque-forming cells on the fourth day after planting. At least two experiments were performed in replicate cultures for each drug concentration. Each experiment represented dispersed spleen cells pooled from three to four mice. Cell concentrations were adjusted to $1{\cdot}5 \times 10^7$ cells per ml of culture fluid.

All drugs used for the experiments were dissolved in Hank's balanced salt solution (Gibco) in such a manner that 10 μl added to 1·0 ml of the culture gave a final concentration as specified in the text. All drugs were added 2 days after cell cultures were started. Sodium amethopterin (MTX) was obtained from Lederle Laboratories, mechlorethamine HCl (HN_2) from Merck, Sharp & Dohme, 6-mercaptopurine (6-MP) from Burroughs Wellcome & Company, vinblastine (VBL) from Eli Lilly & Company, and 5-fluorouracil (5-FU) from Roche.

RESULTS

Studies on the effect of five anti-tumor agents are shown in Figs. 1 and 2. Various concentrations of anti-tumor drugs were tested on cultures of dispersed spleen cells. The measured effect of each drug concentration is given in terms of molarity and also in grams per milliliter of culture fluid. Also listed on the graphs for each drug is the average control value, expressed as hemolysin plaque-forming cells (HPFC) per 10^6 cultured spleen cells. Studies on the effect of amethopterin (MTX) are shown in Fig. 1a. Our previous studies *in vivo* indicated that amethopterin can effectively suppress spleen antibody-producing cells of mice receiving one injection of antigen.[1, 2] Our present studies *in vitro* indicated that 50 per cent reduction of HPFC was obtained at a concentration of about $2{\cdot}5 \times 10^{-6}$ M and that a concentration range of some 400-fold ($0{\cdot}25$ to 100×10^{-6} M) was required to decrease HPFC from 50 per cent to less than 10 per cent of control numbers.

Previous studies *in vivo* indicated the lack of inhibitory effect of 6-mercaptopurine on both direct and developed spleen HPFC at 14 days after antigen injection, although

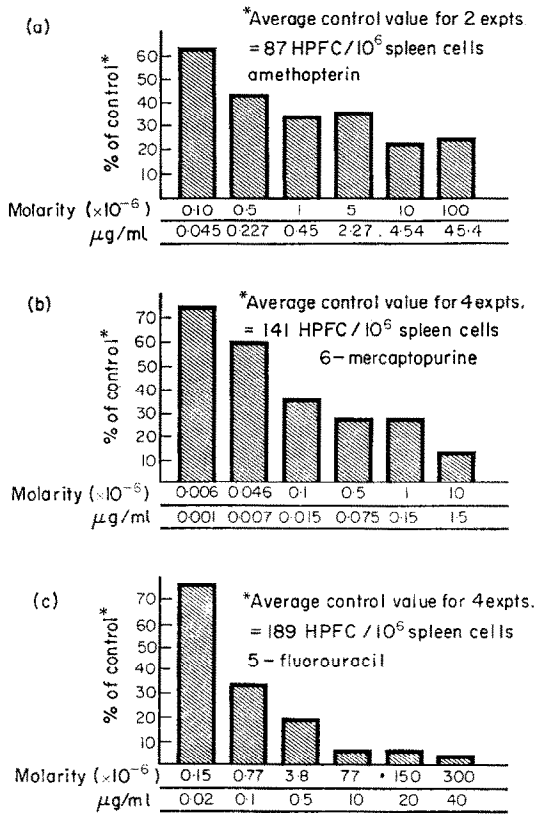

FIG. 1. Drug concentration of (a) amethopterin, (b) 6-mercaptopurine, and (c) 5-fluorouracil inhibiting the production of hemolysin plaque-forming cells (HPFC) *in vitro*. Drug concentrations are expressed in terms of molarity and micrograms per milliliter of culture media. Each bar graph represents the average value of experiments performed at that concentration.

the drug administered 3 days after antigen had a marked inhibitory effect on spleen HPFC and cellularity of the primary response.[1, 2]

Our present findings *in vitro* (Fig. 1b) indicated that 50 per cent inhibition was obtained at a concentration of about $0{\cdot}06 \times 10^{-6}$ M, and that a concentration range of some 160-fold ($0{\cdot}06$ to 10×10^{-6} M) was required to decrease HPFC from 50 per cent to less than 10 per cent of control numbers.

Merrit and Johnson[6] have shown that 5-fluorouracil enhanced the immune response as determined by serum antibody titers. In addition, the adjuvant effect was found to be time dependent; 5-fluorouracil inhibited the antibody response when given 1 or 2 days after antigen administration and enhanced antibody formation when given 1 hr before or 4 days after the antigen. Our present studies *in vitro* (Fig. 1c) indicated that 50 per cent inhibition is obtained at approximately $0{\cdot}10 \times 10^{-6}$ M and that a concentration range of some 100-fold ($0{\cdot}1$ to 10×10^{-6} M) is required to decrease HPFC from 50 per cent to less than 10 per cent of control numbers.

Our studies *in vivo*[1, 2] indicated that vinblastine is effective in suppressing the

formation of direct and developed spleen HPFC of mice when administered at different times after antigen injection. The results of our present experiments *in vitro* (Fig. 2a) indicated that the production of HPFC was inhibited by low concentrations of vinblastine ($0{\cdot}007 \times 10^{-6}$ M or $0{\cdot}006$ μg/ml). This dose was lower than that used by Lettre[7] (0·01 to 0·05 μg/ml) to arrest mitoses in fibroblast cultures after 24 hr of treatment. In our studies, a 2-fold range ($0{\cdot}007$ to $0{\cdot}015 \times 10^{-6}$ M) was required to decrease HPFC from 50 per cent to less than 10 per cent of control numbers.

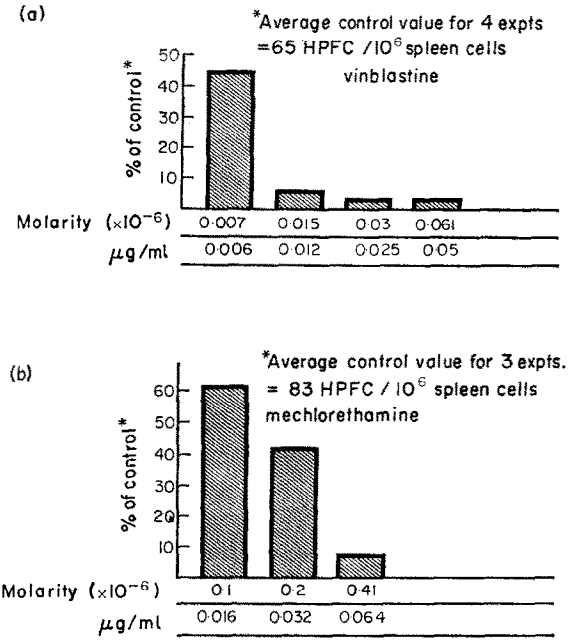

FIG. 2. Drug concentrations of (a) vinblastine and (b) mechlorethamine inhibiting the production of HPFC *in vitro*. See legend of Fig. 1 for other details.

Previous studies *in vivo* have indicated that cyclophosphamide, an alkylating agent, was markedly effective in inhibiting both direct and developed spleen HPFC regardless of the timing of drug injection relative to antigen injection.[2] Since cyclophosphamide must be converted to an active metabolite *in vivo*,[8] mechlorethamine (HN_2) was substituted in our studies *in vitro* to determine the effect of alkylating agents. The results of this study, as shown in Fig. 2b, showed that 50 per cent inhibition was obtained by concentrations of about $0{\cdot}2 \times 10^{-6}$ M and that a dose range of 2- to 3-fold was required to reduce HPFC from 50 per cent to less than 10 per cent of control values.

DISCUSSION

Two observations are worthy of note in our studies on the effects of anti-tumor agents on dispersed spleen cells which were antigenically stimulated *in vitro*. First, the technique provides a sensitive biological test system to study drug alterations of the normal pattern of cytodifferentiation to mature HPFC. Effective drug concentrations

employed in this study, i.e. the dose required to obtain 50 per cent inhibition, are well within the physiological range. Since these drugs were added 2 days after cultures were initiated, we have assumed that they exert their inhibitory effects on the active proliferative stage of the cultured cells. Studies are in progress dealing with the effects of drugs on the early and late stages of the 4-day culture period.

A second and unexpected observation was the finding that the five drugs used in this study can be conveniently, if arbitrarily, classified into two groups on the basis of drug concentration ranges required to inhibit HPFC from 50 per cent to less than 10 per cent of control values. As indicated in Results, vinblastine and mechlorethamine required a narrow range (between 2- to 3-fold), whereas amethopterin 6-mercaptopurine and 5-fluorouracil required a wide dose range (between 100- and 400-fold). This is graphically illustrated in Fig. 3 in which the degree of inhibition (expressed as per cent of control) is plotted against the log of multiples of initial drug concentrations. Thus, vinblastine and mechlorethamine show a steep slope of inhibition, whereas amethopterin, 6-mercaptopurine and 5-fluorouracil shows a gradual slope.

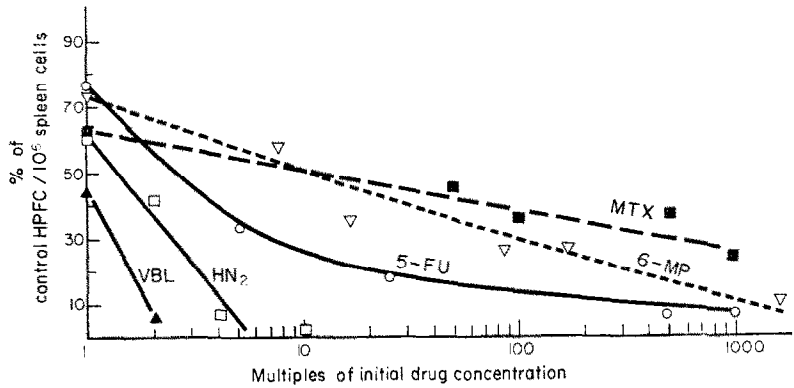

FIG. 3. Comparison of drug concentration ranges required to inhibit HPFC. Drug concentrations of each drug are expressed as multiples of initial drug concentration. Inhibitory effects of the drugs are expressed as per cent of control.

It is tempting to explain the immunosuppressive efficacy of these agents, *in vivo*, in light of these findings. Our previous studies *in vivo* established that mice receiving a second injection of antigen and drugs developed "resistance" to the immunosuppressant effects of 6-mercaptopurine and amethopterin, whereas they did not for vinblastine and cyclophosphamide (a nitrogen mustard analogue). Although alternative explanations are not excluded, a simple interpretation of the data is to suggest that nitrogen mustards and vinblastine can more effectively kill "seeding" cells than either amethopterin or 6-mercaptopurine. Conversely, because of the wide ranges required to kill cells, amethopterin and 6-mercaptopurine cannot effectively kill "seeding" cells and, as drug levels become less than optimal, antibody-producing cells proliferate, resulting in "escape" from immunosuppression. Ideally then, all "seeding" cells must

be destroyed or a clone of immunocompetent cells may arise from a few such cells. This interpretation is analogous to Skipper's[9] to prevent "resistance development" of cancer cells to anti-tumor agents; that is, unless a complete kill of "seeding" cells is accomplished, tumor cells may eventually repopulate the host.

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