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Immunosuppressive effects of (2R,5R)-6-heptyne-2,5-diamine an inhibitor of polyamine synthesis: I. Effects on mitogen-induced immunoglobulin production in human cultured lymphocytes

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SUMMARY

The consequences of specific inhibition of polyamine biosynthesis by (2R,5R)-6-heptyne-2,5-diamine (MAP) a potent inhibitor of L-ornithine decarboxylase (ODC), on immunoglobulin (Ig) production were studied in cultured human peripheral blood lymphocytes stimulated with pokeweed mitogen (PWM). MAP inhibits the usual PWM-induced increase of polyamine (putrescine, spermidine and spermine) concentrations and reduces concomitantly cell replication. In parallel with these biochemical effects, IgG and IgM production are diminished, a 95% decrease being observed at 100 μM MAP concentration. That the suppressive effects of the ODC inhibitor result from polyamine deficiency, and not from unrelated pharmacological effects, is demonstrated by the restoration of normal Ig production when 10 μM putrescine or spermidine are added to the culture medium. These findings established that the cellular immunological response can be affected by specific inhibition of polyamine biosynthesis and deserve further consideration both under *in vitro* and *in vivo* conditions.

Keywords Human lymphocytes (2R,5R)-6-heptyne-2,5-diamine inhibition of polyamines immunoglobulins

INTRODUCTION

The polyamines, spermidine and spermine and their precursor putrescine, have been implicated in numerous processes associated with cell growth and cell differentiation (Pegg, 1986). In particular, induction of L-ornithine decarboxylase (ODC) (EC 4.1.1.17), one of the rate-limiting enzymes of the polyamine biosynthetic pathway, and increased rates of polyamine biosynthesis are among the earliest events associated with T lymphocyte activation induced by mitogens and lymphokines (Kay & Lindsay, 1973; Fillingame, Jorstad & Morris, 1975; Fidelius, Laughter & Twomey, 1984). These biochemical events also occur following induction of proliferation and differentiation of B lymphocytes into antibody-forming cells (Watanabe *et al.*, 1975). Further studies, using DL-α-difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC (Metcalf *et al.*, 1978), provide evidence for the requirement of polyamines in lymphocyte and lymphokine-dependent cell growth (Seyfried & Morris, 1979; Hölltä, Jänne & Hovi, 1979; Bowlin, McKown & Sunkara, 1986) and for stimulated immunoglobulin production *in vitro* (Pasquali *et al.*, 1984). DFMO, administered *in vivo*, causes marked and selective changes in the kinetics of the specific humoral and cellular responses against tumour allograft challenge (Ehrke *et al.*, 1986). Altogether these findings support the notion that the immune responses can be modulated by manipulation of polyamine synthesis.

In our previous *in vitro* studies (Pasquali *et al.*, 1984), DFMO has been shown to block the usual increase of polyamine contents of pokeweed mitogen (PWM)-stimulated human lymphocytes and to reduce concomitantly DNA synthesis and immunoglobulin (Ig) production. However, in those conditions, DFMO does not entirely suppress the elevation of the spermine content and of Ig production. These effects are presently achieved using (2R,5R)-6-heptyne-2,5-diamine (MAP) a more potent and more effective inhibitor of ODC than DFMO *in vitro* (Danzin *et al.*, 1983; Mamont *et al.*, 1984; Casara *et al.*, 1985) and *in vivo* (Danzin *et al.*, 1983; Bartholeyns, Mamont & Casara, 1984). As a consequence, MAP, used at 1/50 the concentration, causes stronger immunosuppression than DFMO. Moreover, it is demonstrated that the suppressive effect of MAP results from its polyamine-depleting activity and not from unrelated pharmacological effects of the drug.

MATERIALS AND METHODS

Cell source and preparation

Peripheral blood specimens from 12 normal donors (medical

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students or employees at the hospital) were drawn into individual vials containing heparin. Mononuclear cells were isolated from these samples by passage through Ficoll-hypaque gradients (Böyum, 1968) and were washed three times in serum-free RPMI-1640 medium. Platelets were then removed by low speed centrifugation.

Culture conditions

After being washed 1×10^6 cells were cultured in 1 ml of medium supplemented with heat-inactivated fetal calf serum (FCS; 56°C for 30 min), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (Eurobio, Paris) in 12×75 mm plastic tubes (Falcon Plastics, Oxnard, CA, USA). To induce cell proliferation, the optimal concentration, 0·1 μg/ml, of PWM (Sigma Chemical, St Louis, MO, USA) was added to the cell culture medium. MAP, synthesized as described by Casara *et al.* (1985), was dissolved in medium without FCS and added to the culture medium simultaneously with PWM. For Ig quantification, duplicate cultures were incubated for 8 days in a humidified incubator under CO_2/air atmosphere (5%, v/v) at 37°C. The cell viability was judged by trypan blue exclusion.

Radioimmunoassay for immunoglobulin quantification

Supernatants from lymphocyte cultures were assayed for IgM and IgG with a solid phase radioimmunoassay, essentially as described by Pasquali *et al.* (1984). Vinyl chloride microtitration plates (Cooke Laboratories, No. 220-24, Alexandria, VA, USA) were coated for 4 h at room temperature with 100 μl/well of 30 μg/ml affinity-purified goat anti-human IgM or anti-human IgG, respectively. The unbound sites were then blocked by the addition of 1% bovine serum albumin (BSA) in borate-buffered saline (BBS). One hundred microlitres of undiluted culture supernatant fluid or 1% BSA in BBS were added to replicate wells. After overnight incubation at 4°C, the wells were washed twice with BBS, and then incubated for 4 h with 100 μl ^{125}I-labelled goat anti-human IgM (10^6 ct/min/ml) or ^{125}I-labelled goat anti-human IgG (10^6 ct/min/ml) (to detect IgM and IgG, respectively) in 1% BSA, iodinated by the chloramine T method. After washing, the wells were cut out and radioactivity measured in a γ-counter. The ct/min bound were converted to mass of IgM or IgG using standard curves derived by assaying monoclonal antibody or a mixture of IgM proteins or affinity purified human IgG.

Proliferation assay

Three days after cultures were started, cells were pulse-labelled for 3 h in the presence of 5 μCi/ml of (methyl-^{3}H)thymidine (37 GBq/mmol; CEA, Saclay, France). The cultures were then filtered through Millipore filters (Millipore, France). The filters were washed twice with saline buffer, twice with 5% trichloracetic acid, twice with 95% ethanol and once with absolute ethanol. The filters were dried and the radioactivity determined (Robbins *et al.*, 1972).

Determination of polyamines

Three days after initiation of the cultures, 10^7 cells (pooled from 10 identical culture tubes) were disrupted by sonication in 0·7 ml of 0·1 N HCl. After precipitation of proteins by 0·2 N $HClO_4$, polyamine analyses were performed directly on 200 μl of the supernatants by reverse-phase ion-pair high performance liquid chromatography as described by Seiler & Knödgen (1980).

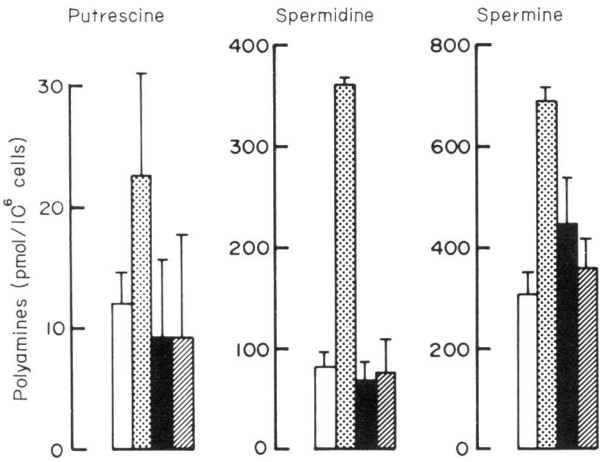

Fig. 1. Effect of MAP on the polyamine content of PWM-stimulated human lymphocytes. Determinations were made 3 days after MAP was added. Results are the averages of three separate experiments ± s.e.m. (□) Control; (▨) PWM; (■) PWM + 50 μM MAP; (▩) PWM + 100 μM MAP.

RESULTS

Effects of MAP on (^{3}H)thymidine incorporation and polyamine content of PWM-stimulated lymphocytes

After the lymphocytes were incubated for 3 days in the presence of PWM, the rate of (^{3}H)thymidine incorporation into lymphocyte DNA was increased 38-fold relative to unstimulated cells (unstimulated, $0{\cdot}02 \times 10^{-5}$ ct/min/10^6 cells; PWM, $0{\cdot}76 \times 10^{-5}$ ct/min/10^6 cells). MAP, added to the culture medium at the time of PWM stimulation, blocked (^{3}H)thymidine incorporation, 80% inhibition being observed at 100 μM concentration after a 3-day incubation period. At the same time, 100 μM MAP completely suppressed the usual increase of intracellular putrescine (1·9-fold), spermidine (4·2-fold) and almost completely that of spermine (2·2-fold) induced by PWM stimulation (Fig. 1).

Effects of MAP on Ig production by PWM-stimulated lymphocytes

The concentration-related effects of MAP on IgG and IgM production, determined at day 8, are illustrated in Fig. 2. The ID_{50} for MAP was about 10 μM. When compared to the concentration of DFMO needed to exert identical effects (Pasquali *et al.*, 1984), MAP was 25 times more effective. Moreover, when used at 100 μM concentration, MAP suppressed entirely the increase of Ig production. This effect is not achieved by DFMO used at 50-times higher concentration (Pasquali *et al.*, 1984). No apparent effect of MAP (100 μM) on cell viability was observed as judged by trypan blue exclusion (control and MAP-treated cells: 70 ± 8 and 72 ± 10 percent viable cells respectively at day 8).

Protection by putrescine or spermidine of the effects of MAP on Ig production

Restoration of the intracellular polyamine content of MAP-induced polyamine deficient rat hepatoma cells by exogenous

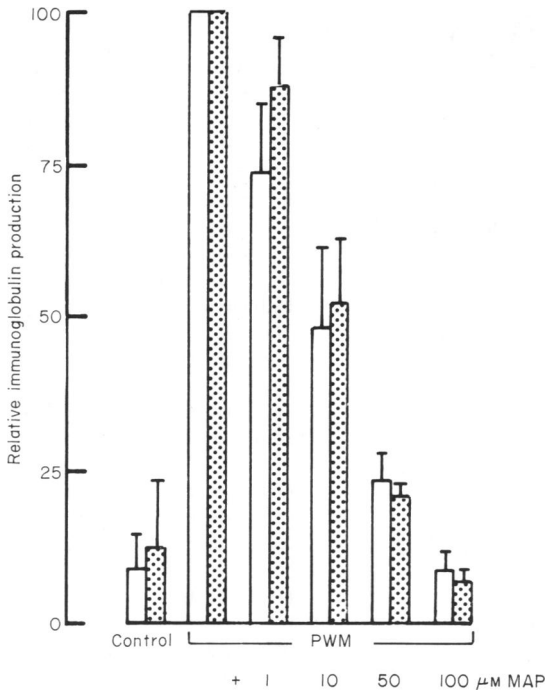

Fig. 2. Effect of MAP on PWM-stimulated IgG and IgM production. Determinations were made 8 days after addition of the drug. Results are expressed as % of maximum IgM and IgG concentrations in media of PWM-stimulated cultures and are the averages of duplicate experiments performed with lymphocytes of 12 normal donors ± 1 s.d. Control refers to IgG and IgM concentrations in media of unstimulated cultures which were 305 ± 80 and 190 ± 70 ng/ml, respectively. (□) IgG; (▨) IgM.

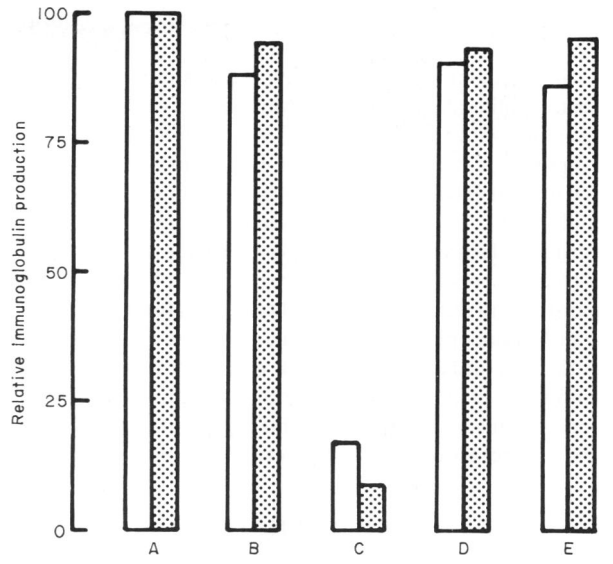

Fig. 3. Protection by putrescine or spermidine of the effects of MAP on PWM-stimulated IgG and IgM production. PWM-supplemented lymphocyte cell cultures were incubated from time 0 in the presence or absence of 50 μM aminoguanidine (AG). 10 μM putrescine (PUT) or 10 μM spermidine (SPD) were added concomitantly with 100 μM MAP. IgM and IgG determinations were performed at day 8. Results are the means of duplicate cultures and are expressed as % of maximum IgG and IgM concentrations in media of PWM-stimulated cultures which were 2540 and 3034 ng/ml, respectively. Identical samples differed by no more than 10%. (□) IgG; (▨) IgM. A, PWM; B, PWM + 50 μM AG; C, PWM + 50 μM AG + 100 μM MAP; D, PWM + 50 μM AG + 100 μM MAP + 10 μM PUT; E, PWM + 50 μM AG + 100 μM MAP + 10 μM SPD.

addition of polyamines to the culture medium abolishes the antiproliferative effects of the ODC inhibitor (Mamont *et al.*, 1984). The same experiment was performed in the present study (Fig. 3). However, it is known that polyamines added to culture media supplemented with FCS inhibit the *in vitro* responses of lymphocyte blastogenesis (Allen *et al.*, 1979). This polyamine toxicity most probably results from oxidation of the amines into aminoaldehydes and hydrogen peroxide, a reaction catalysed by polyamine oxidase activities present in bovine sera (Tabor, Tabor & Bachrach, 1964). To prevent such enzymatic oxidation and its deleterious effects on cells, aminoguanidine (AG), a time-dependent inhibitor of bovine serum polyamine oxidase (Seiler *et al.*, 1983), was added to the culture media. As illustrated in Fig. 3, it was verified that 50 μM AG affected neither the immunological responses to mitogen nor the inhibitory effects of MAP. In those conditions. exogenous addition of 10 μM putrescine, the product of ornithine decarboxylation, or of the polyamine spermidine, completely abrogated the effects of the ODC inhibitor on Ig production.

DISCUSSION

DFMO, an irreversible inhibitor of ODC, blocks polyamine biosynthesis in polyclonal mitogen-stimulated human lymphocytes and concomitantly reduces Ig production (Pasquali *et al.*, 1984). However DFMO suppresses entirely neither the elevation of the intracellular spermine concentrations nor Ig production. These effects are now achieved using MAP, a more potent and more effective inhibitor of ODC than DFMO both *in vitro* and *in vivo*. This new ODC inhibitor not only blocks the mitogen-induced elevation of intracellular putrescine and spermidine concentrations but almost completely inhibits spermine accumulation. Associated with this more profound effect on polyamine biosynthesis, MAP reduces to a greater extent DNA synthesis and Ig production. As shown for other cell culture systems (Mamont *et al.*, 1984), the biological effects of MAP on Ig production can be prevented in putrescine- or spermidine-supplemented media. These results demonstrate that the immunosuppressive effects of MAP do result from polyamine deficiency.

Increase of polyamine biosynthesis is intimately linked to stimulation of macromolecular processes associated with cell multiplication and differentiation. Therefore, it was likely that inhibition of polyamine synthesis should interfere with B lymphocyte activation and differentiation.

B cell activation is a multi-step process in which cells are triggered under the influence of factors and accessory cells to proliferate and differentiate to antibody-forming cells. Since B cell differentiation induced by PWM is dependent on T cell factors (Keightley, Cooper & Lawton, 1976) our present study remains inconclusive with respect to the cellular site of action of the ODC inhibitor. It is known, however, that DFMO reduces the polyclonal induction of cytolytic T lymphocytes by a mechanism which seems not to be entirely dependent on the effects of the ODC inhibitor on T cell proliferation and on IL-2 production (Bowlin, McKown & Sunkara, 1987a). On the

contrary, polyamine biosynthesis is required for IL-2 responsiveness during a primary lymphocytic response (Bowlin *et al.*, 1987b). IL-2, among other factors, induces human B lymphocytes to proliferate and mature (Melchers & Andersson, 1986). By analogy with the observations of Bowlin *et al.* (1987b), one may postulate that inhibition of polyamine biosynthesis by MAP impairs B lymphocyte responses to stimulation by factors, including IL-2. Further investigations, using a more defined experimental system will be needed to provide more insight into the role of polyamines in the regulation of growth, clonal expansion and differentiation of B cells. Whatever the functions that polyamines play in those cellular events, our results indicate that the humoral immune response can be modulated by MAP, an inhibitor of polyamine biosynthesis. In the accompanying report, we will demonstrate that this compond can also produce immunosuppression *in vivo* (Claverie *et al.*, 1988).

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