Elucidation of the Different Effects of Polyamines and Other Naturally-Occurring Inhibitors of Cell Proliferation (Chalones) on T-Lymphocyte and Granulocyte Colony Growth *in vitro*

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Using T-lymphocyte and granulocyte colony assays with truly proliferating cells the effects of the polyamine spermine and of other naturally-occurring inhibitors of cell proliferation have been differentiated. It has been confirmed that spermine, in the presence of fetal calf serum, is a potent inhibitor of cell proliferation. This inhibition could be reversed by the addition of either 3-hydroxybenzyl-oxyamine or 4-bromo-3-hydroxybenzyl-oxyamine, both of which are inhibitors of the polyamine oxidase. In comparison, fractions isolated from calf thymus were shown to inhibit lymphocyte, but not granulocyte colony growth, indicating their tissue specificity and lymphocyte chalone activity. Further this inhibition was not reversed by polyamine oxidase inhibitors demonstrating that polyamines were not the inhibitory principles in this preparation.

Introduction

A number of groups have reported the existence of tissue-specific, species unspecific inhibitors, isolated from lymphoid organs, of mitogen-stimulated and spontaneous lymphocyte proliferation, which act in a reversible, non-cytotoxic manner [1–12]. Factors with such properties meet the definition of chalones [13]. Recently the nature of these inhibitory extracts has been questioned by the demonstration that an inhibitor from porcine thymus was in fact a spermine-protein complex, its inhibitory activity being largely confined to its polyamine moiety [14]. It was suggested that the protein fraction conferred tissue-specificity on the complex which would then also meet the definition of a true chalone. However, this inhibition, also shown by pure spermine, was serum dependent: While human and horse sera were ineffective, the presence of bovine, particularly fetal serum produced drastic inhibition of [^{3}H]-thymidine uptake by lymphocytes [14, 15].

It has been clearly shown that an important prerequisite for the inhibition by spermine is polyamine oxidase [16]. Addition of this enzyme, isolated from bovine serum, to cultures in non-ruminant sera restored the inhibitory effects of exogenous polyamines [16]. It is possible to completely reverse the effect of the enzyme, in the above-mentioned system, by the addition of either 3-hydroxybenzyl-oxyamine or 4-bromo-3-hydroxybenzyloxyamine both of which potently inhibit its action on polyamines [17]. We report here the results of investigations which (a) confirm the effect of polyamines as inhibitors in a truly proliferating *in vitro* cell culture-system; (b) confirm the reversal of this inhibition by inhibitors of polyamine oxidase; (c) demonstrate conclusively that the lymphocyte-specific inhibitor fraction isolated from calf thymus is neither a polyamine, a polyamine-protein complex nor polyamine oxidase.

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Materials and Methods

Chemicals and reagents

Spermine-4-HCl was from Serva, Heidelberg. The sera were from Seromed (München) and Paesel (Frankfurt): Fetal calf serum batch No. 922777; horse serum batch No. 00732. All sera were inactivated at 56 °C for 30 min. Polyamine oxidase inhibitors, 3-hydroxybenzyloxyamine-dihydrogen phosphate (NSD 1024) and 4-bromo-3-hydroxybenzyloxyamine-dihydrogen phosphate (NSD 1055) were purchased from Sandev Ltd. (Harlow, Essex, U.K.).

Assay systems

T-lymphocyte colony assay: The lymphocyte capillary assay was carried out as described previously

[18] with the following modifications. The amount of supplemented autologous plasma was 3 ml and the cell density was adjusted to 1.25×10^6 human lymphocytes/ml and 1.25×10^6 erythrocytes/ml. Phytohemagglutinin (PHA-M, from Difco) was prepared by adding 5 ml saline to one original bottle. After prestimulation the cells were washed only once.

Agar culture

One assay has a total volume of 300 µl including 100 µl "space" for sample or saline; the composition of this space is given separately for each experimental result. The 200 µl culture volume consisted of 23 µl Dulbecco-Eagle medium, 2 µl PHA-M solution, 63 µl autologous plasma, 37 µl lymphocyte suspension and 75 µl bacto agar solution (3.8 ml medium + 1.2 ml 3% liquified bacto agar). The addition followed the above sequence with 100 µl sample or saline being added at the beginning.

Granulocyte colony assay: The granulocyte capillary assay has been described in detail elsewhere [19]. The amounts of the components for a 300 µl assay were: 100 µl sample or saline, 20 µl medium, 80 µl colony stimulating factor (CSF) + horse serum (20 µl CSF + 60 µl horse serum), 25 µl granulocyte suspension (1.2×10^6 cells/ml) and 75 µl agar solution as in the lymphocyte assay. The source of CSF was mouse lung conditioned medium prepared according to [20]. For both assays test cultures were carried out in triplicates, 30 µl/capillary for the lymphocyte assay and 75 µl for the granulocyte assay. At least 6 control capillaries were established for each experiment. Polyamine oxidase inhibitors were added separately to the samples and preincubated for 10 min. Standard error of the mean was well below 5% for both assays and reproducibility was at worst ± 15%. – A colony was defined as an aggregate of > 50 cells. The lymphocyte colonies were counted with the aid of an ocular grid which facilitated differentiation between colonies and clusters.

Preparation of calf thymus extracts: Acetone powder preparation, aqueous extraction, ultrafiltration and gel chromatography were carried out as described previously [21]. In addition the sample was separated on Biogel P6 (from Biorad) in saline and produced a single peak of activity at Kav ~ 0.65. Alternatively an extract was prepared after Houck [25] with ethanol and precipitated with acetone, the precipitate was also chromatographed on Biogel P6 producing a single lymphocyte specific peak at the same Kav. Further details of this procedure will be published elsewhere.

Results

Control experiments

Whilst the assays described can produre reliable results the systems are extremely sensitive, hence the influence of each component was tested.

The addition of fetal calf serum (FCS) alone to our otherwise FCS-free assays had slightly different effects in each assay (Fig. 1). In the lymphocyte (T-LC) assay the addition of FCS produced no significant effects. In the granulocyte (GC) assay the addition of FCS caused significant inhibition of colony growth at volumes of more than 20 µl but there was no inhibition at 20 µl or less. This was acceptable as in the other tests the addition of serum was confined to 20 µl.

The addition of the polyamine oxidase inhibitors 3-hydroxybenzyloxamine (NSD 1024) and 4-bromo-3-hydroxybenzyloxyamine (NSD 1055) individually to the lymphocyte assay produced no significant changes compared to control cultures (Fig. 2).

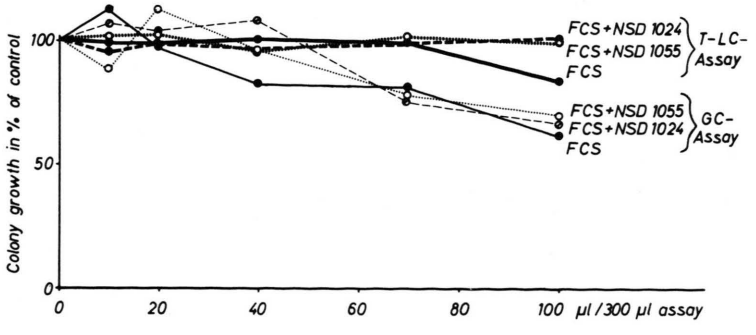

Fig. 1. Effects of fetal calf serum (FCS) and polyamine oxidase inhibitors (NSD 1024 and 1055) added as test samples to the lymphocyte (T-LC) and granulocyte (GC) assay. Results are expressed as a percentage of an FCS-free control assay with a maximum standard error of the mean (sem) of ± 6.0%.

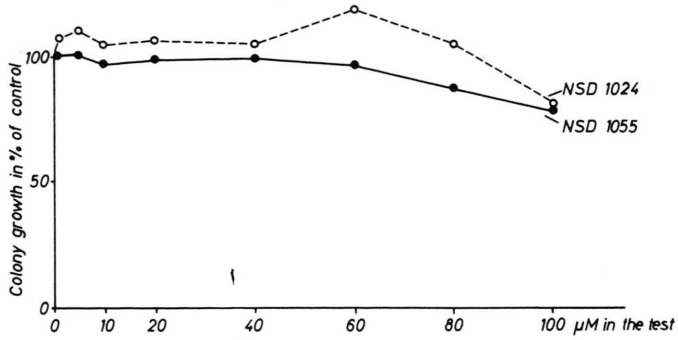

Fig. 2. Effects of polyamine oxidase inhibitors on the fetal calf serum-free lymphocyte assay (max. sem ± 6.0).

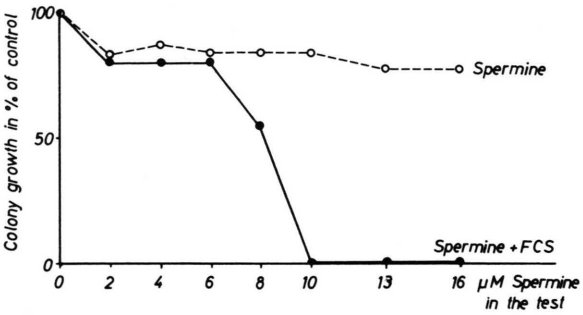

Fig. 3. Effects of spermine on the lymphocyte assay in the presence and absence of fetal calf serum (FCS). A constant volume, 20 µl, FCS was used. FCS was not a normal constituent of the assay medium (max. sem ± 4.6%).

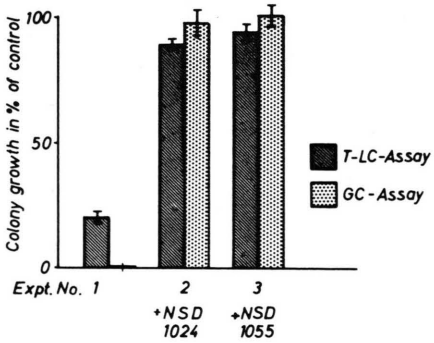

Fig. 4. Combined effects of polyamine oxidase inhibitors (25 µl = 50 µM final conc.), spermine (20 µl = 9 µM final conc.) and fetal calf serum (FCS, 20 µl) on the lymphocyte and granulocyte assay. Experiment No. 1: FCS + spermine + saline (60 µl); No. 2: FCS + spermine + NSD 1024 + saline (35 µl); No. 3: FCS + spermine + NSD 1055 + saline (35 µl).

Taking account of the fact that FCS is a source of polyamine oxidase the combined effect of FCS and enzyme inhibitors was assayed. There was no significant difference between cultures with FCS alone and cultures with both FCS and the enzyme inhibitors (Fig. 1).

The effect of spermine on lymphocyte colony growth in the presence and absence of fetal calf serum

The addition of spermine to the culture system over a range of concentrations up to 16 µM caused inhibition both in the presence and absence of FCS. However, in the absence of FCS inhibition did not exceed 25% at the concentrations studied, whilst in the presence of FCS the inhibition was 100% at concentrations > 10 µM, with an ID_{50} of ~ 8.1 µM (Fig. 3).

Reversal of spermine induced inhibition of cell proliferation by polyamine oxidase inhibitors

Polyamine oxidase inhibitors added to the culture systems at either 50 or 100 µM completely reversed the inhibitory effect of 9 µM spermine in the presence of FCS (Fig. 4). This confirms the essential role of the enzyme in the production of inhibitory compounds from spermine.

Comparison of the effects of a lymphocyte chalone fraction from calf thymus with the effects of spermine on lymphocyte colony growth

To investigate whether the inhibitory activity of a potential lymphocyte chalone was due to adherent polyamines, a fraction was prepared from calf thymus, purified by Biogel P6 chromatography and shown to inhibit lymphocyte but not granulocyte colony growth (Fig. 5A). From the dose-response curve 3 doses were selected which, in the presence of either of the polyamine oxidase inhibitors, inhibited lymphocyte proliferation to the same extent (Fig. 5B). At the concentrations used the inhibitors could, however, completely reverse the inhibition by spermine (Fig. 5C). These results indicate that the inhibition caused by the thymus fraction was independent of polyamine oxidase and therefore of polyamines.

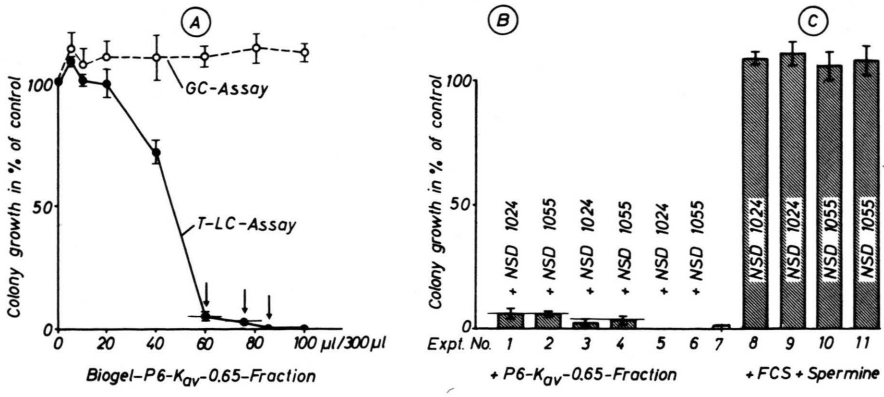

Fig. 5. Comparison of the effects of a lymphocyte chalone fraction from calf thymus and of spermine (20 µl = 9 µM final conc.) on the lymphocyte assay in the presence of FCS (20 µl) and polyamine oxidase inhibitors (25 µl = 50 µM final conc.). A: Dose-response of calf thymus Biogel P6 fraction (Kav ~ 0.65) with the doses selected (arrows) for the experiments of B, No. 1 and 2: 60 µl, No. 3 and 4: 75 µl, No. 5 and 6: 85 µl, in the presence of the oxidase inhibitors. C: Reversal of spermine inhibition (expt. No. 7) by the enzyme inhibitors (experiments No. 8–11) in the presence of FCS and spermine. Each test sample was made up to 100 µl with saline. All experiments were carried out simultaneously with the same batch of lymphocytes.

Discussion

Previous reports which claim to have differentiated between chalone and polyamine-containing fractions have relied upon the reported serum-dependence of polyamine inhibition as the distinguishing feature [9]. However it has been clearly demonstrated, in a truly proliferating system, that this serum dependence is not a reliable index [22]. Indeed in our experimental system we have observed that the inhibition is not serum dependent but batch dependent, *i.e.* certain batches of sera, horse and human, produced inhibitory effects, with polyamines, similar to those observed with fetal calf serum (FCS, unpublished data). In addition, this reliance on serum dependency does not eliminate the possibility of adding the polyamine oxidase to culture systems containing polyamines. Nor does it permit studies using cells which will not grow or grow only poorly in sera other than FCS. We have therefore sought an alternative and more reliable method of distinguishing between the inhibition resulting from the presence of polyamines and that resulting from the action of a true chalone.

In this investigation we have attempted to show conclusively that the method chosen to differentiate between the two systems not only does so, but also that it does not have any other effects in our system. Hence we have shown that the addition, separately or together, of FCS, not normally present in the system, and polyamine oxidase inhibitors has no effect on cell proliferation in the absence of exogenous polyamines. We have also demonstrated that at the concentration of polyamines used the presence of FCS markedly enhances their inhibitory effect.

In the course of establishing the assay system described we have examined the effects of a number of reported inhibitors of polyamine oxidase, the enzyme present in bovine serum and responsible for the inhibitory properties of polyamines [16, 23]. Of these only two, 3-hydroxybenzyloxyamine (NSD 1024) and 4-bromo-3-hydroxybenzyloxyamine (NSD 1055) proved to be suitable for use in our tissue culture system. Other amine oxidase inhibitors including quinacrine, paragylline and aminoguanidine were found to be either cytotoxic or ineffective (results not shown). Both hydroxybenzyloxyamines completely reversed the inhibitory effects of exogenous spermine plus FCS and thus provided two agents suitable for differentiating between polyamines and chalone containing fractions.

Having established our control system we used it to examine a purified chalone extract specific for lymphocytes. An inhibitory fraction may contain polyamines, either free or complexed, polyamine oxidase or another inhibitor *e.g.* a chalone. It is assumed that the aldehyde produced by enzyme oxidation of polyamines would not be present, as it is unstable [17]; nor is the cytotoxic aldehyde break-

down product acrolein present, as the chalone fraction has been shown to be noncytotoxic [21]. For the inhibitory response to be due to polyamine oxidation both polyamine and the oxidase must be present in the culture system. It has been reported that proliferating cells excrete polyamines [24] hence an extract containing the oxidase may produce the observed effects. Alternatively, as serum possibly containing the enzyme was added to the cultures, the inhibition could be due to polyamines added in a tissue extract. By using an enzyme inhibitor essential for the expression of inhibition by polyamines we have eliminated both possibilities. Tests were carried out both in the presence and absence of enzyme inhibitors and no difference could be detected. Thus it is clear that the fraction tested does not contain either polyamines nor polyamine oxidase and may therefore represent a true chalone.

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