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Immunosuppressive activity of angiogenin in comparison with bovine seminal ribonuclease and pancreatic ribonuclease

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Angiogenin, a member of the pancreatic-like ribonuclease family with a special biological action (RISBAses), is a basic protein that induces blood vessel formation. Another member of these special ribonucleases, bovine seminal ribonuclease (BS RNase), displays biological properties, including aspermatogenic, embryotoxic, antitumor and immunosuppressive activities. The effects of two angiogenin preparations tested on the biological activities mentioned above are reported and compared with those of BS RNase and RNase A. In contrast to RNase A, which was ineffective in all biological activities tested, angiogenin suppressed significantly the proliferation of human lymphocytes stimulated by phytohemagglutinin or concanavalin A or by allogenic human lymphocytes (mixed lymphocyte culture). However, angiogenin did not affect the growth of human tumor cell lines, development of cow and mouse embryos and spermatogenicity in mice. On the basis of these results, angiogenin is the first monomeric ribonuclease described so far that displays immunosuppressive activity similar to that of the dimeric BS RNase. The immunosuppressive activity of angiogenin might synergize with the effect on neovascularization of tumor tissues and thus contribute to the development of tumor.

Key words: Angiogenin; Aspermatogenesis; Cancer; Embryotoxicity; Immunosuppression; Lymphocytes; Mice; Ribonucleases.

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Introduction

The isolation, characterization, amino acid sequence and gene structure of angiogenin were reported in recent years (Fett *et al.*, 1985; Strydom *et al.*, 1985; Riordan and Vallee, 1988). Angiogenin was isolated from human HT-29 colon tumor cell-conditioned media (Strydom *et al.*, 1985), and later it was also found in normal human plasma (Shapiro *et al.*, 1987). Angiogenin shares 55% amino acid sequence identity with bovine pancreatic ribonuclease (RNase A), which is a prototype of the secretory ribonuclease superfamily. Angiogenin is a single-chain basic protein of 14 kDa that induces angiogenesis in experimental *in vivo* models such as the rabbit corneal and chick embryo chorioallantoic membrane assays (Fett *et al.*, 1985). Because of this special biological action, angiogenin has been assigned to the RNase group designated as RISBAses (D'Alessio *et al.*, 1991).

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Another substance, bovine seminal ribonuclease (BS RNase), tested in our laboratory, also has been included among RISBAses. BS RNase was isolated from bull seminal vesicle fluid as a native dimeric protein sharing 80% amino acid sequence identity with RNase A (Dostál and Matoušek, 1973). Unlike RNase A, BS RNase displays extraordinary biological properties, including aspermatogenic, embryotoxic, antitumor and immunosuppressive activities (Matoušek, 1973; Matoušek and Grozdanovič, 1973; Matoušek and Staněk, 1977; Vescia *et al.*, 1980; Souček *et al.*, 1981, 1983, 1986; Tamburini *et al.*, 1990; Laccetti *et al.*, 1992). Recently, preparations of BS RNase dimer expressed from synthetic genes in *Escherichia coli* were described (de Nigris *et al.*, 1993; Kim and Raines, 1993). Both catalytic and biological properties of these recombinant proteins were indistinguishable from BS RNase isolated from seminal vesicles.

The most important difference between BS RNase and RNase A seems to be the dimeric form, which is unique among the other secretory ribonucleases. The monomer of BS RNase, similarly to that of RNase A, lacks all biological activities (Tamburini *et al.*, 1990). On the contrary, chemically modified RNase A dimer was reported to exert antitumor activity (Bartholeyns and Baudhuin, 1976). These results led to the assumption that the dimeric form might play an essential role in biological activities. However, our preliminary study showed that angiogenin, a monomeric ribonuclease, also displays an immunosuppressive effect (Matoušek *et al.*, 1992).

We report on the immunosuppressive activities of two angiogenin preparations compared with the effect of BS RNase and RNase A. We also tested the effect of these ribonucleases on the growth of two human tumor cell lines, on development of cow and mouse embryos and on spermatogenicity in mice.

Materials and Methods

Ribonucleases

Two preparations of angiogenin were tested. The first preparation (designated A) was prepared by Professor P. Bünning in Hoechst AG (Frankfurt, Germany) as a recombinant substance from *E. coli*. The cloning and sequence of cDNA and the gene for human angiogenin were described by Kurachi *et al.* (1985). They used a cDNA library prepared from human liver poly(A) mRNA by using a synthetic oligonucleotide as a hybridization probe. The amino acid sequence of angiogenin as predicted from the gene sequence was in complete agreement with that determined by amino acid sequence analysis of the angiogenin purified from the serum-free conditioned medium of a human colon adenocarcinoma cell line (HT-29) (Strydom *et al.*, 1985).

The second angiogenin preparation (designated B) was prepared by expression of a synthetic gene for angiogenin (Tannahill L., Zankel T. and Benner S. A., unpublished data, 1991) in *E. coli*. The sequence of a gene-encoding angiogenin was designed using the program pf (Presnell and Benner, 1988). Overlapping DNA fragments were synthesized using an Applied Biosystems Automated DNA Synthesizer, ligated and the gene cloned into the pUN pJL-502 prokaryotic expression plasmid. To express angiogenin in *E. coli*, medium (adjusted to pH 7.5 with 1 M NaOH) containing (per liter) Bacto-tryptone (Difco, 16 g), yeast extract (Difco, 10 g) and NaCl (10 g) was autoclaved and divided into portions (10 × 450 ml, 2-l shaker flasks). Each was treated with a solution (0.9 ml) containing $MgSO_4$ (1 M), $CaCl_2$ (50 mM) and carbenacillin (50 mg/ml, Sigma). Each flask was then inoculated with an overnight culture (5 ml) (pJ LAngio/lon$^-$), and the cells were allowed to grow at 31°C (300 rpm shaking) until the optical density reached 1.0 (~3.5 hr). The incubator temperature was then increased to 42°C (10 min). After 3 hr, the cells were recovered by centrifugation (20 min, GS3, 6000 rpm) and frozen. Cells (11.5 g, from 4.5 l ferment) were suspended in buffer (10 mM NH_4OAc, pH 7.6, 1 mM PMSF) and lysed with a single pass through a French press.

Angiogenin was isolated from the pellets after centrifugation using the procedure of Stackhouse (Yoon-Raillard S. A., unpublished data, 1993) and purified by a native DNA-cellulose column (Sigma, 0.3 g, pre-equilibrated in 50 mM NaOAc, pH 5.2). The protein was eluted with a gradient (50 mM NaOAc, pH 5.2, 0–500 mM NaCl). The fractions containing angiogenin were identified by gel electrophoresis. This procedure provides pure protein as judged by SDS-polyacrylamide gel electrophoresis (PAGE). Using conditions described by Fett *et al.*, (1985), angiogenin was analyzed by high-performance liquid chromatography (C-18 column, stored in 50% aqueous acetonitrile), where is behaved as authentic material.

BS RNase was isolated from bull seminal vesicle fluid by precipitation with acetic acid and ammonium sulphate and subjected to CM Sephadex C-50 and Sephadex G-100 column chromatography. The purity of BS RNase was determined by disc electrophoresis using an acrylamide gel, starch-gel electrophoresis and immunoelectrophoresis in agar gel by peak homogeneity at the last step of separation and

by the ultracentrifugation pattern (Dostál and Matoušek, 1973). This dimeric native protein co-migrated during non-reducing SDS-PAGE with recombinant BS RNase purified from *E. coli* (Kim and Raines, 1993).

RNase A was obtained from Boehringer-Mannheim (Hamburg, Germany) ~70 Kunitz units/mg 5× cryst. lyophil. Lysozyme, which is recommended to be used for the stability of angiogenin (Bond, 1988), was obtained from Serva (Heidelberg, Germany) 150,000 units/mg cryst.

Aspermatogenic activity assay

The effect of various ribonucleases on the production of sperm in ICR mice was estimated as follows. Left testes of mice (five animals per group) were injected with 100 μg of each RNase. Ten days after the treatment, the mice were killed, isolated testes were weighed, stained with hematoxilin and eosin and subjected to histological examination. Aspermatogenic activity was expressed by the index weight (i.e., testes weight/body weight $\times 10^3$) by measuring the diameter of semiferous tubules and the width of spermatogenic layers. Results were recorded as the mean ± SEM and compared with the untreated right testes of the same mouse. The spectrum of spermatogenic cells was also investigated.

Embryotoxic activity assay

The cytotoxic effect of various ribonucleases on mouse or cow cryoconserved embryos (8–64 blastomers) was assessed according to Říha and Landa (1989). Briefly, five to seven embryos washed free of the cryoprotecting substance were incubated with various ribonucleases in 1 ml of MEMD medium supplemented with fetal calf serum (FCS) (20% v/v). The cultivation Falcon plates (NUNC, Denmark) covered with paraffin oil were kept in the atmosphere of N_2 (90% v/v), CO_2 (5% v/v) at 37°C. The mortality of embryos was assessed 6, 24, 48 and 72 hr after addition of a ribonuclease (100 μg/well); mortality was indicated by a stopping of cell division and subsequent degranulation.

Leukemic cells proliferation assay

The effect of various ribonucleases on the growth of human leukemic cell lines K-562 and HL-60 (derived from human erythroid leukemia and human myeloid leukemia) was tested. Briefly, 0.1 ml of tumor cells (10^5/ml) suspended in RPMI 1640 and supplemented with 10% FCS were cultivated in microtiter plates (FB type) for 48 hr at 37°C under humidified atmosphere containing CO_2 (5% v/v). The inhibitory effect of RNases on the cell growth was assessed by measuring the incorporation of ^{3}H-thymidine into the newly synthetized DNA. Each sample was pulsed 4 hr before the termination of incubation with 24 kBq of ^{3}H-thymidine of specific activity 980 GBq/m/mol. The mean value of triplicate samples was evaluated as cpm, and suppression of DNA synthesis caused by RNases was expressed as percentage of control values (Souček *et al.*, 1986).

Immunosuppressive activity assay

The effect of various RNases on the proliferation of normal human lymphocytes stimulated in mixed lymphocyte culture (MLC) was assessed by the method described earlier (Souček *et al.*, 1986). Briefly, lymphocytes were isolated on a Ficoll-paque (Pharmacia, Uppsala, Sweden) solution gradient from the heparinized blood of two unrelated humans. Both cell preparations were mixed (1:1) and resuspended in RPMI 1640 medium supplemented with inactivated human AB serum (20% v/v). One hundred microliters of lymphocyte mixture (10^5 cells) was set up in microtiter plates (U type), and 100 μl of various concentrations of each ribonuclease was added in three triplicate samples. After 6 days of cultivation, the proliferation of stimulated cells was estimated by measuring the incorporation of ^{3}H-thymidine into the newly synthesized DNA. The labeling of cells was done under the same conditions as mentioned in the antitumor activity assay. The mean value of three replicate samples was evaluated as cpm, and suppression of DNA synthesis caused by RNases was expressed as percentage of control values. In experiments where the stimulation of lymphocytes was induced by mitogens, 10^5 lymphocytes from one donor were mixed either with 5 μg of phytohemagglutinin (PHA) or with 5 μg of concanavalin A (Con A), and the known concentration of RNase was added in a total volume of 200 μl of cultivation medium. After 3 days of cultivation, the immunosuppressive effect of RNase was evaluated under the same conditions as mentioned in MLC test.

Statistical analysis

Results in tables are recorded as the mean ± SEM. The data were analyzed statistically using Fisher's *t*-test.

Results

Aspermatogenic activity

In this article, we determined the effect of angiogenin on spermatogenicity in mice compared with that of BS RNase and RNase A.

Table 1. Effect of angiogenin, BS RNase and RNase A on spermatogenicity in mice

Substances injected	Index weight of testes: Injected testes	Index weight of testes: Non-injected testes	Width of spermatogenic layers (μm): Injected testes	Width of spermatogenic layers (μm): Non-injected testes	Diameter of seminiferous tubules (μm): Injected testes	Diameter of seminiferous tubules (μm): Non-injected testes
Angiogenin						
One injection	3.56 ± 0.93	3.30 ± 0.78	48 ± 5	46 ± 7	136 ± 19	142 ± 36
Two injections	3.86 ± 0.60	3.55 ± 0.75	53 ± 4	51 ± 3	156 ± 13	154 ± 18
BS RNase	1.26 ± 0.41*	3.33 ± 0.66	29 ± 3*	47 ± 3	111 ± 19*	143 ± 33
RNase A	3.36 ± 0.63	3.46 ± 0.63	42 ± 5	45 ± 7	133 ± 31	136 ± 9

Values are means ± SEM.
*$P < 0.01$.

The mean values of three parameters (diameter of seminiferous tubules, the weight of the testes and the width of spermatogenic layers) are recorded in Table 1 relative to that of the non-injected right testes of the same mice. Although BS RNase (100 μg/mouse) decreased significantly, all of the three parameters tested (mostly spermatogonia and primary spermatocytes were present) both angiogenins and/or RNase A did not affect any of these tests. When the double dose of angiogenin was injected (2 × 100 μg/mouse), it did not affect the spermatogenicity in mice. It did cause non-significant increase in the weight of testes, in the thickness of spermatogenic layers and in the diameter of seminiferous tubules.

Embryotoxic activity

We determined also the effect of angiogenin on the development of mouse and bovine embryos in comparison with the effect of BS RNase. The results of this testing are shown in Table 2. The effect of angiogenin on embryo mortality during 72-hr incubation did not markedly differ from the control group, whereas BS RNase caused 100% mortality of bovine or mouse embryos after 24-hr incubation.

Immunosuppressive activity

Previously, we reported that BS RNase displayed immunosuppressive activity in both *in vivo* and *in vitro* experiments, whereas RNase A was ineffective (Souček *et al.*, 1983, 1986). Here, we assessed the immunosuppressive activity of two angiogenin preparations on mitogen- and antigen-stimulated normal human lymphocytes and compared their effect with that of BS RNase and RNase A. As is shown in Figs. 1–3, both angiogenin preparations displayed remarkable immunosuppressive activity assessed on PHA, Con A- or MLC-stimulated lymphocytes, activity very close to that of BS RNase. RNase A exerted only negligible activity at the highest concentration (100 μg/ml). It was reported that lysozyme can prevent inactivation of angiogenin caused by its adsorption to container surfaces (Bond, 1988). Addition of lysozyme to the incubation did not influence the activity of angiogenin (Table 3). Lysozyme alone also did not affect the proliferation of stimulated lymphocytes (unpublished data).

Leukemic cells proliferation activity

We reported earlier that BS RNase potently inhibits the growth of human tumor cells of a

Table 2. Effect of angiogenin, BS RNase and RNase A on embryo development

Development after	Species	Normal medium (control): No. of developing embryos	Normal medium (control): Embryo mortality†	Medium with angiogenin: No. of developing embryos	Medium with angiogenin: Embryo mortality†	Medium with BS RNase: No. of developing embryos	Medium with BS RNase: Embryo mortality†	Medium with RNase A: No. of developing embryos	Medium with RNase A: Embryo mortality†
0 hr*	Mice	20	0 (0)	12	0 (0)	5	0 (0)	6	0 (0)
	Bovine	13	0 (0)	35	0 (0)	24	0 (0)	11	0 (0)
6 hr	Mice	20	0 (0)	12	0 (0)	5	0 (0)	6	0 (0)
	Bovine	11	2 (15)	30	5 (14)	21	3 (13)	9	2 (18)
24 hr	Mice	19	1 (5)	12	0 (0)	0	5 (100)	5	1 (17)
	Bovine	10	3 (23)	24	11 (32)	0	24 (100)	7	4 (36)
48 hr	Mice	19	1 (5)	11	1 (9)	0	5 (100)	4	2 (33)
	Bovine	8	5 (39)	19	16 (46)	0	24 (100)	6	5 (45)
72 hr	Mice	19	1 (5)	11	1 (9)	0	5 (100)	4	2 (33)
	Bovine	8	5 (39)	19	16 (46)	0	24 (100)	5	6 (55)

*Beginning of cultivation.
†Values are number of incidences with percents in parentheses.

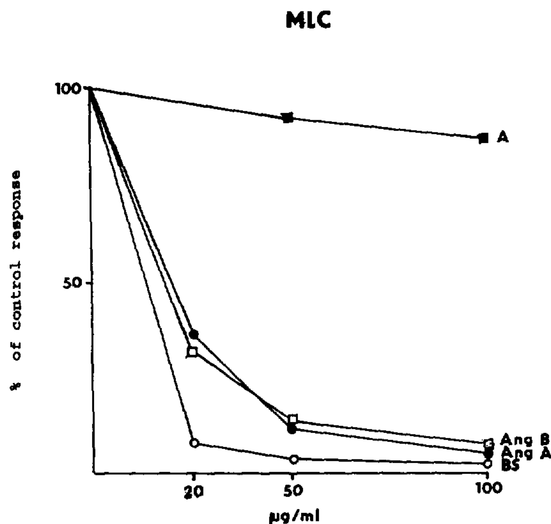

Fig. 1. In two-way mixed lymphocyte cultures (MLC), 10^5 human lymphocytes from each of two allogeneic unrelated donors were mixed and cultured in a total volume of 0.2 ml RPMI 1640 medium at 37°C in an atmosphere of 5% CO_2 for 6 days. The cultures were set up in triplicate. ■ A, RNase A; ● AngA, angiogenin (Frankfurt); □ AngB, angiogenin (Zurich); ○ BS, BS RNase.

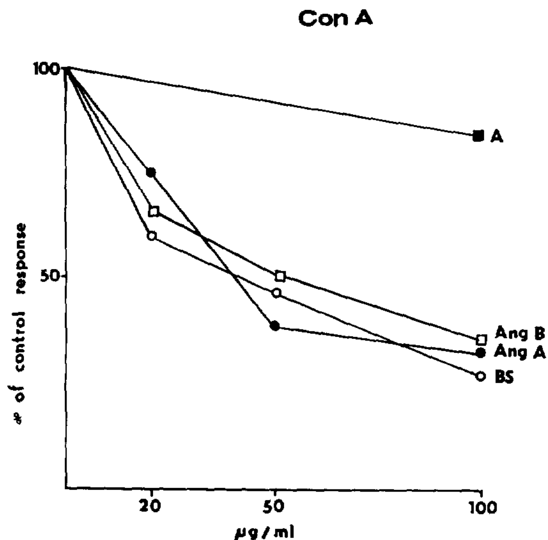

Fig. 3. Human lymphocytes 10^5 per sample were cultured with 5 µg Con A in a total volume of 0.2 ml RPMI 1640 medium at 37°C in an atmosphere of 5% CO_2 for 3 days. The cultures were set up in triplicate. ■ A, RNase A; ● AngA, angiogenin (Frankfurt); □ AngB, angiogenin (Zurich); ○ BS, BS RNase.

lymphoid type in culture (Souček *et al.*, 1981). Recently, we found that the inhibitory effect of BS RNase on various types of 20 human tumor cell lines occurs to different extents (Souček J. and Matoušek J., unpublished data 1993). Here, we chose two representatives of these cell lines, that is, K-562, a more sensitive, and HL-60, a less sensitive, tumor cell line for testing the proliferation activity. The results, recorded in Table 4, show the efficiency of BS RNase on the growth of the both tumor cell lines, whereas angiogenin and RNase A were ineffective. Adding of lysozyme to angiogenin did not change this result.

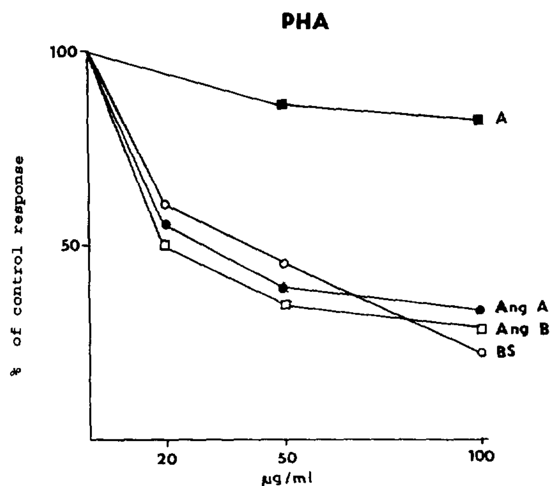

Fig. 2. Human lymphocytes 10^5 per sample were cultured with 5 µg PHA in a total volume of 0.2 ml RPMI 1640 medium at 37°C in an atmosphere of 5% CO_2 for 3 days. The cultures were set up in triplicate. ■ A, RNase A; ● AngA, angiogenin (Frankfurt); □ AngB, angiogenin (Zurich); ○ BS, BS RNase.

Discussion

Angiogenin and BS RNase belong to a group of RNases, designated as RISBAses (ribonucleases with Special Biological Action) (D'Alessio *et al.*, 1991). Regarding the biological effect, each of these RISBAses possesses a special activity. Angiogenin induces neovascularization, the process leading to the development of a vascular network in normal and malignant tissues (Fett *et al.*, 1985; Vallee *et al.*, 1985). The non-significant increase in the weight of testes and in the thickness of spermatogenic layers and diameter of seminiferous tubules observed after intratesticular injection in injected (and non-injected) testes (colateral effect) might support testicle vascularization. BS RNase has been effective in aspermatogenic, embryotoxic, antitumor and immunosuppressive activities (Dostál and Matoušek, 1973; Matoušek and Grozdanovič, 1973; Matoušek, *et al.*, 1973; Matoušek, 1975; Matoušek and Staněk, 1977; Souček *et al.*, 1983, 1986), whereas RNase A, a prototype of the superfamily of secretory ribonucleases, is ineffective in all of these activities. Immunosuppressive activity of angiogenin prepared in Hoechst Aktiengesellschaft was reported earlier (Matoušek *et al.*, 1992). Now we assessed

Table 3. Effect of lysozyme on angiogenin immunosuppressive activity

Angiogenin (μg/ml)	MLC		PHA		Con A	
	−Lys	+Lys	+Lys	+Lys	−Lys	+Lys
0	100	—	100	—	100	—
10	76	68	79	73	92	93
20	41	43	61	43	72	56
50	—	—	37	22	32	37
100	28	24	—	—	—	—

MLC-stimulated lymphocytes were cultivated for 6 days and PHA- or Con A-stimulated lymphocytes for 3 days with various concentrations of angiogenin or its combination with 100 μ/ml of lysozyme (Lys). Values evaluated by incorporation of ^{3}H-thymidine incorporation into the newly synthetized cell DNA are the mean of three replicates in two experiments and represent the percentage of the control values.

another sample of angiogenin obtained from a Swiss laboratory (ETH Zentrum, Zurich). We found identical immunosuppressive activity of both angiogenin preparations with that of BS RNase. BS RNase is much closer in structure to RNase A than to angiogenin. The difference in effectiveness of BS RNase and RNase A was believed to be due to the dimeric form of BS RNase. This hypothesis was confirmed in other experiments with monomeric BS RNase substance (Tamburini *et al.*, 1990). Nevertheless, the immunosuppressive activity of angiogenin, a monomeric ribonuclease, shows that the dimeric form of RNase molecule may not be essential for this activity.

The effect of addition of lysozyme to angiogenin, done to prevent the adsorption of angiogenin to container surfaces (Bond, 1988), was also tested. Lysozyme was highly toxic for cow or mouse embryos (data not shown). However, in human tumor cells or lymphocyte cultures, this toxic effect was not observed. Nevertheless, the presence of lysozyme in the incubation mixture did not increase markedly the ability of angiogenin to inhibit the biological reactions tested. This suggests that a low stability of angiogenin, observed earlier (Bond, 1988), is not related to its immunosuppressive efficiency.

Our findings of a new biological property of angiogenin may initiate further studies with this very interesting RNase derivative, because both activities, induction of vascularization and immunosuppressive effect, could generate synergic effect on tumor development.

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Table 4. Effect of angiogenin, BS RNase and RNase A on the human tumor cell proliferation

Concentrations (μ/ml)	K-562						HL-60					
	Angiogenin		BS RNase		RNase A		Angiogenin		BS RNase		RNase A	
	+Lys	−Lys	+Lys	−Lys	+Lys	−Lys	+Lys	−Lys	+Lys	−Lys	+Lys	−Lys
20	89	96	56	78	—	—	116	110	91	71	—	—
100	87	102	23	34	80	118	113	115	63	58	104	98

Human tumor cell lines K-562 and HL-60 were cultivated in microtiter plates for 48 hr with two concentrations of angiogenin A, BS RNase and RNase A. Lysozyme (Lys) was added at the concentration of 100 μg/ml. The proliferative activity was evaluated by ^{3}H-thymidine incorporation into the newly synthetized DNA. Each value is a mean of three replicates in two experiments and represents the percentage of the control value.

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