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Potentiation of the Bleomycin, Arabinofuranosylcytosine and Adriamycin-caused Inhibition of DNA Synthesis in Lymphocytes by Bestatin *in Vitro**†

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Abstract—*Combinations of* 1-β-D-*arabinofuranosylcytosine (araC), bleomycin (BLM) or adriamycin (ADM) with the dipeptide bestatin do not result in an enhanced* in vitro *cytotoxicity in the macrophage-free L*5178y *mouse lymphoma cell system. However, in macrophage-containing murine spleen lymphocytes bestatin causes a potentiating effect of the cytostatic drugs araC, BLM and ADM with respect to their potencies to inhibit DNA synthesis. In the presence of* 1 μg *bestatin/*ml, *the* ED_{50} *concentrations causing a* 50% *reduction of* [^{3}H]*dThd incorporation were significantly lowered;* 4.3*-fold in the studies with araC and BLM, and* 1.8*-fold in the experiments with ADM. Bestatin, given alone, displays a stimulating effect on* [^{3}H]*dThd incorporation into macrophage-containing lymphocyte cultures within the concentration range* 0.1–10 μg/ml. *In contrast to the bestatin-stimulated lymphocytes, ConA-stimulated as well as LPS-stimulated lymphocytes do not show a higher sensitivity to the selected drugs inhibiting DNA synthesis. These data should encourage the practical use of bestatin in combination with araC, BLM or ADM in cancer treatment.*

INTRODUCTION

In vitro studies indicate that bleomycin (BLM) [1], 1-β-D-arabinofuranosylcytosine (araC) [2] and adriamycin (ADM) [3] are lethal to those cells passing the DNA-synthetic phase of the cell cycle. Biochemical data suggest that BLM produces this lethality by DNA fragmentation [4]. On the other hand, araC (converted to the 5′-triphosphate derivative) acts as an inhibitor of DNA polymerases [1, 5], while ADM was found to bind to the DNA [6]. The antitumor effects of these antibiotics *in vivo* are enhanced by a simultaneous administration of bestatin [7]. This dipeptide [8] was found to interact with the cell surface-bound leucine aminopeptidase, preferentially in macrophage systems [9], resulting in a mitogenic response in T-lymphocytes via an as yet unknown mechanism [10, 11].

The studies reported here examine the effect of bestatin *in vitro* on the cytotoxicity of BLM, araC and ADM in the L5178y mouse lymphoma cell and murine spleen cell systems.

MATERIALS AND METHODS

Compounds

The clinical mixtures of BLM (containing 55–70% A_2; 25–32% B_2; ⩽7% A_2; and ⩽1% B_4) and araC were obtained from H. Mack, Illertissen (F.R.G.); ADM was from Deutsche Farmitalia, Freiburg (F.R.G.); bestatin was prepared by Nippon Kayaku Co., following the instructions of one of the authors [8].

Concanavalin A (ConA; No. C7275) and lipopolysaccharide (LPS; No. L4130) were obtained from Sigma, St. Louis, MO (U.S.A.), and

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[methyl-^{3}H]thymidine (sp. act. 78 Ci/mmol) was from the Radiochemical Centre, Amersham (U.K.).

Lymphoma cells and cultivation

L5178y mouse lymphoma cells, a Thy-1,1-bearing, Fc receptor-positive T cell lymphoma line [12], were obtained through the courtesy of Dr G. A. Fischer, Yale University (New Haven, CT, U.S.A.). The cells were grown in minimum essential medium (Spinner modification), supplemented with 10% horse serum [13]. For dose-response experiments the cultures (5 ml) were initiated by inoculation of 5×10^3 cells/ml. They were incubated at 37°C in roller tubes for 72 hr; during this period the controls reached a cell concentration of 5.7×10^5/ml. The ED_{50} was estimated by logit regression [14]. In some experiments the cell proliferation is given in doubling steps [15].

Cultivation of lymphocytes from spleen

Spleen cells were prepared from 5- to 6-week-old male outbread NMRI mice. They were suspended at a density of 1.5×10^7 cells/ml of RPMI 1640 medium, supplemented with 20% fetal calf serum, as described [16]. Two populations of spleen cells were tested: (a) macrophage-containing cell populations; and (b) macrophage-depleted cultures; they were prepared as described [10]. Cells (5×10^5) were placed in a final volume of 200 μl in cups of sterile flat-bottomed microtiter plates (Costar No. 3596) and incubated for 72 hr; 18 hr prior to the end of the incubation 0.2 μCi of [^{3}H]dThd was added to each cup [16]. Where indicated 2 μg/ml of ConA or 20 μg/ml of LPS were added to the cultures. The cytostatic agents and bestatin were added at time 0. Incorporation of dThd was determined as described [16].

Each experiment was done in quadriplicate. The ED_{50} concentrations causing a 50% reduction of [^{3}H]dThd incorporation were estimated by logit regression [14].

Statistical evaluation

t-tests to determine the significance of the growth inhibition effects at different drug combinations were performed according to Student [14].

RESULTS

Cytostatic effect of araC, BLM and ADM on L5178y cells in combination with bestatin

As known from previous *in vitro* studies [17], bestatin increases RNA synthesis in L5178y cells cultivated in the presence of macrophages. In the present investigation it was found that bestatin had no influence on proliferation of L5178y cells grown in the absence of macrophages. Within the concentration range 0–10 μg bestatin/ml the extent of the cell proliferation, expressed in doubling steps, varied insignificantly between 6.83 ± 0.48 and 6.75 ± 0.41 (data not shown).

The inhibitory potencies of the cytostatic agents araC, BLM and ADM on growth of L5178y cells (in the absence of macrophages) were found not to be influenced by the presence of different concentrations of bestatin (Table 1). The ED_{50} concentrations estimated from dose-response experiments (72 hr) in the case of araC varied insignificantly around a value of 0.050 μg/ml ($P \geqslant 0.1$), in the case of BLM around 1.40 μg/ml ($P \geqslant 0.1$) and for ADM around 0.038 μg/ml ($P \geqslant 0.1$).

Differential effect of bestatin on the inhibitory potency of araC, BLM and ADM on murine lymphocytes in vitro

The effect of bestatin, added in combination with the cytostatic agents araC, BLM and ADM, on DNA metabolism of lymphocytes was determined both in the presence and in the absence of macrophages. The dThd incorporation rate was chosen as a parameter for the activation state of the cells.

In the presence of macrophages, bestatin activated cell metabolism within the concentration range 0.1–10 μg/ml (Fig. 1A). In the absence of bestatin, the dThd incorporation rate was only 40.4×10^3 cpm/18 hr, while in the presence of 1 μg bestatin/ml the incorporation rate increased to 56.0×10^3 cpm/18 hr. In contrast to these results, incorporation studies with lymphocyte

Table 1. Influence of araC, BLM and ADM on growth of L5178y mouse lymphoma cells in the absence or the presence of different concentrations of bestatin

Cytostatic agent	Addition of bestatin (μg/ml)	ED_{50} on cell growth (μg/ml)
araC	0	0.051 ± 0.004
	0.1	0.052 ± 0.004
	1.0	0.047 ± 0.003
	10.0	0.049 ± 0.004
BLM	0	1.42 ± 0.09
	0.1	1.34 ± 0.08
	1.0	1.39 ± 0.08
	10.0	1.43 ± 0.10
ADM	0	0.034 ± 0.003
	0.1	0.042 ± 0.003
	1.0	0.039 ± 0.003
	10.0	0.035 ± 0.003

Each value represents the mean of 10 independent experiments (the S.D. is indicated).

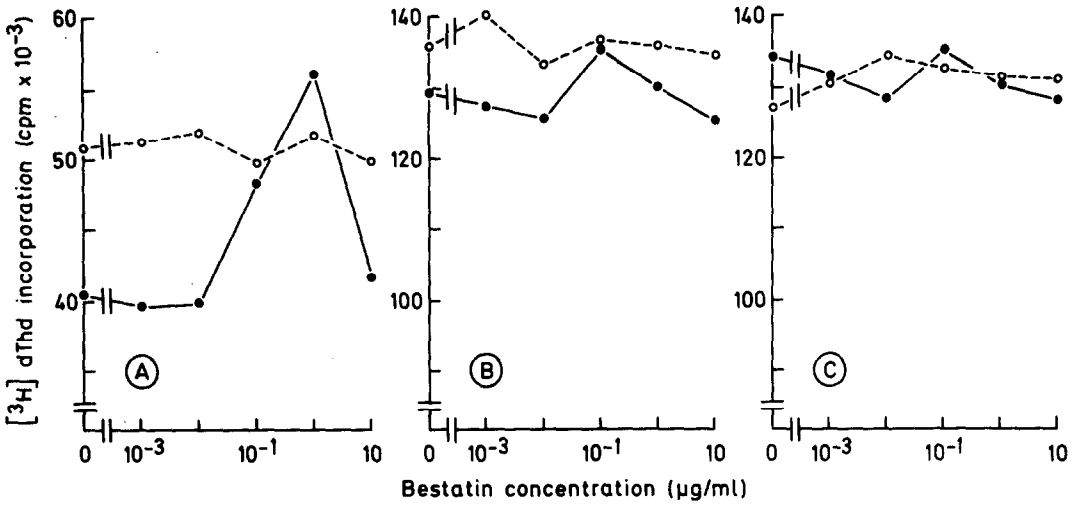

Fig. 1. Effect on different bestatin concentrations on [³H]dThd incorporation (cpm/assay) into lymphocytes, treated in the absence [A] or the presence of mitogens (ConA [B]; LPS [C]). Lymphocyte cultures were used which were either depleted from macrophages (O — — O) or contained macrophages (●———●). The values given are means of 4 independent determinations; the standard deviation did not exceed 10%.

cultures, depleted from macrophages, revealed no significant changes in dependence on the presence of bestatin (Fig. 1A). As in previous experiments, using the same experimental techniques, the dThd incorporation rate was greater in macrophage-depleted cultures than in macrophage-containing cultures [18, 19]. The incorporation rates of lymphocytes, stimulated by the mitogens ConA or LPS, vary only insignificantly in response to bestatin, irrespectively of the presence or the absence of macrophages (Fig. 1B, C).

Detailed dose-response experiments were performed with bestatin in combination with the selected cytostatic agent. As shown in Fig. 2(A), the ED_{50} inhibitory concentration for araC in macrophage-containing lymphocytes was determined to be 0.034 ± 0.002 µg/ml in the absence of bestatin. However, addition of increasing concentrations of bestatin resulted in a significant decrease of the concentrations, causing a 50% reduction of dThd incorporation rates (0.1 µg bestatin/ml: 0.016 ± 0.001 µg/ml; *P* value vs control: ≤0.001; 1 µg/ml: 0.008 ± 0.001 µg/ml; *P* value vs control: ≤0.001); see Table 2. The same effects of bestatin on the dThd incorporation rates of lymphocytes were also determined for the two other cytostatic agents chosen, BLM and ADM. In

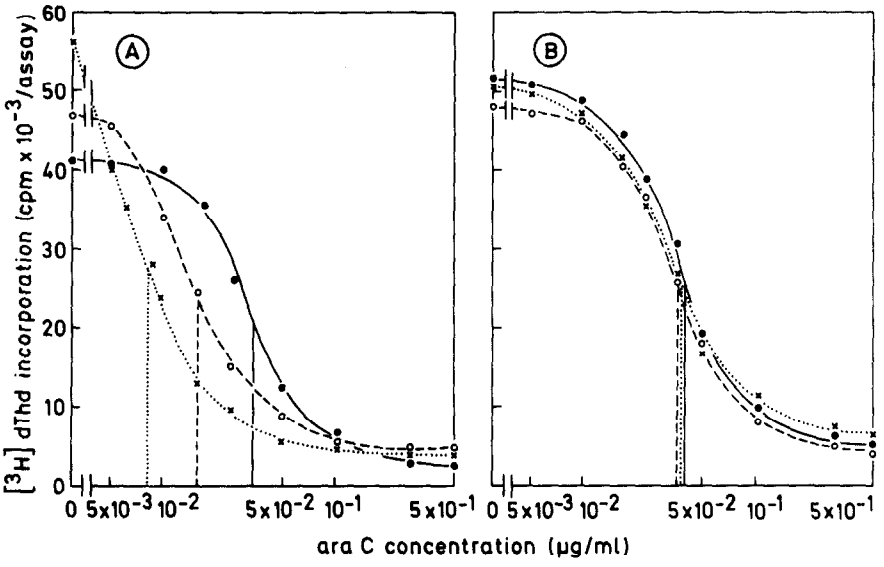

Fig. 2. Effect of araC on [³H]dThd incorporation into lymphocytes in the presence of 0 (●———●), 0.1 (O — — O) and 1 µg bestatin/ml (×·····×). (A) Influence on macrophage-containing lymphocyte cultures; (B) influence on macrophage-depleted cultures. Means of quadriplicate experiments are presented; the S.D. were less than 10%. The values for a 50% inhibition of [³H]dThd incorporation of the corresponding dose-response experiments are given as vertical lines; araC in the presence of 0 (———), 0.1 (— — —) or 1 µg bestatin/ml (.....).

the case of BLM the inhibitory potency (expressed by the ED_{50} concentration of dThd incorporation) increased from 0.61 ± 0.05 μg/ml (in the absence of bestatin) to 0.14 ± 0.1 μg/ml (in the presence of 1 μg/ml of bestatin); the differences are highly significant, with a P value vs control of ≤0.001 (Table 2). In the experiments with ADM a similar significant increase of the inhibition was determined (P value vs control: ≤0.001). In macrophage-depleted cultures the ED_{50} values remained unchanged even in the presence of bestatin, and were determined to be: 0.039 ± 0.003 μg/ml [in the dose-response experiments with araC; Fig. 2(B)], 0.64 ± 0.05 μg/ml (for BLM) and 0.14 ± 0.01 μg/ml (for ADM) (data not shown).

The observed increase of the inhibitory potency of the cytostatic agents in the presence of bestatin was not found in those macrophage-containing lymphocyte cultures which were activated with the mitogens ConA or LPS (Table 2). The P values vs controls in the different combinations were estimated to be greater than 0.1.

DISCUSSION

After administration of bestatin to mice an increase of [^{3}H]dThd incorporation into DNA in T lymphocytes has been observed [20]. This effect has been attributed to a bestatin-caused induction of DNA polymerase α activity, as determined by both *in vitro* [11] and *in vivo* studies [20]. The increase of [^{3}H]dThd incorporation by bestatin is observed only in those cultures which contain macrophages [10]. Based on these results it can be expected that bestatin positively influences the antitumor effect of those cytostatic agents which interfere with DNA synthesis, especially in slow-growing tumors. *In vivo* studies of Ishizuka *et al.* [18] support this assumption by the demonstration of bestatin displaying a marked antitumor effect on IMC carcinoma and Gardner's lymphosarcoma.

The presented data on the effect of bestatin on L5178y mouse lymphoma cells *in vitro* (in the absence of macrophages) revealed no significant influence on growth of these cells. This result is in agreement with earlier findings [17] showing that the activity of bestatin against L5178y cells requires macrophages. Given bestatin in combination with the cytostatic agents BLM, araC or ADM, no additional lethality on L5178y cells to that observed for the drugs alone was found. These data imply that bestatin displays no growth effect on L5178y cells.

The interaction of bestatin with the drugs inhibiting DNA synthesis was investigated in a second cellular system whose DNA metabolism is known to be modulated by bestatin [10]. These published data, which are confirmed in the present study, establish that bestatin causes a mitogenic effect on spleen lymphocytes in the presence of macrophages. Previous *in vitro* [10, 19] and also *in vivo* experiments [21] have clearly demonstrated that the mitogenic action of bestatin is directed towards T cells, and the generation of this action requires macrophages. As now reported, the bestatin-activated lymphocytes are more susceptible to an inhibitory influence by the cytostatic agents BLM, araC and ADM than those lymphocyte cultures which are not treated with bestatin. It seems to be important to note that in contrast to bestatin-stimulated lymphocytes, ConA- as well as LPS-stimulated lymphocytes do not show a higher sensitivity to these drugs. Thus the mitogenic response of T or B lymphocytes to

Table 2. Determinations of the inhibitory potencies of araC, BLM and ADM on macrophage-containing lymphocyte cultures, in the absence or the presence of two selected bestatin concentrations

Cytostatic agent	Addition of bestatin (μg/ml)	ED_{50} on [^{3}H]dThd incorporation (μg/ml) No mitogen	+ConA	+LPS
araC	0	0.034 ± 0.002	0.073 ± 0.005	0.081 ± 0.006
	0.1	0.016 ± 0.001	0.069 ± 0.004	0.075 ± 0.005
	1.0	0.008 ± 0.001	0.072 ± 0.005	0.082 ± 0.006
BLM	0	0.61 ± 0.05	0.93 ± 0.07	0.88 ± 0.07
	0.1	0.27 ± 0.02	0.87 ± 0.06	0.92 ± 0.07
	1.0	0.14 ± 0.01	0.88 ± 0.06	0.87 ± 0.07
ADM	0	0.14 ± 0.01	0.20 ± 0.01	0.19 ± 0.01
	0.1	0.10 ± 0.01	0.19 ± 0.01	0.18 ± 0.02
	1.0	0.08 ± 0.01	0.18 ± 0.02	0.19 ± 0.02

The values for the 50% reduction of [^{3}H]dThd incorporation into lymphocytes were calculated as described under Materials and Methods and in the legend to Fig. 2. The lymphocyte cultures were assayed in the absence or the presence of the two mitogens ConA and LPS.

the specific mitogens ConA or LPS is distinguished, at least in this respect, from the T lymphocyte activation caused by the bestatin/macrophage-triggering system. First experimental evidence already indicates that bestatin causes a stimulation of interleukin production in macrophages [22].

The reported fact that bestatin amplifies the cytostatic activity of BLM, araC and ADM in those cell systems which respond to bestatin with an increase of DNA synthesis should encourage the practical application of this dipeptide in combination with inhibitors of DNA synthesis in cancer treatment.

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