

The Influence of Spermine, Spermidine and Various Sera on T-Lymphocyte and Granulocyte Colony Growth *in vitro*

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Spermine, Spermidine, Human Serum, Lymphocyte, Granulocyte Colony Growth

Using T-lymphocyte (T-LC) and granulocyte colony (GC) assays with truly proliferating cells, the dose-response relationships of spermine and spermidine, of fetal calf (FCS), calf (CS), horse (HS) and human AB serum (ABS), and of the polyamines in the presence of selected doses of the sera were analyzed. In contrast to earlier observations using [^3H]thymidine uptake measurements it was found that the polyamines as well as the sera themselves inhibit T-LC and GC growth in the presence of autologous HS and ABS, respectively. The polyamines also inhibit in the presence of additional HS and ABS, yet most effectively with added FCS and CS. Thus the previously reported species-specificity of the serum-dependent polyamine inhibition has here been shown to be more quantitative than qualitative. These studies stress the significance of assays utilizing truly dividing cells and broad dose-response relationships in order to assess specific biological effects *in vitro*.

Introduction

Several groups have reported on the existence of tissue-specific inhibitors, from lymphoid organs, of mitogen-stimulated and spontaneous lymphocyte proliferation, acting in a reversible, non-cytotoxic manner [1–12]. Factors with such properties meet the definition of chalones [13]. In spite of several years effort, progress in the isolation and chemical identification of a pure lymphocyte chalone has been hampered for various reasons. Indeed reported molecular weights of the presumed polypeptide range from 5–70,000. An inhibitor from porcine thymus has been shown to be a spermine-protein complex, its inhibitory activity being largely confined to its polyamine moiety [14]. In addition, pure spermine was found to simulate this effect *in vitro*. The inhibition was, however, serum-dependent: while human and mouse sera were ineffective, only the presence of bovine serum, particularly fetal calf serum (FCS), produced drastic inhibition of [^3H]thymidine uptake by the lymphocytes [14, 15]. Recently these findings have been questioned: a bovine thymus factor was extracted which inhibits the apparent *in vitro* proliferation of human and murine lymphoid cells in the absence of FCS [9].

Abbreviations: CS, calf serum; FCS, fetal calf serum; HS, horse serum; ABS, human AB serum; T-LC, T-lymphocyte colony; GC, granulocyte colony; Cl, clusters of granulocytes; PHA, phytohemagglutinin; DEM, Dulbecco's modification of Eagle's medium; CSA, colony stimulating activity; [^3H]-Tdr, [^3H]-thymidine.

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Apart from this still unsolved controversy, it is worth-while noting that all *in vitro* measurements of lymphocyte proliferation in the above reports are based on the uptake or incorporation of [^3H]thymidine ([^3H]Tdr) into DNA. However several possible artifacts due to the careless use of [^3H]Tdr have been analyzed [17–20], which may possibly render conclusions, obtained with this method on the inhibition of proliferation, illusive.

It seemed therefore mandatory, to analyze the polyamine effects on truly *in vitro* dividing lymphocytes. We have established culture conditions to grow T [21] – and B [22] – lymphocyte colonies as well as granulocyte-macrophage colonies [23] in agar contained in glass capillaries. This offers a number of advantages over other culture methods.

Among these may be stressed the possibility of analyzing dose-response relationships on a broad basis. Here we report the effects of spermine, spermidine, several sera and the polyamines in the presence of the sera on dividing human peripheral T-lymphocytes. In addition, the same polyamines and sera were tested on myelomonocytic cell colonies developing from stimulated mouse bone marrow cells, similarly grown in agar capillaries. The ratio of granulocyte clusters to colonies as a suggested measure of toxicity [24] was determined.

Materials and Methods

1. Chemicals and reagents

Spermine·4 HCl (Serva, Heidelberg, No. 35300), Spermidine·3 HCl (Serva, No. 35285). The sera



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were from Seromed (München): fetal calf serum (FCS) No. 0113, batch 711385; calf serum (CS) No. 0123, batch 711216; horse serum (HS) No. 0133, batch 004; and pooled human AB-serum (ABS). All sera were inactivated at 56 °C for 30 min. See [19, 21, 25] for further details.

2. Assay systems:

2.1. T-lymphocyte colony assay (T-LC)

Isolation and stimulation of lymphocytes: The lymphocyte capillary assay was carried out as described previously [21] 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. PHA-M solution (Difco) was prepared by adding 5 ml saline to one original bottle from Difco. After prestimulation the cells were washed once only.

Agar culture: One assay has a volume of 300 μ l with 100 μ l "space" for sample or saline. Composition of the 200 μ l rest volume: a) for FCS-tests 19.25 μ l DEM, 50 μ l FCS; b) for CS-tests 39.25 μ l DEM, 30 μ l CS; c) for HS-tests 29.25 μ l DEM, 40 μ l HS; d) for ABS-tests 39.25 μ l DEM, 30 μ l ABS. For controls, sera were replaced by saline. Further supplements were equal for all tests: 68.75 μ l PHA-M + autologous plasma (2 μ l PHA + 66.75 μ l plasma), 37 μ l lymphocytes suspension (1.2×10^6 /ml) and 75 μ l Bacto agar solution (3.8 ml DEM + 1.2 ml 3% liquified Bacto agar).

The addition followed the above sequence with 50 μ l sample or saline at the beginning.

2.2. Granulocyte colony assay

The granulocyte capillary assay has been described in detail [23]. The amounts of components for a 300 μ l assay were: 50 μ l sample or saline, 20 μ l FCS (CS, HS or ABS) or saline, 62.5 μ l medium, 67.5 μ l CSA + HS (5 μ l CSA + 62.5 μ l HS), 25 μ l granulocyte suspension (1.2×10^6 /ml) and 75 μ l agar/solution as in the lymphocyte assay.

The source of colony stimulating activity (CSA) was lung conditioned medium prepared according to [26].

For both assays, 3 capillaries with 30 μ l for the T-LC-assay and 75 μ l for the GC-assay were incubated for each dose, and at least 6 capillaries for control. Standard error of the mean (sem) was well below $\pm 5\%$ for both assays. Reproducibility of assays was at worst $\pm 15\%$.

A colony was defined as > 50 cells; clusters consisted of 5–50 cells. The lymphocyte assay was measured by the aid of an ocular grid which facilitated discerning between colonies and clusters.

Results

1. Dose-response relationships of spermine and spermidine on the standard colony assays

Both polyamines inhibit the colony growth of both assays to different extent (Fig. 1). The T-LC assay is more readily inhibited by spermine ($ID_{50} = 10 \mu M$) than by spermidine ($ID_{50} = 60 \mu M$),

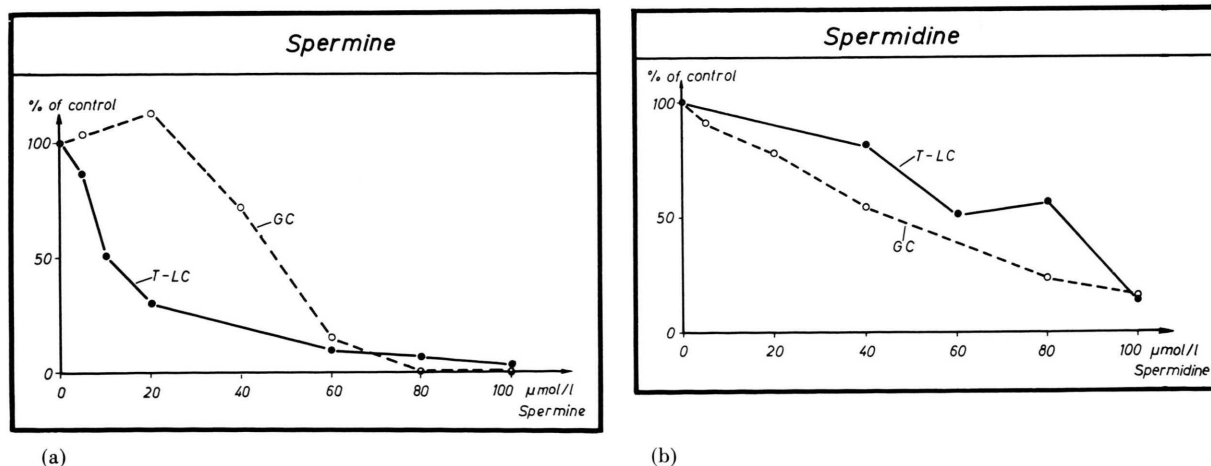


Fig. 1. Dose-response curves of spermine (a) and spermidine (b) using T-LC and GC assays. Polyamine concentrations are given in μM in the assays. For statistics see Materials and Methods.

probably due to its additional amino group, while the reverse relationship is found on the GC assay. Interestingly, at low doses spermine exerts a slight stimulation on GC growth (Fig. 1 a), a phenomenon also found with some sera (see below). The shape of the curves is different: the immediate inhibition by spermine on T-lymphocyte proliferation as compared with the GC assay may indicate some inherent differences of the assay systems (see Discussion). The rather sigmoidal spermine curves in comparison with the rather straight spermidine curves suggest different mechanisms of inhibition.

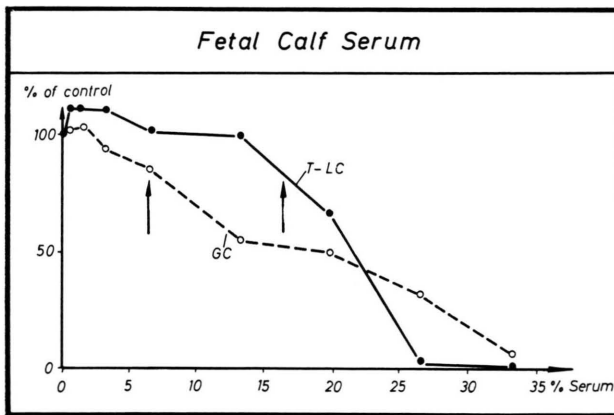
2. The effects of various sera on the standard colony assays

In order to evaluate the effects of the polyamines on colony growth in view of the reported strong

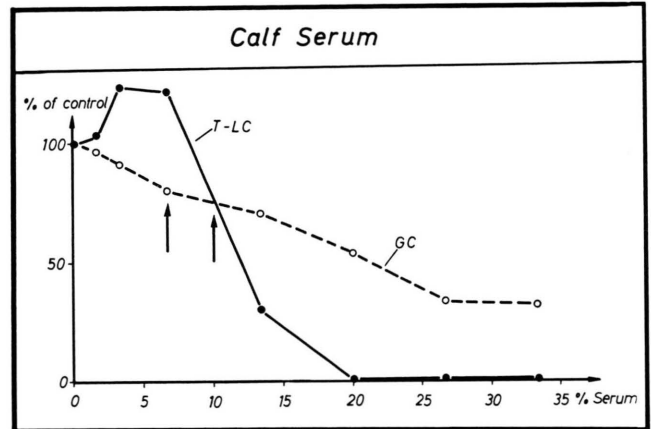
serum dependence [14, 15], it was first necessary to determine the influence of various commonly used sera, over certain dose ranges (Fig. 2). From the curves serum doses were selected which were easy to handle but only slightly inhibitory themselves. Thus, any inhibition by the added polyamines should be readily detected (see below).

Fetal calf serum (FCS, Fig. 2 a) and *calf serum* (CS, Fig. 2 b) showed a more immediate inhibition on GC than on T-LC growth. Interestingly, CS exerts some stimulation at low doses on T-LC, but more inhibition at high doses than on GC growth. Selected doses for the T-LC assay with FCS: 50 μ l, CS: 30 μ l; in the GC assay with FCS: 20 μ l, CS: 20 μ l.

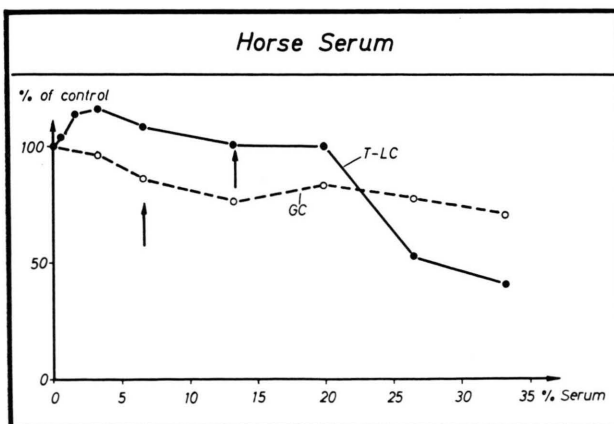
Horse serum (HS) showed some inhibition only at a concentration above 20% of the total incubation



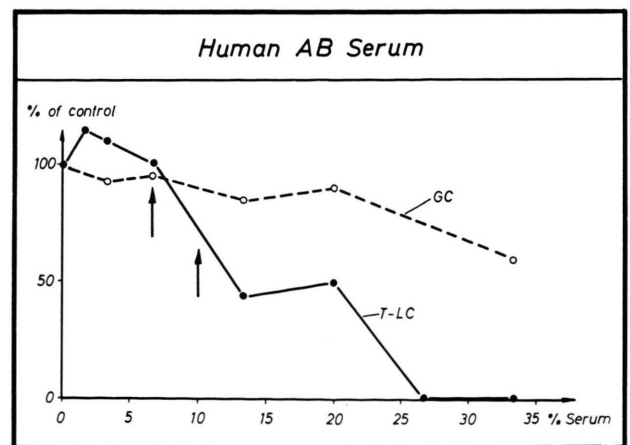
(a)



(b)



(c)



(d)

Fig. 2. Dose-response curves of fetal calf (a), calf (b), horse (c) and human AB serum (d) using T-LC and GC assays. The arrows indicate the serum doses selected for the experiments of Figs 3 and 4.

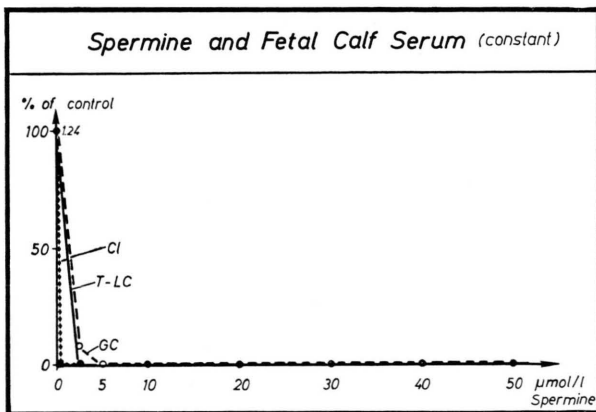
volume of the T-LC assay, while almost no inhibition was found on the GC assay (Fig. 2 c). It should be mentioned that the T-LC assay uses autologous human plasma, while the GC assay is based on horse serum. Selected doses for the T-LC assay: 40 μ l; for the GC assay: 20 μ l.

Human AB serum (ABS) revealed a clear inhibition on T-LC growth ($ID_{50} = 50 \mu M$), but much less on GC growth (Fig. 2 d). Selected doses for the T-LC assay: 30 μ l; for the GC assay: 20 μ l.

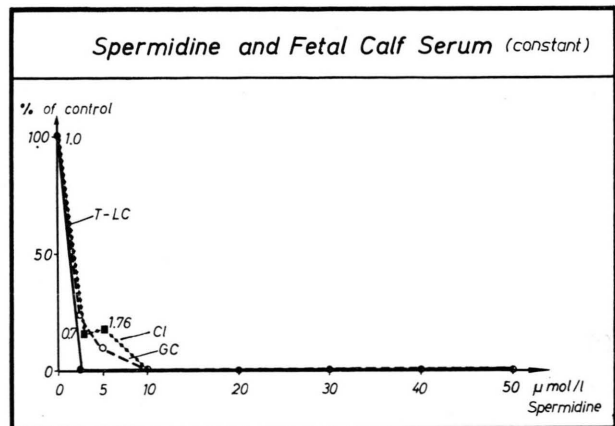
These serum effects evidently depend largely on the type of batch being tested. Therefore any generalizations from the data are likely unjustified. The data do however indicate how important it is to establish dose-response relationships for defined conditions.

3. The effects of spermine and spermidine on T-lymphocyte and granulocyte colony growth in the presence of selected doses of various sera

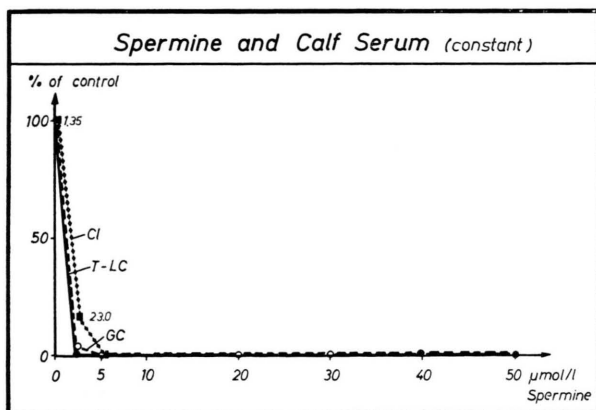
A concentration of as little as 2.5 μM spermine drastically inhibited T-LC and GC growth of T-lymphocytes in the presence of 50 μ l fetal calf serum (Fig. 3 a). Spermidine inhibited T-LC growth to a similar extent, but GC growth less (Fig. 3 b). A cluster/colony ratio above 1 was found. This is also true for calf serum, which showed strong inhibition by spermine (Fig. 3 c) and spermidine (Fig. 3 d) on T-LC, but less on GC growth; in fact more clusters were found, indicated also by the granulocyte cluster/colony ratio above 2 when compared with the normal ratio (0.65).



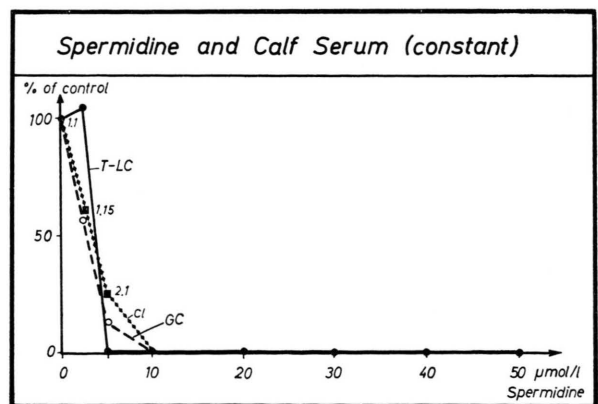
(a)



(b)



(c)



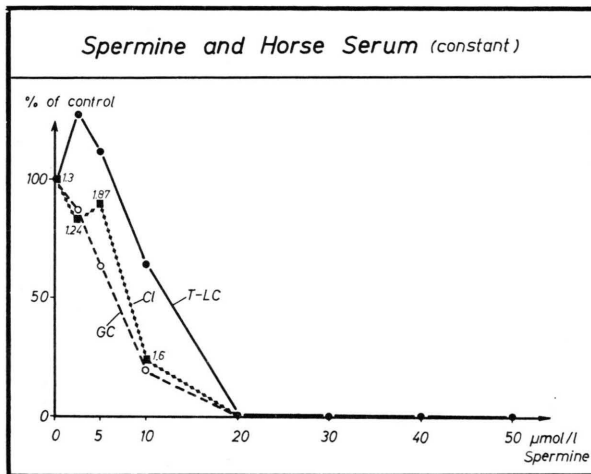
(d)

Fig. 3. Dose-response curves of spermine (a, c) and spermidine (b, d) using T-LC and GC assays in the presence of fetal calf (a, b) and calf serum (c, d) at doses selected from the experiments of Fig. 2. Cl, granulocyte clusters; numbers indicate the granulocyte cluster/colony ratios.

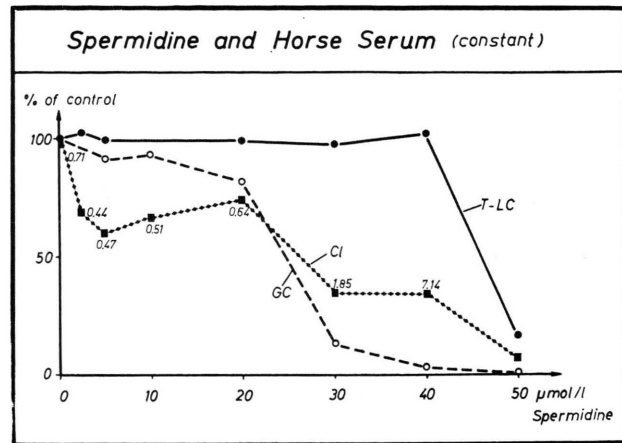
Horse serum revealed quite different effects with varying doses of the polyamines. While $12 \mu\text{M}$ spermine were the ID_{50} for T-LC growth (Fig. 4 a), more than $40 \mu\text{M}$ spermidine were required to show any inhibition (Fig. 4 b). This pattern agrees with the differential inhibition found without additional serum (Fig. 1). Spermine also produced a considerable inhibition in the GC assay ($\text{ID}_{50} = 7 \mu\text{M}$), whereas the ID_{50} of spermidine amounted to about $30 \mu\text{M}$. Interestingly, the granulocyte cluster/colony ratio ranged between 1.24 – 1.87 with spermine,

while it showed normal values (0.44 – 0.71) with spermidine up to $20 \mu\text{M}$; above that dose the values increased up to 7. Moreover, at doses up to $20 \mu\text{M}$ a significant depression by spermidine of cluster growth was observed with little effect on colony growth. Whether this involves some specific inhibitory effect of low spermidine doses on clusters in contrast to colonies remains to be investigated.

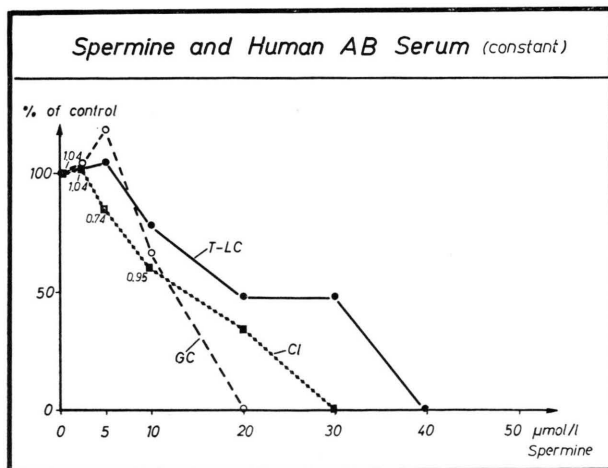
In the presence of human AB serum spermine also exerted a clear-cut inhibition of T-LC growth ($\text{ID}_{50} = 20 \mu\text{M}$, Fig. 4 c), while spermidine had only a



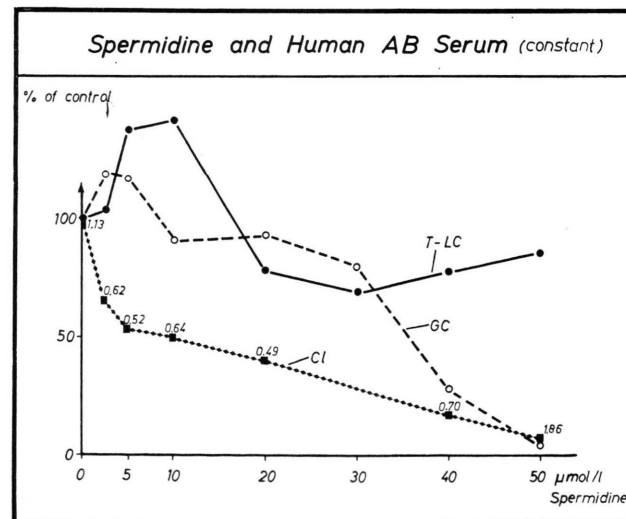
(a)



(b)



(c)



(d)

Fig. 4. Dose-response curves of spermine (a, c) and spermidine (b, d) using T-LC and GC assays in the presence of horse (a, b) and human AB serum (c, d) at doses selected from the experiments of Fig. 2. CI, granulocyte clusters; numbers indicate the granulocyte cluster/colony ratios.

moderate inhibitory effect above $20\text{ }\mu\text{M}$ with a remarkable stimulation at low doses (Fig. 4 d).

There was also a pronounced inhibition of GC growth by spermine ($\text{ID}_{50} = 12\text{ }\mu\text{M}$, Fig. 4 c) and spermidine ($\text{ID}_{50} = 35\text{ }\mu\text{M}$, Fig. 4 d). The granulocyte cluster/colony ratio was slightly above normal (0.75 to 1.05) for spermine, yet clearly in the normal range (0.5–0.7) up to $40\text{ }\mu\text{M}$ of spermidine. As in the case of horse serum with spermidine, the clusters were surprisingly depressed with low doses of spermidine up to $40\text{ }\mu\text{M}$, while colony growth was little affected up to $30\text{ }\mu\text{M}$. This remarkable difference is inconsistent with recent findings by Lehmann *et al.* [24] that the polyamines reduce the colonies without affecting the clusters. The apparent differential inhibition on clusters versus colonies seems worthy of further analysis.

Discussion

For the *in vitro* demonstration of a specific inhibitor of lymphocyte proliferation, it is mostly the uptake and incorporation of [^3H]thymidine ([^3H]-Tdr) into lymphocyte DNA which has been measured. However, at least a dozen possible [^3H]Tdr artifacts can be extracted from the literature [17], and in view of the risk of serious pitfalls one wonders why this method is still widely used. Indeed, it was observed, among others, that about 70% of mitogen responding lymphocytes in culture, while actively synthesizing DNA, were not in the cell cycle for division [27]; DNA turnover involving synthesis and exchange of newly synthesized material was occurring. The authors concluded that “it is misleading to simply equate rates of [^3H]Tdr incorporation with proliferation of stimulated lymphocytes in culture, and caution is required in considering such cultures as models for cell growth analysis”.

Using our T-lymphocyte and granulocyte colony assays in glass capillaries with truly proliferating cells we have analyzed the dose-response relationships of spermine and spermidine, of different sera and of the polyamines in the presence of selected doses of the sera. We found a clear inhibition, by the polyamines, of PHA-stimulated lymphocyte proliferation in the presence of human autologous serum. This seems to contradict reported observations [9, 14] using the [^3H]Tdr method on mitogen-stimulated lymphocytes. However, it is difficult to assess other data, since the experimental conditions

are not directly comparable. Interestingly, spontaneous and mitogen-stimulated DNA synthesis differ when spermine and FCS are present together: Lymphocytes spontaneously incorporating [^3H]Tdr are not inhibited, whereas mitogen-stimulated mouse spleen and human blood lymphocytes are drastically depressed as measured by the [^3H]Tdr uptake [9] and colony growth.

A clear serum dependence of inhibition by spermine and spermidine on [^3H]Tdr uptake has been reported [14, 15]. FCS and CS generally inhibit, in the presence of the polyamines, both [^3H]Tdr uptake and lymphocyte colony growth, but only when mitogen-stimulated. Human and mouse sera lack this effect in the [^3H]Tdr assays. Our colony data, however, show that inhibitions may be found in virtually all combinations, although to different extent. Our data support the view, that FCS and CS are most effective, but that ABS is clearly inhibitory, also in the absence of the polyamines. This raises the question of whether there really exists a species-specificity of the sera, as suggested by reports so far [14, 15], or whether this is rather a dependence on some serum factor(s) with different levels in various sera. In fact, it was indicated that the inhibition by the polyamines is closely linked to plasma amine oxidases abundant in ruminant sera but little in human sera, the aminoaldehydes produced being toxic to mammalian cells in culture [28, 29]. Thus the different degrees of inhibitory activity should be attributed to different levels of these enzymes. This seems unlikely, as we used heat-inactivated sera, thus excluding the possible presence of heat-labile enzymes which could convert the polyamines to cytotoxic amino aldehydes [30]. Moreover the inhibitory effect by the polyamines has been found to be reversible rather than toxic [15].

The role of the polyamines in cell growth regulation is still not clear. Increased cellular levels are required for optimal DNA synthesis in ConA-stimulated lymphocytes [31], probably to organize t-RNA structure and activity [32]. In fact, the addition of small doses of external spermine up to $20\text{ }\mu\text{M}$ appears to stimulate granulocyte colony growth (Fig. 1 a). Conversely, polyamine deficiency reduces the rate of DNA synthesis [33].

Interestingly, the polyamines at high doses were found inhibitory, unspecifically, on lymphocytes, fibroblasts and granulocytes, while a thymus fraction containing spermine in a tightly bound com-

plex was relatively specific, the ligand conferring the specificity [14]. This finding has been questioned on the observation that a thymus crude fraction could inhibit [^3H]Tdr uptake of mouse thymocytes and stimulated spleen cells even in the absence of FCS, *i. e.* without any serum-dependency [9]. This discrepancy can only be solved, if the various factors involved in the inhibition of lymphocyte proliferation are chemically defined and can be added in balanced quantities to follow dose-response relationships. In any case, some of the differences, particularly the FCS-spermine effects on the DNA synthesis of thymocytes and mitogen stimulated spleen cells [9] may well have derived from the [^3H]Tdr artifacts cited.

Thus the findings mentioned above must be re-evaluated using truly dividing lymphocyte cultures. In addition to show any cell specificity of inhibition a closely related cell colony assay seems recommendable. Utilizing this approach we attempted first to demonstrate the effects of the polyamines, various sera and combinations thereof, prior to analyzing the influence of a thymus inhibitor as a possible lymphocyte chalone.

Our data in essence suggest that also in the absence of FCS inhibition of T-LC growth by both spermine and human serum alone may be observed, which disagrees with previous findings [9, 14]. The differential inhibition on the GC assay is interesting, but difficult to evaluate. It appears that each assay exerts specific sensitivities depending on the test compound [3, 4]. It should be mentioned that

the T-LC assay uses prestimulated lymphocytes, *i. e.* after the antigen recognition stage at the onset of DNA-synthesis, when brought into contact with the test sample. On the other hand, the GC assay utilizes various bone marrow stem cells, some of which may be stimulated to differentiate to granulocytes after a few cell divisions; however, the test sample is added with the CSA simultaneously to the stem cells, *i. e.* at an early stage of differentiation.

It was conjectured, that the granulocyte cluster/colony ratio may permit a distinction between the effects of toxic and physiological, reversible proliferation inhibitors [24]. Polyamines were found to drastically change this ratio with horse or human sera, in contrast to untreated or granulocyte chalone-treated bone marrow cell cultures. However, this is inconsistent with our data, indicating insignificant effects in the lower dose range of spermine and, particularly, of spermidine. It remains to be shown whether the ratio is indeed a clear-cut measure of a distinct biological activity or not.

Summarizing, the use of proliferation assays involving truly dividing T-lymphocytes and myelopoietic cells for specificity testing provides a more reliable basis than those presently used to investigate the mechanism of inhibition of lymphocyte proliferation by polyamines and other natural factors.

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