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Thymidine secretion by hybridoma and myeloma cells

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Abstract

Secretion of thymidine appeared to be a common property of hybridoma and myeloma cells, but not of other cell types, which were tested. Of three hybridoma cell lines tested, all secreted thymidine in amounts resulting in the accumulation of thymidine to concentrations of 10–20 μM in the culture medium. Also three of five myeloma cell lines that were analyzed secrete thymidine, but none of the other cell types that were studied. Thymidine was purified to homogeneity (4 mg purified from 3 l of culture medium) and identified as such by nuclear magnetic resonance spectroscopy. The cells that secreted thymidine showed high resistance to the growth inhibitory effect of thymidine.
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Keywords: Thymidine; Hybridoma; Myeloma; Growth inhibitory factor

Hybridoma and myeloma cells have been reported to secrete a cell growth inhibitory factor (referred to in the following as ITI: inhibitor of radioactive thymidine incorporation) which inhibits incorporation of radioactive thymidine into cells [1,2]. Other cell types (human lymphoma and mouse embryo fibroblasts) which have been studied do not produce the inhibitory factor [2]. It has been suggested that the factor prevents growth of hybridoma cells to high cell densities and thus limits the amount of monoclonal antibodies that may be obtained from batch cultures of hybridoma cells [1,2]. In this study, ITI has been purified to homogeneity and unequivocally identified as thymidine by use of nuclear magnetic resonance (NMR), circular dichroism (CD), and mass spectrometry (MS). The results indicate that hybridoma and (some) myeloma cells—but not several other cell types that have been tested—secrete relatively large amounts of thymidine to their surroundings.[1]

Materials and methods

Cells and culture conditions. The following cells (American Type Culture Collection number in parenthesis) were used in this study: 1B9, 6D11 [4], and 5c8 (HB-10916) hybridoma cells; P3X63Ag8.653 (CRL-1580) [5], OURI, and NSO [6] mouse myeloma cells; U266 (TIB-196) [7] and RPMI 8226 (CCL-155) [8] human myeloma like-cells; Jurkat human T-lymphocytes (TIB-152) [9]; MOLT-4 human T-lymphoblasts (CRL-1582) [10]; TOM-1 human pre-B-cells [11]; Raji human B-lymphoblasts (CCL-86) [12]; U698 human B-cells [13]; K-562 human erythroleukemia cells (CCL-243) [14]; MDCK II canine epithelial cells [15]; monkey CV-1 fibroblasts (CCL-70) [16]; and HD11 chicken macrophages [17].

The cells were maintained at 37 °C as stationary cultures in tissue culture flasks (25, 75 or 225 cm^2; Costar) with either (for 1B9, 6D11, NSO, MDCK II, CV-1, and HD11 cells) Dulbecco's modified Eagle's medium (DMEM; Bio-Whittaker) or (for 5c8, P3X63Ag8.653, OURI, U266, RPMI 8226, Jurkat, MOLT-4, TOM-1, Raji, U698, and K-562) RPMI 1640 medium (Gibco/BRL). The medium was supplemented with 20 mM Hepes buffer (Gibco/BRL), 2 mM L-glutamine (Bio-Whittaker), and 0.1 mg/ml kanamycin (Gibco/BRL) or (for MDCK II, CV-1 and HD11) 100 U/ml penicillin (Bio-Whittaker) and 0.1 mg/ml streptomycin (Bio-Whittaker). The medium also contained 10% (for 1B9, 6D11, 5c8, P3X63Ag8.653, OURI, NSO, Jurkat, MOLT-4, TOM-1, Raji, U698, K-562, MDCK II, CV-1, and HD11 cells), 15% (for U266 cells), or 20% (for RPMI 8226 cells) heat inactivated fetal calf serum (Gibco/BRL). The cell cultures were diluted in fresh medium to a density of about 10^4 cells/ml twice each week, after they had reached a density of about 10^6 cells/ml. The cell number and viability were determined by dye exclusion with 0.2% (v/v) Trypan Blue using a hemocytometer chamber.

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[1] Some of the findings reported here were presented at the Xth International Symposium on Purines and Pyrimidines in Man and in the proceedings from that symposium [3].

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Assay for ITI-activity. The hybridoma 1B9 cells were used as target cells. Varying amounts (1–20 μl) of fractions to be assayed for inhibitory activity were added to 6 mm tissue culture wells (type 3595; Costar) containing 2×10^3 cells in a final volume of 200 μl DMEM supplemented with 25 mM Hepes buffer, 2 mM L-glutamine, 0.1 mg/ml kanamycin, and 10% (v/v) heat inactivated fetal calf serum. After incubation at 37 °C for 48 h, 1 μCi of (methyl-^{3}H)thymidine (specific activity: 5 Ci/mmol; New England Nuclear) was added, and the cultures were harvested with a Skatron cell harvester after 2 h of (methyl-^{3}H)thymidine incorporation [18]. The results were calculated as percent inhibition of target cell (methyl-^{3}H)thymidine incorporation (%TI), as follows:

$$\%\mathrm{TI} = 100 - \frac{\text{cpm in cells exposed to fractions assayed for inhibitor}}{\text{cpm in control cultures without inhibitor}} \times 100.$$

The fractions were assayed in triplicate or quadruplicate, and the standard deviations obtained for %TI were on the average less than 10% of %TI.

Quantification of thymidine in cell culture supernatants. The amount of thymidine that was secreted into the cell culture medium by various cell types was assayed using a short-term isotope dilution assay similar to that described by Stadecker et al. [19]. Hybridoma 1B9 cells were added to 6 mm tissue culture wells (type 3595; Costar) such that each well contained 5000 cells in 100 μl DMEM supplemented with 25 mM Hepes buffer, 2 mM L-glutamine, 0.1 mg/ml kanamycin, and 10% (v/v) heat inactivated fetal calf serum. After incubation at 37 °C for 4 h, varying amounts of thymidine (in order to obtain a standard curve) or culture supernatants to be quantified for thymidine were added. The volume in each well was then adjusted to 200 μl with culture medium and 1 μCi of (methyl-^{3}H)thymidine (specific activity: 5 Ci/mmol; New England Nuclear) was added to each well. The cultures were harvested with a Skatron cell harvester after 4 h of (methyl-^{3}H)thymidine incorporation. The concentration of thymidine in culture supernatants was determined by using the calculated standard curve.

Purification of ITI/thymidine. ITI in 3 l culture supernatant collected about day 7 after passage of hybridoma 1B9 cells was passed through filters (Diaflo YM3 ultra filters; Amicon) with a molecular weight cut-off of 3000. The supernatant was then concentrated about 10-fold—to about 400 ml—by reverse dialysis using dialysis tubing (Spectra/Por CE tubular membrane; Spectrum) with a molecular weight cut-off of 100 and subsequently applied (in 10 ml aliquots) to a 3 ml Resource RPC reverse phase column (Amersham Biosciences) equilibrated with 0.1% trifluoroacetic acid (TFA). ITI was eluted by applying a 0–20% (v/v) linear isopropanol (in 0.1% TFA) gradient to the column. ITI was subsequently applied to a PepRPC HR 10/16 silica-C18 reverse phase column (Amersham Biosciences) equilibrated with 20 mM potassium phosphate buffer, pH 6.8, containing 0.25 M KCl and eluted by applying a 0–10% linear isopropanol (in phosphate buffer, pH 6.8, and 0.25 M KCl) gradient to the column. ITI was finally reapplied to the PepRPC reverse phase column, but this time at pH 2 (in 0.1% TFA), and it was eluted by applying a 0–10% linear isopropanol (in 0.1% TFA) gradient.

NMR spectroscopy. NMR spectra of ITI and thymidine were recorded on a Bruker Spectrospin Avance DRX 500 instrument equipped with a TXI 5 mm sample holder. All spectra were acquired and processed with Xwinnmr 2.6 on Indy computers. The ITI sample was first dissolved in deuterium oxide (Cambridge Isotope Labs) and then in dimethyl-d_6-sulfoxide (Cambridge Isotope Labs) and ^{1}H (pulse program: zg30), ^{13}C (zgpg30), DEPT (dept90, dept135, and dept45), COSY (cosygs), TOCSY (mlevtp), HSQC (invieagssi), HMBC (inv4gslplrnd), and NOESY (noesytp) spectra were recorded with both solvents. NOESY spectra were obtained in degassed solution with a mixing time of 1100 ms.

Circular dichroism and mass spectrometry. CD spectra of ITI and thymidine were recorded using a Jasco J-810 spectropolarimeter calibrated with ammonium D-camphor-10-sulfonate. Measurements were performed at room temperature using a quartz cuvette with path-length of 0.1 cm.

The molecular mass of ITI and thymidine was determined using an electrospray FTICR (fouriertransform-ionsynclotronresonance) mass spectrometer (Bio Apex 4.7). Purified ITI and thymidine in 49% (v/v) methanol and 2% (v/v) acetic acid was injected into the spectrometer.

Results and discussion

Production and purification of ITI (thymidine)

Fig. 1 shows the amount of ITI (thymidine), which accumulated extracellularly as a function of the time after passage of hybridoma cells to fresh medium. The number of viable cells reached a maximum value (about 1×10^6 cells/ml) at day four after passage and significant amounts (3–6 μM) of ITI (thymidine) had accumulated in the media by day 4, but few dead cells were detected before day 5.

In order to identify ITI by NMR spectroscopy, a procedure was developed for purification of ITI to homogeneity and in quantities that enabled structural characterization of ITI. The procedure was developed on the basis of an earlier reported micro-preparative purification procedure that showed that ITI had a molecular mass less than 500 Da, that it did not bind to ion exchangers (i.e., had no apparent charge), and that it was retarded on reverse phase columns [1]. Macromolecules in culture supernatants containing ITI were first removed by ultra-filtration using filters with a molecular weight cut-off of 3000. ITI passed through the filters and it was subsequently concentrated about 10-fold by reverse dialysis. The recovery after these two purification steps was greater than 90%. In the following reverse phase chromatography step using the Resource RPC column (Amersham Biosciences), most of the contaminating material passed through the column (as judged by the high optical density of the flow through fraction), whereas most of the inhibitor bound to the column as long as the sample

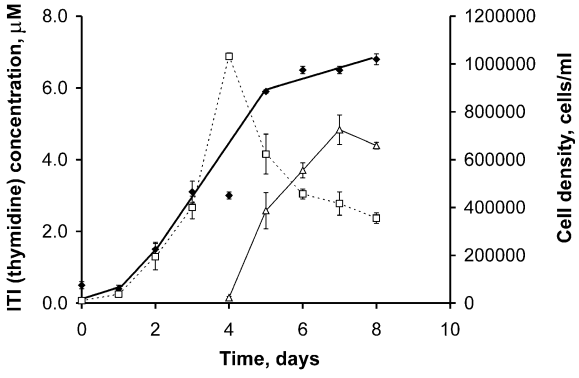

Fig. 1. Thymidine concentration (♦) and the number of viable (□) and dead (△) cells per milliliter in the cell culture medium, all as a function of the time after passage of hybridoma cells (1B9) to fresh medium. After having reached a cell density of about 5×10^5, the hybridoma cells were on day 0 diluted in fresh medium to a density of about 1×10^4 cells/ml and cultured for 14 days. Culture samples were taken at the indicated days after passage and the thymidine concentration was determined by measuring the ITI-activity using the short-term isotope dilution assay essentially as described in [19]. Briefly, 5000 cells were cultured 4 h in 6 mm tissue culture wells, after which an aliquot of culture supernatant (sample to be assayed for thymidine) or a known amount of thymidine (for generating a standard curve) was added to each well together with 1.0 μCi ^{3}H-thymidine (5.0 Ci/mmol), and the amount radioactivity incorporated into the cells was determined after 4 h labeling as described in [1]. The number of viable and dead cells was determined by exclusion of Trypan Blue using a hemocytometer chamber.

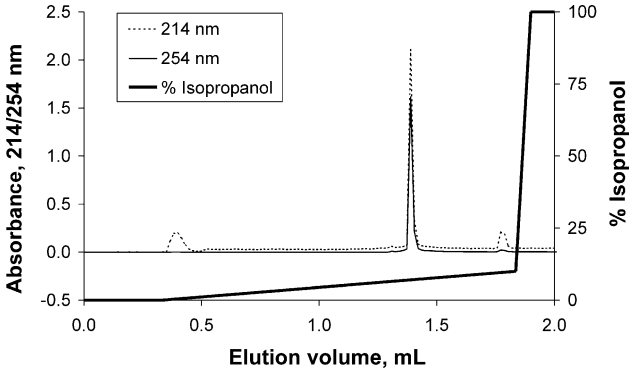

Fig. 2. Analysis of purified ITI (thymidine) by analytical reverse phase chromatography on a Sephasil C18 SC 2.1/10 column (Amersham Biosciences) using the SMART chromatography system (Amersham Biosciences).

volume applied to the column did not exceed 10–15 ml. ITI eluted from the column at 5–7% isopropanol with a recovery of 60–80%. In the two subsequent reverse phase chromatography steps where the PepRPC column (Amersham Biosciences) was used under neutral and acidic conditions, ITI eluted at about 2–5% isopropanol. ITI appeared at this stage to be more than 90% pure as judged by analytical reverse phase chromatography (Fig. 2) and gel filtration, and the overall recovery was estimated to be 30–50%. ITI was identified as thymidine by NMR spectroscopy (see next section) and based on the extinction coefficient of thymidine and the absorbance of the purified sample, the amount of ITI/thymidine isolated from 3 l of supernatant was estimated to be 3.6 mg. This implies (taking into account that the overall recovery was in the range 30–50%) that the ITI/thymidine concentration in the cell culture supernatant was 2–4 μg/ml or about 10 μM. A similar value for the concentration was obtained by comparing the ITI-activity in culture supernatant with the ITI-activity in a commercially obtained thymidine sample of known concentration (data not shown) and upon quantifying the amount of thymidine in the culture supernatants using the thymidine quantification assay described in Materials and methods (Table 1).

Structural identification of ITI as thymidine

Upon determining the structure of ITI by NMR spectroscopy, ITI was uniquely identified as thymidine. NMR spectra were obtained as described in Materials and methods and the resonances were assigned to thymidine: ^{1}H NMR (D_2O, 500 MHz) δ: 1.86 (d, 3H, CH_3-5), 2.34 (m, 2H, H-2′), 3.77 (o, 2H, H-5′), 3.98 (m, 1H, H-4′), 4.43 (q, 1H, H-3′), 6.26 (t, 1H, H-1′), 7.61 (d, 1H, H-6) and ^{13}C NMR (D_2O, 125 MHz) δ 14.4 (CH_3-5), 41.3 (C-2′), 64.0 (C-5′), 73.3 (C-3′), 87.9 (C-1′), 89.3 (C-4′), 114.3 (C-5), 140.3 (C-6), 154.5 (C-2), 169.3 (C-4). The identification was confirmed by results showing that identical NMR spectra were obtained upon analyzing ITI and commercially obtained thymidine (Fig. 3). Moreover, identical UV-absorbance spectra (220–320 nm) and CD-spectra (190–310 nm) were obtained for ITI and thymidine (data not shown) and identical molecular masses were obtained (242.1 Da for both, theoretical mass for thymidine is 242.2) upon ESI FTICR mass spectrometry.

Thymidine is secreted by hybridoma and myeloma cells, but not by other cells tested

Three hybridoma cell lines—1B9, 6D11, and 5c8—were tested for secretion of ITI/thymidine. The 1B9 cells were initially formed by fusing lymphocytes with mouse myeloma BALB/c cells, whereas the 6D11 [4] and 5c8 cells were formed by fusing lymphocytes with mouse myeloma Sp2/0 cells. All three hybridoma cell lines were shown to secreted ITI-activity (Table 1). ITI was purified to homogeneity from two of these cell lines (1B9 and 6D11) and was from one of the cell lines (1B9) structurally identified as thymidine by NMR spectroscopy. From all three hybridoma cells, thymidine was secreted in amounts that resulted in concentrations of 5–20 μM in the culture medium within 4–8 days of culture (Table 1).

All three mouse myeloma cell lines that were analyzed also secreted thymidine (Table 1). None of these myeloma cell lines were used as fusion partners upon producing any of the three hybridoma cell lines. Of the 11 other cell types that were tested—which included two human myeloma-like cell types—none secreted detectable amounts of thymidine, although they reached cell densities that were generally higher than the densities reached by the hybridoma and mouse myeloma cells (Table 1). The results suggest that the hybridoma cells had obtained the thymidine-secretion characteristic from their myeloma cell fusion partner and that secretion of thymidine may be common property of myeloma and hybridoma cells. All the cells were also tested for secretion of deoxycytidine using an assay similar to that used for quantifying thymidine—except that radioactive thymidine was replaced with radioactive deoxycytidine. No secretion of this nucleoside was, however, detected from any of the cells (results not shown).

Macrophages have been reported to release thymidine ($2–4\times 10^{-9}$ moles thymidine pr. 10^7 macrophages in 1 ml for 24 h) [19,20], but the biological role or significance of this thymidine release remains unclear. It has been suggested that thymidine may be an effector molecule in macrophage-mediated regulation of cell growth, since some cells are particularly sensitive to thymidine and may be inhibited by micro-molar concentrations (4–10 μM) [19,21,22]. Moreover, in vivo secretion of thymidine by a cell may of course result in higher local in vivo concentrations than that obtained in cell culture supernatants.

The hybridoma and mouse myeloma cells had high tolerance for thymidine

Thymidine at concentration of 0.1–2 mM is known to cause cell growth arrest and is used to synchronize cell

Table 1
Secretion of thymidine from various cell types

Cell type[a]	Thymidine concentration[b] (μM)		Highest cell density (10^6)[c]	Day of highest cell density[d]
	Day 4	Day 8		
Hybridoma cells				
1B9	5 ± 1	7 ± 1	1.0	4
5c8	5 ± 1	17 ± 1	1.7	4
6D11	5 ± 3	20 ± 10	0.5	4
Mouse myeloma cells				
P3X63Ag8.653	5 ± 2	10 ± 3	0.6	4
OURI	0.4 ± 0.2	0.9 ± 0.2	1.1	4
NSO	0.6 ± 0.2	0.8 ± 0.1	0.9	4
Human myeloma cells				
U266	ND[e]	ND[e]	2.3	8
RPMI 8226	ND[e]	ND[e]	1.6	5
Human T-cells				
T-lymphocyte, Jurkat	ND[e]	ND[e]	3.4	4
T-lymphoblast, MOLT-4	ND[e]	ND[e]	2.3	4
Human B-cells				
Pre-B-cells, TOM-1	ND[e]	ND[e]	2.5	8
B-lymphoblasts, Raji	ND[e]	ND[e]	1.3	5
Pre-B-cells, U698	ND[e]	ND[e]	2.4	4
Human erythroleukemia cells				
K-562	ND[e]	ND[e]	1.4	4
Dog epithelial cells				
MDCK II	ND[e]	ND[e]	—[f]	—[f]
Monkey fibroblasts				
CV-1	ND[e]	ND[e]	—[f]	—[f]
Chicken macrophage				
HD 11	ND[e]	ND[e]	—[f]	—[f]

[a] See Materials and methods for the American Type Culture Collection number and/or references for the various cells.
[b] For the 6D11 cells the assay for ITI-activity was used to determine the thymidine concentration. For all the other cells the assay for quantification of thymidine in cell culture supernatants was used, and the values given are the averages of four measurements ± standard deviations.
[c] The highest cell density obtained during the 8 day culture period. The cell density was determined by counting the cells in a hemocytometer. The values are the averages of two measurements and the deviation between the two measurements was less than 15%. The cell density at start (day 0) of culture was in the range 10^4–10^5 cells/ml.
[d] The day in the culture period at which the highest cell density was attained.
[e] ND, not detectable (<0.5 μM).
[f] These are adherent cells and they reached confluence at day 3–4.

growth [23]. The thymidine-secreting hybridoma and myeloma cells (1B9, 5C8, and P3x63Ag8.653 were analyzed) showed high tolerance for thymidine. Even 10 mM thymidine did not entirely block the growth of these cells (1B9 shown in Fig. 4), whereas thymidine-concentrations less than 1 mM not only caused cell cycle arrest of the MOLT-4 human T-cells, but also killed a large percentage of these cells (Fig. 4). Thus, thymidine cannot account for the cytotoxic activity reported to be released by hybridoma cells [2].

Conclusion and perspectives

The results suggest that secretion of thymidine is a common, if not a general, property of hybridoma and (some) myeloma cells, but apparently not of other cell types. It is, however, not clear why hybridoma and the mouse myeloma cells release thymidine, and the in vivo role or the biological significance of the release remains unclear. It has been suggested that the release of thymidine by macrophages regulates growth of neighboring cells and that the release is due to lack of thymidine kinase in macrophages [24]. It has also been shown that fibroblasts with a non-functional thymidine kinase release thymidine, whereas fibroblasts with a functional kinase do not [25]. It is, however, unlikely that the hybridoma cells lack thymidine kinase, since the cells incorporated radioactive thymidine. Moreover, establishment of the hybridoma cells involves selection of cells that can utilize thymine or thymidine in HAT medium and thus it involves selection of cells that have thymidine kinase. It is also unlikely that the release of thymidine is the result of a general degradation of nucleic acid, since thymidine was detected extracellularly when the cells were in a rapidly dividing phase, with few dead cells in the culture. Moreover, deoxycytidine could not be detected in culture supernatants.

If many, if not all, myeloma cells—but not their normal counterparts—secrete thymidine, thymidine secretion might

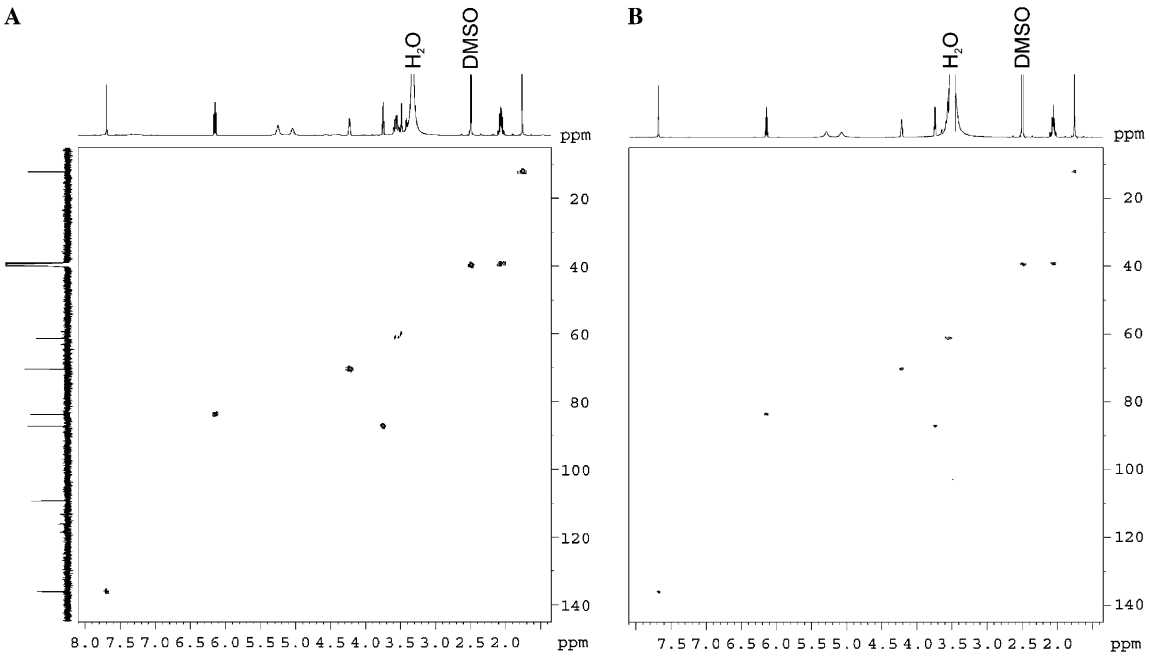

Fig. 3. HSQC spectra of purified ITI (A) and commercially obtained thymidine (B) obtained on a Bruker Spectrospin Avance DRX 500 instrument equipped with a TXI 5 mm sample holder. The samples were dissolved in DMSO-d_6.

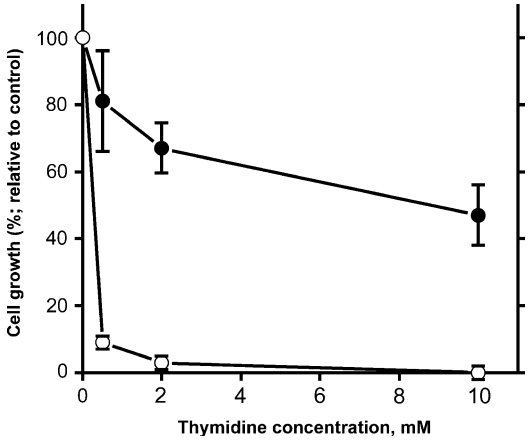

Fig. 4. Effect of thymidine on growth of the 1B9 hybridoma (●) and MOLT-4 cells (○). Cells were grown with the indicated amount of thymidine for 48 h after which the relative number of cells was determined by exclusion of Trypan Blue using a hemocytometer chamber. 100% cell growth is defined as cell growth in a culture without thymidine.

somehow correlate to the formation of myeloma cells. If also some normal cells secrete thymidine, extracellular thymidine might have an important biological function as an effector molecule. Thus, it is of interest to screen more cell types, including myeloma and normal plasma cells and clinical cell samples, for secretion of thymidine.

Acknowledgments

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