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Lysozyme stimulates immunoglobulin production by human-human hybridoma and human peripheral blood lymphocytes

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Abstract

Lysozyme [EC 3.2.1.17] derived from hen egg white stimulated immunoglobulin production by human-human hybridoma, HB4C5 cells producing human lung cancer specific monoclonal IgM. IgM production by HB4C5 cells was enhanced more than 13-fold by the addition of lysozyme at 380 μg/ml in a serum-free medium. The immunoglobulin production stimulating effect of lysozyme was observed immediately after inoculation and maintained for 5 days. Lysozyme enhanced immunoglobulin production by the hybridoma line without growth promotion. This enzyme also accelerated IgM and IgG production of human peripheral blood lymphocytes 5.3-fold and 2.3-fold, respectively. These results suggest that lysozyme stimulates immunoglobuling production of not only specific hybridoma line, but also non-specific immunoglobulin producers. However, although the enzymatic activity of lysozyme was almost lost by heat-treatment at 100 °C for 30 min, the IPSF activity was retained. This fact suggests that IPSF activity of lysozyme does not come from its enzymatic activity or reaction products. All these findings clearly indicate that lysozyme has a novel function as an immunoglobulin production stimulating factor.

Abbreviations: GAPDH – glyceraldehyde-3-phosphate dehydrogenase; Ig – immunoglobulin; IPSF – immunoglobulin production stimulating factor; PBL – peripheral blood lymphocytes; HPLC – high-performance liquid chromatography.

Introduction

Monoclonal antibodies (MAbs) are applied to various fields, such as therapy, diagnosis, immunohistochemistry and also used for industrial applications to purify products by immunoaffinity chromatography. There are two strategies to produce MAbs in large quantities under serum-free conditions. One is the high-density large-scale culture of hybridomas, and the other is the enhancement of immunoglobulin (Ig) productivity of hybridomas. We have demonstrated that there are substances termed Ig production stimulating factor (IPSF) which enhance Ig production by hybridomas and lymphocytes. IPSF-I purified from culture medium of human lymphoblastoid cell line, HO-323 was a 410 kDa macro-protein (Toyoda *et al*., 1990). IPSF-II was found in cell extracts of Namalwa cells (Sugahara *et al*., 1991a). IPSF-II contained two IPSF active substances, i.e. IPSF-IIα and -IIβ. IPSF-IIα and IIβ were completely purified and characterized as glyceraldehyde-3-phosphate dehydrogenase (GARPH) and enolase, respectively (Sugahara *et al*., 1991b and 1992). IPSF-IIα stimulated Ig production by various human-human and mouse-mouse hybridomas 4- to 15-fold under serum-free conditions. Enzymatic activity in the glycolysis of GAPDH was not indispensable for expression of IPSF activity. Moreover, it was suggested that GAPDH stimulates post-transcriptional processes in protein synthesis to accelerate Ig production by hybridomas (Sugahara *et al*., 1995). Enolase identified as IPSF-IIβ also stimulated Ig production by various hybridomas and human peripheral blood lymphocytes (PBL). These facts indicated that both GAPDH and enolase possess a novel

biochemical function as IPSF, besider their enzymatic activity.

In addition, it was revealed that several basic proteins and poly-basic amino acids facilitated Ig production by hybridoma, HB4C5 cells (Sugahara *et al*., 1994). These findings urged us to investigate IPSF activity of other basic proteins. As a result of screening, we found that lysozyme has IPSF activity against human-human hybridomas and human PBL. Lysozyme is a very simple and stable protein which has lower molecular weight (14 kDa) than other IPSF's, and is easily separated from antibodies in culture medium by gel filtration. Moreover, this enzyme is so cheap that in practice it can be used for mass production of monoclonal antibodies. For these reasons, we investigated Ig production stimulating activity of lysozyme derived from hen egg white with a pI 10.5–11.0.

Material and methods

Lysozyme and its substrate

Lysozyme from hen egg white was purchased from Boehringer Mannheim (Germany), and dissolved in 10 mM sodium phosphate buffer (pH 7.4). This product gave single peak at 280 nm on HPLC gel filtration. Enzyme solution was sterilized by filtration before use. *Micrococcus luteus*, a substrate of lysozyme, was purchased from Sigma (USA).

Cells and cell culture

Human-human hybridoma HB4C5 cells which produce human lung cancer specific monoclonal IgM were used to assay IPSF activity of lysozyme. HB4C5 cells were fusion product of a human lymphocyte from lung cancer patient and a human fusion partner, NAT-30 (Murakami *et al*., 1985). HB4C5 cells were cultured in ERDF medium (Kyokuto Pharmaceutical, Japan) supplemented with 10 μg/ml of insulin, 20 μg/ml of transferrin, 20 μM of ethanolamine and 25 nM of selenite (ITES-ERDF) at 37 °C under humidified 5% CO_2-95% air (Murakami *et al*., 1982).

Human PBL were freshly obtained from peripheral blood of a healthy donor before use. Peripheral blood diluted with equal volume of phosphate buffered saline was centrifuged at room temperature for 30 min on lymphocyte separation medium (Organon Teknika, USA). Following the washing with ERDF medium 3 times, PBL were pre-cultured in ERDF supplemented with 10% of fetal bovine serum for 1 day to remove ahesion-dependent cells such as monocytes. After preculture, PBL were inoculated in ITES-ERDF medium containing lysozyme to determine IPSF activity of lysozyme against human PBL.

Assay of IPSF activity of lysozyme

IPSF activity was examined 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 containing lysozyme. The assay of IPSF activity was performed in a 96-well culture plate of which culture volume is 200 μl/well. HB4C5 cells and human PBL were inoculated at 1×10^5 cells/ml and 1×10^6 cells/ml, respectively. After cultivation in CO_2-incubator at 37 °C, the amount of Ig secreted in each culture medium was determined by enzyme-linked immunosorbent assay (ELISA) using anti-human IgM or IgG antibody (TAGO, USA) as mentioned in previous report (Sugahara *et al*., 1994). Determination of IPSF activity of lysozyme was triplicated.

Assay of enzymatic activity of lysozyme

Enzymatic activity of lysozyme against *Micrococcus luteus*, a substrate of lysozyme, was turbidimetrically determined. *M. luteus* was suspended in 0.05 M sodium phosphate buffer (pH 7.0) to give an approximate absorbance 1.0 at 530 nm. Lysozyme was added to the substrate solution at 16 μg/ml, and the enzymatic reaction was performed for 5 min at room temperature. The decrease of absorbance at 530 nm due to digestion of *M. luteus* by lysozyme was determined.

Results

Effect of lysozyme on IgM production by HB4C5 cells

Hybridoma HB4C5 cells were cultured in ITES-ERDF medium supplemented with lysozyme at various concentrations for 6 h to investigate a dose-response effect of lysozyme. After cultivation in a CO_2-incubator, the amount of IgM secreted by HB4C5 cells in each culture medium was measured by ELISA. As shown in Fig. 1, IgM production by HB4C5 cells was stimulated dose-dependently by the addition of lysozyme. HB4C5 cells were allowed to produce about 72 ng/ml of IgM in the presence of 380 μg/ml of lysozyme dur-

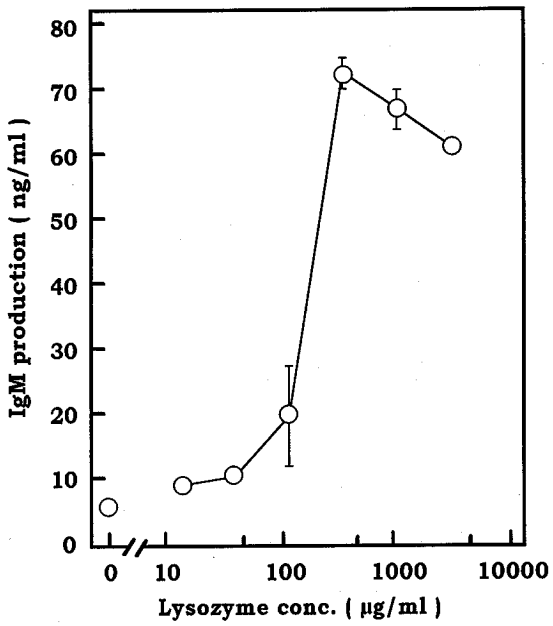

Figure 1. Effect of lysozyme from hen egg white on IgM production by HB4C5 cells. Hybridoma HB4C5 cells were inoculated at 1×10^5 cells/ml in ITES-ERDF medium supplmented with lysozyme at various concentrations. After 6 h-cultivation, the amount of IgM in each culture medium was measured by ELISA. Results are expressed as the means $\pm$ S.D. of three independent measurements.

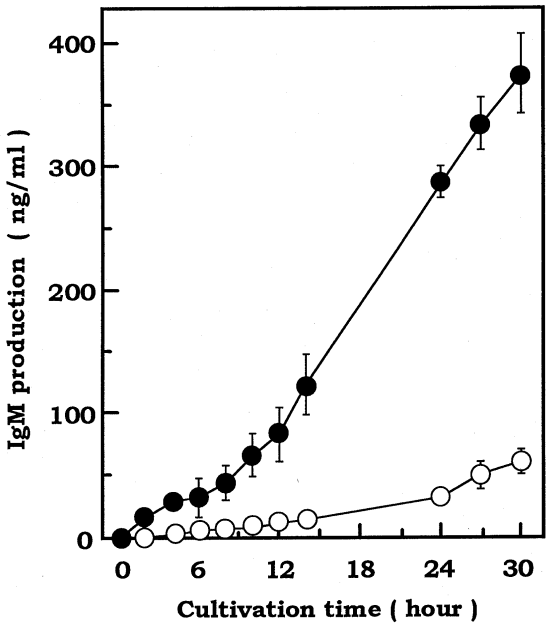

Figure 2. Time-course of IPSF activity of lysozyme on IgM production by HB4C5 cells. HB4C5 cells were inoculated at 1×10^5 cells/ml in 96-well culture plate. IgM production by human-human hybridoma HB4C5 cells was measured in ITES-ERDF medium under the presence of 380 μg/ml of lysozyme (●) on each sampling time. Accumulated IgM amount in the culture medium was measured by ELISA. Open circle (○) represents IgM production by hybridoma cultured in ITES-ERDF medium without lysozyme. Results represent the means $\pm$ S.D. of three independent measurements.

ing 6 h. This result discloses that lysozyme facilitates IgM production more than 13-fold as compared with that in ITES-ERDF medium.

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

It was clearly demonstrated that lysozyme stimulates IgM production by HB4C5 cells during 6-h cultivation. Then, the time-course of the IPSF effect of lysozyme was examined. HB4C5 cells were inoculated in ITES-ERDF medium containing 380 μg/ml of lysozyme. As indicated in Fig. 2, lysozyme immediately started to enhance IgM production by HB4C5 soon after inoculation. Then the amount of IgM in the culture medium was linearly increased, and the IPSF effect was maintained throughout the culture period.

Moreover, a long-term effect of lysozyme on cell growth and IgM production of HB4C5 cells were investigated. HB4C5 cells were inoculated at 1×10^5 cells/ml in ITES-ERDF medium with or without 380 μg/ml of lysozyme for 5 days. Lysozyme, however, showed no significant growth promoting activity toward HB4C5 cells, despite accelerated IgM production as shown in Fig. 3. IgM production was facilitated more than 10-fold by this enzyme at the 4th day after inoculation. This result also suggests that lysozyme stimulates specific IgM productivity of HB4C5 cells.

Effect of lysozyme on Ig production by human PBL

Human PBL were inoculated at 1×10^6 cells/ml in ITES-ERDF medium supplemented with lysozyme to investigate the IPSF effect on IgM and IgG production by human PBL. Table 1 indicates the cell numbers and Ig levels in the culture medium after 4 days. The addition of 380 μg/ml of lysozyme in the serum-free medium resulted in an increase in IgM and IgG production by human PBL. Production of IgM by PBL was enhanced 5.3-fold, and that of IgG by 2.3-fold. These findings indicate that this enzyme has an aptitude to facilitate Ig production by not only for specific hybridomas, but also for non-specific Ig producers.

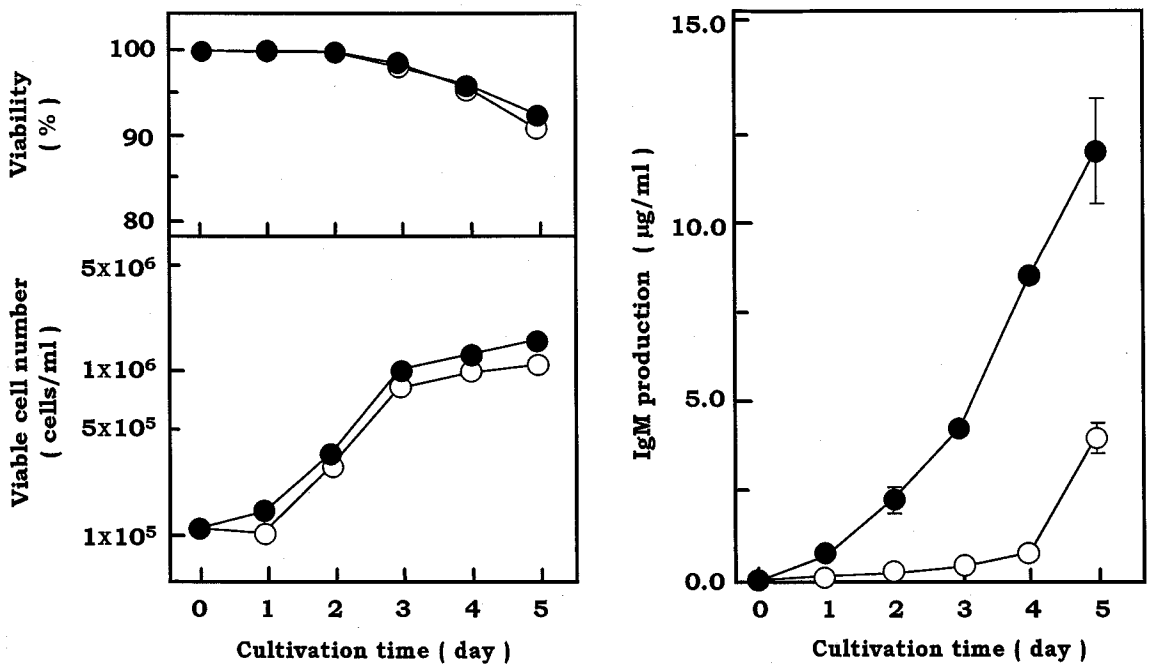

Figure 3. Effect of lysozyme on cell growth and IgM production of HB4C5 cells. HB4C5 cells were incubated at 1×10^5 cells/ml in ITES-ERDF medium supplemented with (●) or without (○) 380 μg/ml of lysozyme, and the amount of IgM production, cell densities, and cell viabilities were measured on each time. Results are expressed as the means ± S.D. of three independent measurements.

Table 1. Effects of lysozyme on cell growth and immunoglobulin production by human peripheral blood lymphocytes

	Viable cell No. (cells/ml)	Immunoglobulin production (ng/ml)	
		IgM	IgG
None	$4.5\pm0.3\times10^5$	2.9±0.6	33.5±2.3
Lysozyme	$4.7\pm0.6\times10^5$	15.3±3.5	77.5±10.0

Human peripheral blood lymphocytes were inoculated at 1×10^6 cells/ml in ITES-ERDF medium supplemented with lysozyme at 380 μg/ml or not. After 4 days-cultivation, viable cell number and immunoglobulin concentration (IgM and IgG) in the culture medium were determined. Viable cell numbers were measured by hemocytometer after trypan blue staining. These values are expressed as the means ± S.D. of four independent measurements.

Table 2. Effect of heat-treatment on IPSF and enzymatic activities of lysozyme

	Native	Heat-treated
IPSF activity	100.0%	81.4%
Enzymatic activity	100.0%	10.0%

Lysozyme was heat-treated at 100 °C for 30 min. For assay of IPSF activity, HB4C5 cells were inoculated at 1×10^5 cells/ml in ITES-ERDF medium containing 380 μg/ml of each lysozyme sample, and cultured for 6 h. For assay of enzymatic activity, each lysozyme sample was added to substrate solution at 16 μg/ml. After 5 min-incubation at room temperature, absorbance at 530 nm was measured. IPSF activity and enzymatic activity are expressed as relative activity against native lysozyme.

Heat and pH stability of lysozyme as IPSF

Lysozyme in 10 mM sodium phosphate buffer (pH 7.4) was boiled for 30 min to determine heat stability of IPSF and enzymatic activities. After ice-cooling, the enzymatic and IPSF activities were assayed. HB4C5 cells were cultured at 1×10^5 cells/ml for 6 h in ITES-ERDF medium supplemented with heat-treated or non-treated lysozyme. Lysozyme, however, lost its enzymatic activity by boiling, IPSF activity was retained as shown in Table 2.

For analysis of pH stability, lysozyme was incubated in various pH buffer solutions for 12 h at 4 °C. As indicated in Fig. 4, both IPSF activity and enzymatic activity were remarkably stable in a wide pH range, and showed the same features against pH treatment.

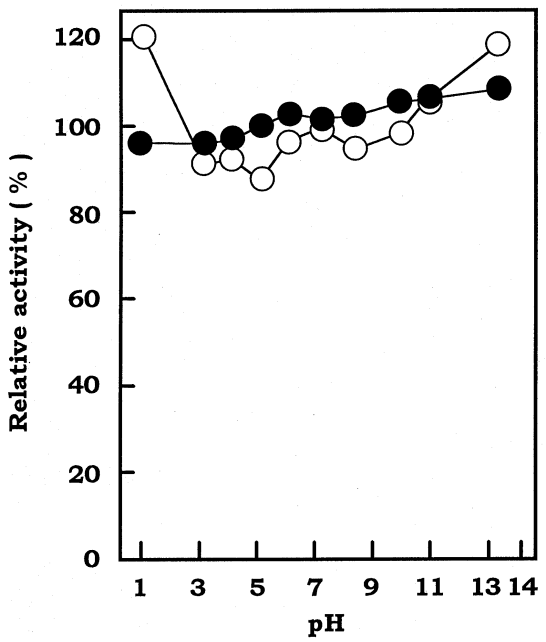

Figure 4. pH stability of lysozyme. Lysozyme was treated with pH buffer solutions of various pHs for 12 h at 4 °C. Following dialysis against 10 mM sodium phosphate buffer (pH 7.4), IPSF activity and enzymatic activity were assayed. Results which indicated as relative activities against native lysozyme represent the means of three independent measurements. Closed circle (●) and open circle (○) denote relative IPSF activity and relative enzymatic activity, respectively.

This fact simultaneously suggests that the IPSF activity of the lysozyme preparation used here is not derived from contaminants except for lysozyme.

Discussion

IPSF stimulates Ig production of hybrisomas and lymphocytes under serum-free conditions. Ig production by hybridomas in serum-free media supplemented with IPSF is superior to that in serum-supplemented media with or without IPSF (Murakami, *et al*., 1993). Some substances have been identified as IPSF. We previously demonstrated that IPSF-IIα and -IIβ in Namalwa cell lysate were glycolysis enzymes, GAPDH and enolase, respectively. GAPDH has been studied by many workers for its functions other than enzymatic activity (Sabath *et al*., 1990, Nagy *et al*., 1995 and Singh *et al*., 1995). GAPDH is also a protein having a binding affinity with nucleic acids, such as single stranded DNA, RNA, and mono- and polyribosomes (Perucho *et al*., 1977, Perucho *et al*., 1980, and Ryazanov *et al*., 1988). Moreover, GAPDH is a one of the three major RNA-binding proteins in rabbit reticulocyte lysate (Ryazanov, 1985). We predicted from these findings that a relationship exists between IPSF activity and nucleic acid binding activity. The IPSF activity of some nucleic acid binding proteins were assessed. The result was that lysine-rich histone (H1, H2A and H2B) and poly-lysine stimulated IgM production by hybridomas (Sugahara *et al*., 1994).

Another common feature of these IPSF's including GAPDH and enolase, is basicity of the proteins. We thus paid attention to basic proteins as Ig production stimulating factor, and screened various basic proteins. Here we demonstrated the Ig production stimulating activity of lysozyme [EC 3.2.1.17]. Lysozyme from hen egg white remarkably enhanced Ig production by human-human hybridoma HB4C5 cells and human PBL under the serum-free conditions. Lysozyme accelerated IgM production by HB4C5 cells in ITES-ERDF medium more than 13-fold. HB4C5 cells were facilitated in their IgM production by lysozyme immediately after inoculation, and the IPSF effect was maintained for 5 days without growth promotion. These facts clearly indicate that lysozyme stimulates specific IgM productivity of the cell. Lysozyme also enhanced IgM and IgG productivity of human PBL 5.3-fold and 2.3-fold, respectively. It is assumed from this result that lysozyme in serum contributes not only to digestion of infectious microorganisms, but also accelerates immune responses *in vivo*.

Enzymatic and IPSF activities of lysozyme were examined after 100 °C treatment for 30 min. However the enzymatic activity was almost lost, lysozyme stimulated IgM production by HB4C5 cells. This fact makes it clear that the IPSF activity of lysozyme is derived from neither its enzymatic activity nor reaction products. This result also provides an indication that this enzyme has a novel function other than enzymatic one.

Lysozyme is a extremely stable, and lower molecular weight protein in comparison with other IPSF proteins. This feature of lysozyme contributes not only to practical use for mass-production of monoclonal antibodies, but also to investigation of the mode of action as IPSF. We have some data implying that lysozyme stimulates post-transcriptional process in the same was as GAPDH and enolase (unpublished data). Uncovering the mode of action of lysozyme as IPSF will contribute to the effective enhancement of cellular productivity of Ig by hybridomas and lymphocytes.

References

Murakami H, Hasui H, Sato GH, Sueoka N, Chow TP and Kono-Sueoka T (1982) Growth of hybridoma cells in serum-free medium. Proc Natl Acad Sci USA 79: 1158–1162

Murakami H, Hashizume S, Ohashi S, Ohashi H, Shinohara K, Yasumoto K, Nomoto K and Omura H (1985) Human-human hybridomas secreting antibodies specific to human lung carcinoma. *In Vitro* Cell Develop Biol 21: 593–596

Murakami H, Sugahara T and Nakajima H (1993) Immunoglobulin production stimulating factors. In: Oka MS and Rupp RG (eds.) Cell Biology and Biotechnology. (pp. 35–62) Springer-Verlag, New York

Nagy E and Rigby WFC (1995) Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD^+-binding region (Rossmann fold). J Biol Chem 270: 2755–2763

Perucho M, Salas J and Salas ML (1977) Identification of the mammalian DNA-binding protein P8 as glyceraldehyde-3-phosphate dehydrogenase. Eur J Biochem 81: 557–562

Perucho M, Salas J and Salas ML (1989) Study of the interaction of glyceraldehyde-3-phosphate dehydrogenase with DNA. Biochim Biophys Acta 606: 181–195

Ryazanov AG (1985) Glyceraldehyde-3-phosphate dehydrogenase is one of the three major RNA-binding proteins of rabbit reticulocytes. FEBS letters 192: 131–134

Ryazanov AG, Ashmarina LI and Muronetz VI (1988) Association of glyceraldehyde-3-phosphate dehydrogenase with mono- and polyribosomes of rabbit reticulocytes. Eur J Biochem 171: 301–305

Sabath DE, Broome HE and Prystowsky MB (1990) Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte. Gene 91: 185–191

Singh R and Green MR (1995) Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. Science 259: 365–368

Sugahara T, Shirahata S, Yamada K and Murakami H (1991a) Purification of immunoglobulin production stimulating factor-IIα derived from Namalwa cells. Cytotechnology 5: 255–263

Sugahara T, Shirahata S, Akiyoshi K, Isobe T, Okuyama T and Murakami H (1991b) Immunoglobulin production stimulating factor-IIα (IPSF-IIα) is glyceraldehyde-3-phosphate dehydrogenase like protein. Cytotechnology 6: 115–120

Sugahara T, Nakajima H, Shirahata S and Murakami H (1992) Purification and characterization of immunoglobulin production stimulating factor-IIβ derived from namalwa cells. Cytotechnology 10: 137–146

Sugahara T, Sasaki T and Murakami H (1994) Enhancement of immuniglobulin productivity of human-human hybridoma HB4C5 cells by basic proteins and polY-basic amino acids. Biosci Biotech Biochem 58: 2212–2214

Sugahara T, Shirahata S, Sasaki T and Murakami H (1995) The mode of actions of glyceraldehyde-3-phosphate dehydrogenase identified as an immunoglobulin production stimulating factor. FEBS Lett. 368: 92–96

Toyoda K, Sugahara T, Inoue K, Yamada K, Shirahata S and Murakami H (1990) Purification and characterization of the immunoglobulin production stimulating factor derived from human B lymphoblastoid cell HO-323. Cytotechnology 3: 189–197

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