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Spermine enhances IgM productivity of human-human hybridoma HB4C5 cells and human peripheral blood lymphocytes

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Abstract

The polyamine spermine was assessed for enhancement of IgM production by human-human hybridoma, HB4C5 cells, under serum-free conditions. IgM production of HB4C5 cells was stimulated approximately 6-fold by the addition of 7.3 mM of spermine. The facilitating effect was observed soon after inoculation. In spite of suppression of cell growth, the IgM production rate was accelerated for at least 5 days without medium change. Moreover, laser confocal microscopic analysis revealed that the IgM content inside HB4C5 cells was increased by spermine treatment. These findings suggest that spermine enhances specific IgM productivity of the hybridoma line. Spermine also facilitated IgM production by human peripheral blood lymphocytes under serum-free conditions. This result implies that spermine enhances immunoglobulin production of not only specific hybridoma lines, but also non-specific immunoglobulin producers. Immunoglobulin production stimulating activity of spermine was accelerated 2-fold by the addition of DNA whith a chain length of about 400–7000 base pairs (bp). However, degraded short-chain DNA fragments (less than 200 bp) did not facilitate the immunoglobulin production stimulating activity of spermine.

Abbreviations: BSA – bovine serum albumin; DNA – deoxyribonucleic acid; ELISA – enzyme-linked immunosorbent assay; FITC – fluorescein isothiocyanate; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; Ig – immunoglobulin; IPSF – immunoglobulin production stimulating factor; PBL – peripheral blood lymphocytes; PBS – phosphate buffered saline

Introduction

Monoclonal antibody has high specificity against an antigen. This is the reason why monoclonal antibodies have been employed in various areas, such as diagnosis, therapy, purification by immunoaffinity chromatography, and immunohistochemistry. Therefore, mass production of monoclonal antibody using hybridoma cell culture in serum-free media has been improved. There are two approaches applied for efficient production of monoclonal antibodies by hybridomas and genetically engineered cell lines. One is the high-density large-scale culture of cells, and the other is enhancement of cellular productivity of monoclonal antibody. For achievement of efficient production of immunoglobulin (Ig), we have searched for factors which stimulate Ig production by hybridoma cell lines and lymphocytes. As a result of screening, several protein factors termed Ig production stimulating factors (IPSF) have been discovered. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase and lysozyme have been identified as IPSF's (Sugahara *et al*., 1992 and 1995, and Murakami *et al*., 1997). On the other hand, protein-free media are necessary for efficient production of bioactive substances, such as monoclonal antibody, because of the difficulty of purification of those from culture media supplemented with serum or protein factors. For that reason, we tried to discover new low molecular weight substances which stimulate Ig production by hybrido-

ma cell lines and peripheral blood lymphocytes.

Many IPSFs are categorized as basic proteins or basic poly-amino acids, such as histone (H1, H2A and H2B), poly-lysine, GAPDH, enolase and lysozyme. We focused on spermine as a candidate for a novel low molecular weight IPSF. Spermine, a polyamine, is an important factor which affects nucleic acid and protein synthesis (Tabor *et al.*, 1976) and proliferation and differentiation of cells (Pegg *et al.*, 1982). Here we report the Ig production stimulating activity of spermine under serum-free conditions, and the mode of action of spermine as IPSF.

Materials and methods

Spermine

Spermine (N,N-bis[3-Aminopropyl]-1,4-butanediamine) was purchased from SIGMA, U.S.A.. Spermine was dissolved in 10 mM sodium phosphate buffer (pH 7.4), and stored at –80 °C until needed. After neutralization by 6 N HCl, it was diluted to 780 mM with 10 mM sodium phosphate buffer (pH 7.4).

Deoxyribonucleic acids

Deoxyribonucleic acid (DNA) from salmon sperm was purchased from Wako Pure Chemical (Japan) and degraded DNA from herring sperm was from Sigma (U.S.A.). Agar gel electrophoresis revealed that the chain length of DNA from salmon sperm was about 400–7000 base pairs (bp), and that from herring sperm was less than 200 bp. In this paper, we term the former DNA preparation as long-chain DNA and the latter as degraded short-chain DNA, respectively. Each DNA preparation was dissolved in 10 mM sodium phosphate buffer (pH 7.4), and boiled for 5 min before use.

Cells and cell culture

Ig production stimulating activity of spermine was examined with a human-human hybridoma cell line, HB4C5, which produces monoclonal IgM antibody (Murakami *et al.*, 1985). HB4C5 cells were maintained in ERDF medium (Kyokuto Pharmaceutical, Japan) supplemented with 5 μg ml^{-1} insulin, 20 μg ml^{-1} transferrin, 20 μM ethanolamine and 25 nM sodium selenite (ITES-ERDF) at 37 °C under humidified atmosphere of 5% CO_2/95% air (Murakami *et al.*, 1982).

Human PBL obtained from peripheral blood of a healthy donor were also applied for assay of the IPSF effect of spermine on IgM and IgG production. Peripheral blood diluted with the same volume of sterilized phosphate buffered saline (PBS) was placed on lymphocyte separation medium (LSM; Organon Teknika, U.S.A.) in centrifuge tubes and centrifuged at 600 xg for 30 min. The thin layer of lymphocytes at the blood/LSM interface was collected and the PBL fraction was washed with ERDF medium 3 times by centrifugation at 700 xg for 5 min. The retrieved human PBL were pre-cultured in ERDF medium containing 10% of newborn calf serum for 1 day to remove adhesion-dependent cells such as monocytes. After pre-culture, the assay of IPSF effect on human PBL was performed using ITES-ERDF medium supplemented with spermine.

Assay of IPSF activity

The IPSF activity of spermine was assessed by measuring the amount of Ig secreted by HB4C5 cells or human PBL in culture media. HB4C5 cells and human PBL were inoculated in ITES-ERDF medium supplemented with or without spermine at 1×10^5 cells ml^{-1} and 1×10^6 cells ml^{-1}, respectively. Cultivation was performed in 96-well culture plates whose cultivation volume was 200 μl/well. After cultivation, the amount of Ig in each culture medium was measured by enzyme-linked immunosorbent assay (ELISA) using anti-human IgM or anti-human IgG antibodies (TAGO, U.S.A.).

Laser confocal microscopic analysis of intracellular IgM.

HB4C5 cells were inoculated at 1×10^6 cells ml^{-1} in ITES-ERDF medium with or without spermine and cultured for 8 h. After cultivation, cells were fixed with 95% ethanol and incubated in phosphate PBS containing 3% bovine serum albumin (BSA) for 1 h. After washing 3 times with PBS, cells were treated with FITC conjugated anti-human IgM antibody (TAGO, U.S.A.) in 3% BSA-PBS for 1 h. Then, HB4C5 cells were washed 3 times with PBS and mounted on slides using Permafluor (Immunothech S.A., France) containing 50% glycerol. Fluorescence derived from FITC was determined and visualized with a laser scanning confocal microscope with an argon ion laser coupled with Meridian InSIGHT plus^{TM} confocal unit attached to an Olympus IMT-2 inverted research microscope with

a SPlanApo ×100 objective lens.

Results

Effect of spermine on IgM production by HB4C5 cells

HB4C5 cells were inoculated at 1×10^5 cells ml^{-1} in ITES-ERDF medium supplemented with various concentrations of spermine. After cultivation for 6 h, the amount of IgM in each culture medium was measured by ELISA. As shown in Fig. 1, the amount of IgM secreted by HB4C5 cells was 37.5 ng ml^{-1} for 6 h when spermine was added at 7.3 mM. IgM production in ITES-ERDF medium without spermine was about 6 ng ml^{-1}. IgM production is enhanced about 6-fold by spermine. However, the optimal range of spermine for stimulation of IgM production was very narrow because of the toxicity of spermine. For this reason, the optimal concentration of spermine for subsequent experiments was selected as 2.4 mM at which IgM production was enhanced 5-fold.

Time-course effect of spermine on IgM production by HB4C5 cells

At first, the time-course effect of spermine on IgM production was examined to investigate the mode of action of spermine. HB4C5 cells were inoculated at 1×10^5 cells ml^{-1} in ITES-ERDF medium with or without 2.4 mM spermine, and the amount of IgM secreted into the culture medium was measured at each sampling period. As demonstrated in Fig. 2, IgM production by HB4C5 cells was facilitated 2- to 3-fold within 6 h after inoculation, and the IPSF effect was maintained throughout 48 h. Finally, the IgM production was enhanced 6-fold at 48 h after inoculation.

Effect of spermine on proliferation and specific IgM productivity of HB4C5 cells

As indicated above, spermine stimulated the IgM production of HB4C5 cells dose-dependently and the IPSF activity was maintained for 48 h. Next the long-term culture of HB4C5 cells was performed to investigate the effect of spermine on cell proliferation, viability, and specific IgM productivity. HB4C5 cells were inoculated at 5.6×10^4 cells ml^{-1} in ITES-ERDF medium with or without 2.4 mM spermine and cultured for 5 days. Cell density was measured using hemocytometer and viability was determined by trypan blue staining.

As shown in Fig. 3, spermine suppressed the proliferation of HB4C5 cells. However, the decline of viability was not significant for 3 days. On the other hand, the specific IgM productivity was significantly enhanced by the addition of spermine. The specific IgM productivity between the 2nd day and the 3rd day after inoculation was about 54 ng/10^4 cells/d when spermine was supplemented at 2.4 mM in ITES-ERDF medium, and that in ITES-ERDF was 7 ng/10^4 cells/d. This result means that the specific IgM productivity of HB4C5 cells was enhanced 7.7-fold by spermine. All these results suggest that spermine contributes to enhancement of specific IgM productivity of HB4C5 cells without growth promotion.

Fluorescence confocal microscopic analysis of intracellular IgM

HB4C5 cells were cultured in ITES-ERDF medium supplemented with or without 2.4 mM spermine for 8 h. After cultivation, these cells were fixed with 95% ethanol, and intracellular IgM was stained with FITC-conjugated anti-human IgM antibody. Then the samples were visualized using laser scanning confocal microscope with an argon ion laser. As shown in Fig. 4, the fluorescence intensity of spermine-treated cells was greater than that of control cells. This result clearly demonstrates that the amount of intracellular IgM was increased by spermine. It is supposed from these findings that spermine enhances IgM productivity of HB4C5 cells by acceleration of IgM synthesis.

Effect of spermine on Ig production by human PBL

Human PBL derived from peripheral blood of a healthy donor were pre-cultured in ERDF medium containing 10% newborn calf serum for 1 day. After pre-culture, human PBL were inoculated at 1×10^6 cells ml^{-1} in ITES-ERDF medium supplemented with each concentration of spermine, and cultured for 5 days. After cultivation, the amount of IgM and IgG secreted in each culture medium was determined by ELISA. As shown in Table 1, IgG production was not affected by spermine at all. The IgM production by human PBL was enhanced 1.8-fold when 0.8 mM of spermine was supplemented in ITES-ERDF medium. The cell density was not affected by the addition of 0.8 mM spermine (data not shown). This fact reveals that spermine stimulates specific Ig productivity of IgM producing PBL. However, IgM productivity was suppressed at higher dose of spermine, even though IgG productivity

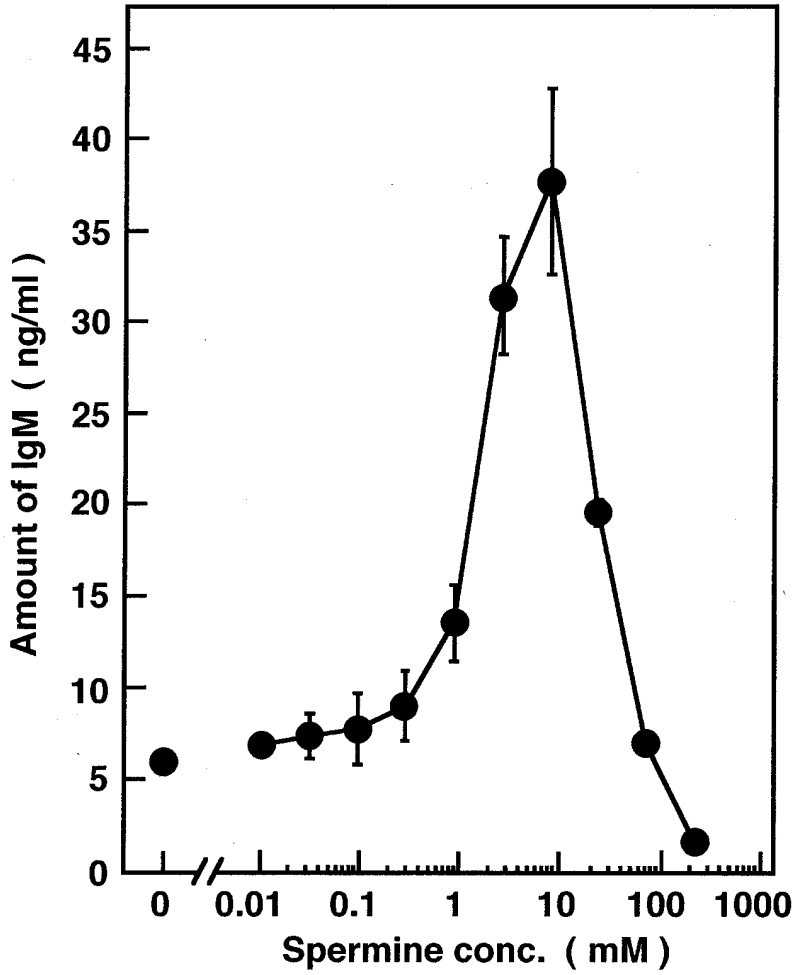

Figure 1. Effect of spermine on IgM production by HB4C5 cells. HB4C5 cells were inoculated at 1×10^5 cells ml^{-1} in ITES-ERDF medium supplemented with various concentrations of spermine. HB4C5 cells were maintained at 37 °C under humidified atmosphere of 5% CO_2/95% air. After 6 h cultivation, the amount of IgM in each culture medium was measured by ELISA. Results are expressed as the means± S.D. of three independent measurements.

Table 1. Effect of spermine on Ig production by human peripheral blood lymphocytes

	IgG production		IgM production	
Spermine conc. (mM)	Amount of IgG (ng ml^{-1})	Relative production	Amount of IgM (ng ml^{-1})	Relative production
0.0	15.7±0.8	1.0	1.8±0.1	1.0
0.8	15.8±2.0	1.0	3.2±0.3	1.8
2.4	12.4±0.6	0.8	0.8±0.0	0.4
7.2	12.5±0.6	0.8	0.5±0.1	0.3
21.8	13.8±0.5	0.9	0.3±0.0	0.2

Human peripheral blood lymphocytes (PBL) were obtained from peripheral blood of a healthy donor. Human PBL were inoculated at 1×10^6 cells ml^{-1} in ITES-ERDF medium supplemented with spermine at various concentrations. After cultivation for 5 days, the amounts of IgG and IgM in the culture medium was measured by ELISA. Results are represented as the means±S.D. of four independent measurements.

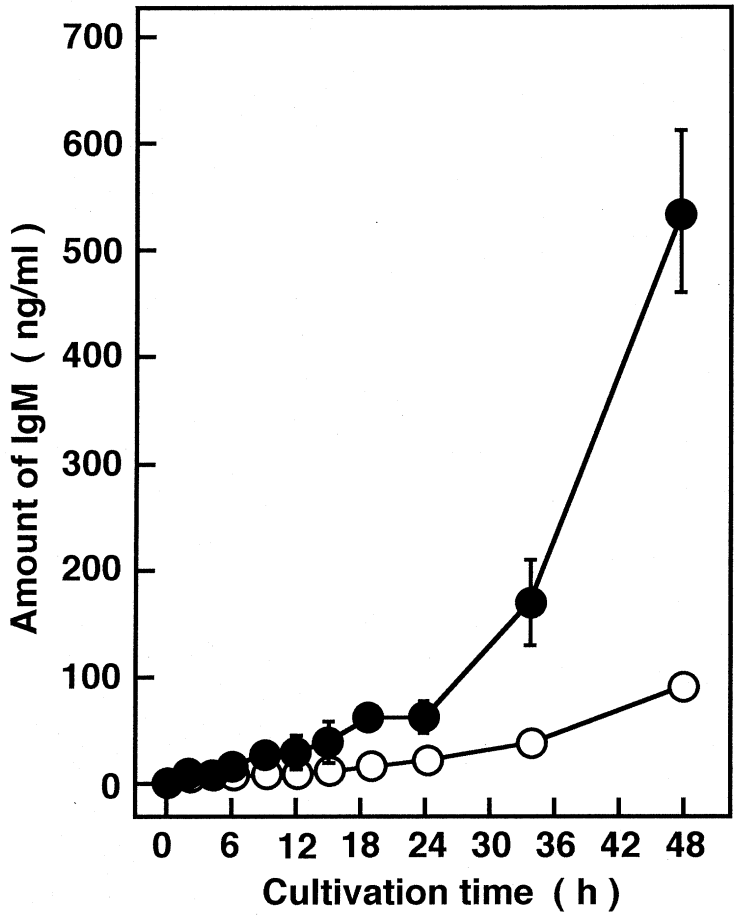

Figure 2. Time-course effect of spermine on IgM production by HB4C5 cells. HB4C5 cells were inoculated in ITES-ERDF medium with (●) or without (○) 2.4 mM of spermine at 1×10^5 cells ml^{-1}. After cultivation, the amount of IgM secreted in culture medium was measured by ELISA. The error bars denote S.D. calculated from four independent measurements.

was not suppressed, These results suggest that spermine accelerates IgM production not only of a specific hybridoma line, but also of non-specific IgM producing cells at appropriate dose.

Effect of DNA on IPSF activity of spermine

It is well known that spermine binds to, and interacts with nucleic acids (Igarashi *et al.*, 1977, and Flink *et al.*, 1975). Then, the effect of DNA on IPSF activity of spermine was investigated. Long-chain and degraded short-chain DNA preparations were boiled for 5 min. Following immediate ice-cooling, these DNA preparations were diluted at various concentrations with 10 mM sodium phosphate buffer (pH 7.4). These DNA solutions and the spermine preparation were mixed at the same volume, and immediately added to ITES-ERDF medium for assay of IPSF activity of spermine. The final concentration of spermine in ITES-ERDF medium was 1.2 mM. HB4C5 cells were inoculated at 1×10^5 cells ml^{-1} in these media and cultured for 6 h. After cultivation, the effect of DNA on IPSF activity of spermine was examined by measuring the amount of IgM in the culture media. As shown in Fig. 5A, the IPSF activity of spermine was enhanced about 2-fold by the addition of 70 μg ml^{-1} of long-chain DNA from salmon sperm. However, degraded short-chain DNA from herring sperm did not accelerate IPSF activity of spermine at all as indicated in Fig. 5B. On the other hand, synthetic poly-nucleic acids such as poly (U), poly (C) and poly (I)(C) suppressed IPSF activity of spermine (data not shown). All these facts show that the IPSF activity of spermine is strongly associated with nucleic acids.

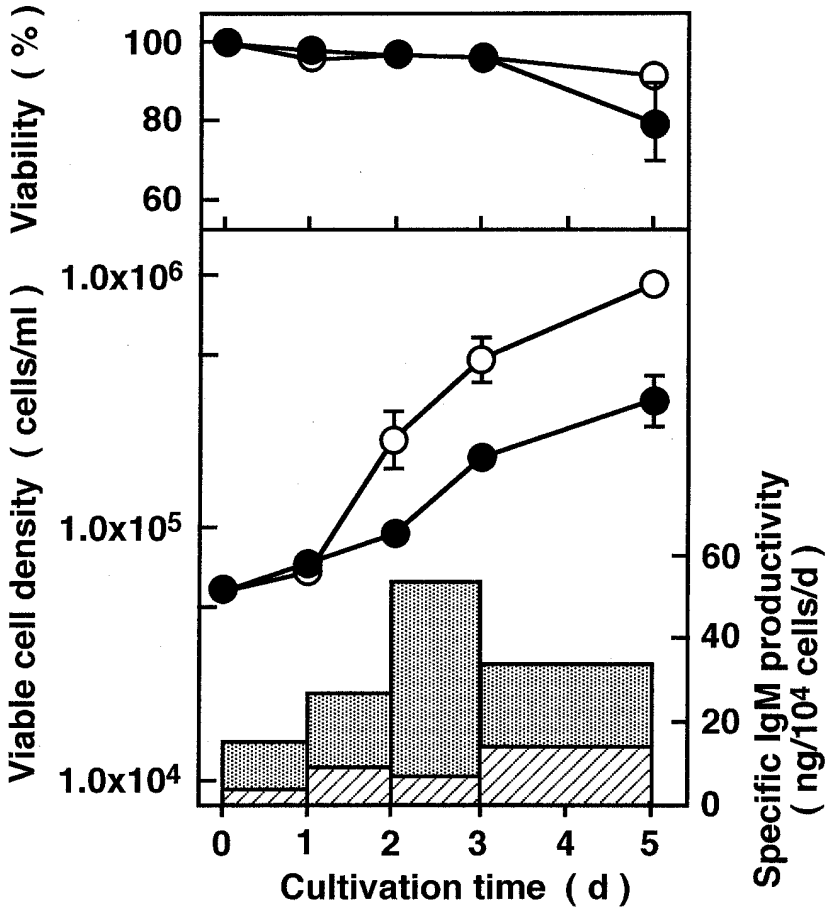

Figure 3. Effect of spermine on proliferation and specific IgM productivity of HB4C5 cells. HB4C5 cells were inoculated in ITES-ERDF medium containing 2.4 mM spermine (● and meshed bars) or not (○ and oblique lined bars) at 5.6×10^4 cells ml^{-1}. The viable cell density were determined using hemocytometer after trypan blue staining. The amount of IgM in culture medium was measured by ELISA. The measurement was performed with duplicate culture series. Specific IgM productivity between day 1 (d_1) and day 2 (d_2) was calculated by the following formula (Merten, 1988): Specific IgM productivity = $\frac{(C_2-C_1)(\ln X_2-\ln X_1)}{(d_2-d_1)(X_2-X_1)} \times 10^4$ (ng/10^4 cells/d), where C_1 and C_2 are the IgM concentrations (ng ml^{-1}) at day 1 and day 2, respectively. X_1 and X_2 are the viable cell densities (cells ml^{-1}) at day 1 and day 2. The measurements of cell density, viability and the amount of IgM in culture medium were duplicated.

Discussion

Low Ig productivity of hybridomas in serum-free medium compared to that in serum supplemented medium prompted us to screen Ig production stimulating factors. As the result of screening, we have found several proteins which stimulate Ig production by hybridomas and lymphocytes. Some glycolytic pathway enzymes, such as GAPDH, enolase, and aldolase stimulate Ig production by hybridomas and lymphocytes (Sugahara *et al*., 1991 and 1992). Alcohol dehydrogenase-I and lysozyme also accelerate Ig production of HB4C5 cells and PBL. IPSF activities of these enzymes were irrelevant to their enzymatic activity or reaction products (Sugahara *et al*., 1995). As a matter of interest, these enzymes have a common feature, namely, basic protein. Therefore, we focused on basic proteins and poly-basic amino acids. As might have been expected, lysine-rich histones (H1, H2A and H2B), and poly-lysine had IPSF activity (Sugahara *et al*., 1994).

Protein-free media are necessary for the efficient production of bioactive materials by animal cells. Low molecular and non-protein IPSF is indispensable to develop protein-free high-productivity media. Thus, we screened low molecular weight IPSF paying attention to basicity of substances. As the result of screening, spermine was identified as an IPSF. Spermine is a polyamine whose

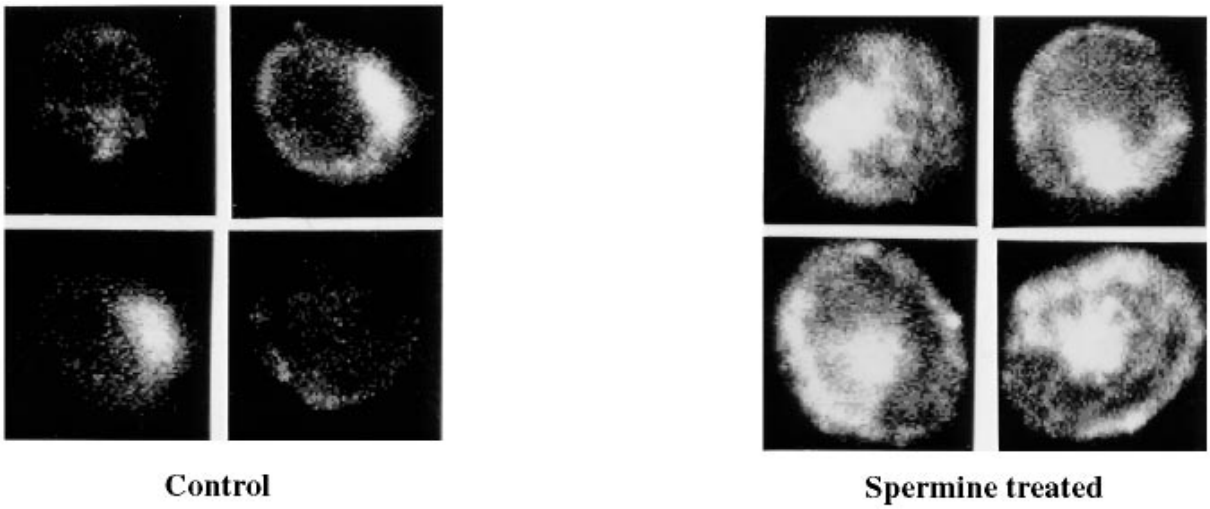

Figure 4. Laser confocal microscopic analysis of intracellular IgM. HB4C5 cells were inoculated at 1×10^6 cells ml^{-1} in ITES-ERDF medium containing 2.4 mM spermine or not, and cultured for 8 h. After cultivation, intracellular IgM were labeled by anti-human IgM antibodies conjugated with FITC. Fluorescence of FITC was monitored by an argon ion laser scanning confocal microscope.

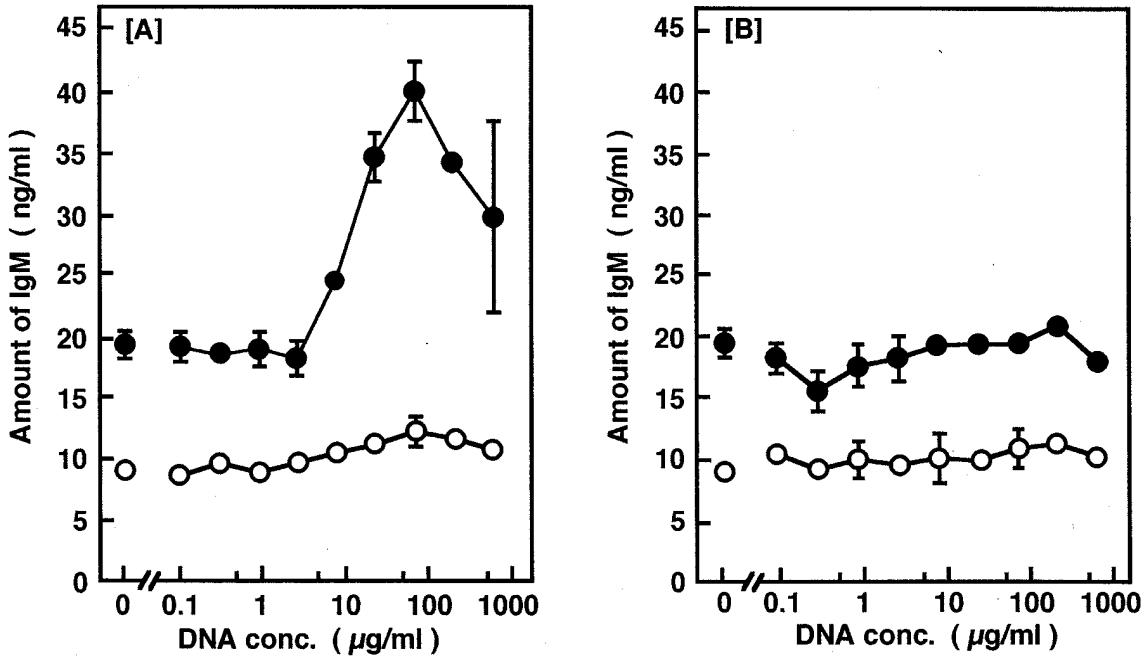

Figure 5. Effect of DNA on IPSF activity of spermine. Section [A]: Long-chain DNA from salmon sperm was mixed with the same volume of spermine (●) or not (○) at various concentrations just before supplement to ITES-ERDF medium. Section [B]: Degraded short-chain DNA from herring sperm was mixed with the same volume of spermine (●) or not (○) at various concentrations. HB4C5 cells were inoculated at 1×10^5 cells ml^{-1} in ITES-ERDF media containing DNA with or without 1.2 mM spermine. After cultivation for 6 h, the amount of IgM in culture medium was measured by ELISA. Results are expressed as the means ± S.D. of three independent measurements.

molecular weight is 202.3 composed of 2 imino groups, 2 amino groups, and 10 methylene groups [$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$; $C_{10}H_{26}N_4$]. Spermine stimulated IgM production by human hybridoma line HB4C5 cells 6-fold at 7.3 mM in serum-free medium. The Ig production stimulating

activity against HB4C5 cells was observed soon after inoculation in ITES-ERDF medium with spermine, and the effect was maintained for 5 days. Though the cell growth of HB4C5 cells was suppressed by spermine, it enhanced Ig production of the hybridoma line. This result suggests that spermine enhances specific IgM productivity of the cell. This hypothesis was clearly proved by quantitative analysis of intracellular IgM using a laser scanning confocal microscope. Spermine also facilitated IgM production by human PBL. This fact implies that spermine can be applied as IPSF for non-specified cell lines. These findings suggest that spermine is useful as a low molecular weight IPSF which is absolutely necessary for improving protein-free high-productivity media.

Several important physiological features of polyamine analogs including spermine have been reported. Spermine stimulates DNA synthesis in concanavalin A treated lymphocytes (Fillingame *et al*., 1975). Spermine binds to synthetic polynucleotides (Igarashi *et al*., 1977 and Ikemura, 1969), and stabilizes DNA folds (Flink *et al*., 1975). Moreover, high concentrations of spermine inhibit cleavage of DNA by restriction endonuclease (Pingoud *et al*., 1984). In regard to these findings, the effect of DNA binding affinity on Ig production stimulating activity of spermine was examined. Thus, the IPSF activity of spermine was examined in coexistence with DNA and found to enhance the activity of spermine more than 2-fold by coexistence with long-chain DNA. However, degraded short-chain DNA did not accelerate IPSF activity of spermine. The reason why long-chain DNA stimulated the IPSF activity of spermine is not known. Discovering the mechanisms of the actions of spermine will contribute not only to the development of protein-free medium, but also to the improvement of more useful low molecular weight IPSF.

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