Effect of Nitrite on Cell Growth and Antibody Production in Mouse Splenic B Cells Stimulated with Lipopolysaccharide and B Cell Hybridomas

Kuniaki TAKAGI,* Shotaro AE and Yasunobu SUKETA

Department of Environmental Biochemistry, School of Pharmaceutical Science, University of Shizuoka, 395 Yada, Shizuoka 422, Japan.
Received July 4, 1991

Nitric oxide, nitrite and nitrate are released by activated macrophages in an immune response. We showed here that nitrite influenced cell growth and antibody production in mouse lipopolysaccharide (LPS)-stimulated splenic B cells and B cell hybridomas. The addition of 10^{-7} and 10^{-6} M nitrite enhanced deoxyribonucleic acid (DNA) synthesis of LPS-stimulated splenic B cells. However, DNA synthesis and antibody production in the case of total spleen cells stimulated with LPS were suppressed by nitrite in a dose dependent-manner. These phenomena were also observed in a similar experiment involving mouse B cell hybridomas. Antibody production of all B cell hybridomas was significantly suppressed by the addition of nitrite. This suppressing effect could not be explained by changes in viable cell yields. This data suggests that the antibody production and cell proliferation of B cells may be influenced by nitrite from activated macrophages in the immune response.

Keywords B cell growth; antibody production; nitrite; nitric oxide

Introduction

The antibody production system is regulated by many cytokines, such as interferon-γ (IFN-γ),[1] and by interleukins (IL).[2-4] The mechanisms for differentiation and activation of B cells by these protein factors have been well studied.

Macrophages and macrophage like cells activated by IFN-γ, IL-6 and lipopolysaccharide (LPS) have been reported to produce nitric oxide (NO), nitrite (NO_2^-) and nitrate (NO_3^-).[5-7] NO exerts anti-tumor activity by inhibiting mitochondria respiration,[8] the tricarboxylic acid cycle enzyme aconitase[9] and deoxyribonucleic acid (DNA) synthesis.[10]

Recently, we reported that mouse splenic macrophages produced NO_2^- in an immune response by regulation with T cells.[11] From the evidence, nitrogen oxides produced by activated macrophages were supposed to have other physiological functions and to affect immune responses. In this report, we show that NO_2^- influences cell growth and antibody production of mouse LPS-stimulated spleen cells and B cell hybridomas.

Materials and Methods

Materials Reagents and their sources were as follows. Endotoxin-screening fetal calf serum (FCS) was obtained from HyClone Co. (Logan, UT) and Mitubishi Kasei Co. (Japan). [^{3}H]Thymidine was from ICN Biomedicals Inc. (Costa Mesa, CA). Horse radish peroxidase (HRP)-labeled goat-anti mouse immunoglobulin G (IgG) was from Bio-Rad. HRP-labeled goat anti mouse IgM was from Cappel Co. Rabbit complement (low-Tox-M) was from Cedarlene Co. Sodium nitrite, sodium nitrate and GIT medium (containing growth factors and 4.47×10^{-9} M NO_3^-, but a serum-free medium) was purchased from Wako Pure Chemical Co. (Osaka, Japan). Eagle's minimum essential medium (MEM) was from Nissui (Tokyo, Japan). *E. coli* LPS (0127: B8 and 0111: B4) was from Sigma Chemical Co. (St. Louis, MO).

Animals Balb/c and ddY (specific pathogen free) mice were purchased from SLC Co. Female mice (ddY, 8 weeks old) were used in this study. Balb/c mice were used for making hybridomas.

Preparation of B Cells and Cell Culture Mouse spleen cells were prepared as described before.[11] The spleen cells were incubated on horse serum-coated plastic dishes for 14 h, then non-adherent cells were collected. In order to deplete T cells from the spleen cells, the cells were incubated with 2 ml of culture supernatants of monoclonal anti-Thy1.2 antibody for 1 h at 4 °C, washed three times with MEM, and then incubated with an appropriately diluted complement for 1 h at 37 °C. After incubation, the cells were washed three times with MEM and used for further studies. About ninety percent of the cells were surface immunoglobulin (sIg)-positive and the cells did not produce nitrite by stimulation with LPS, nor did they incorporate [^{3}H]thymidine by stimulation with Con A. The cells were plated in 48-well culture plates (Sumilon MS-80480) at a density of 1.0×10^6 cells/well in the presence of LPS and sodium nitrite (10^{-7}—10^{-3} M). After 1—5 d, 2 μCi of [^{3}H]thymidine (specific activity 40—60 Ci/mmol) were added to each well and incubated for 15 h for measurement of DNA synthesis.

B Cell Hybridomas and Cell Culture 30H12 (anti-Thy1.2 antibody producing hybridoma) was kindly supplied by Dr. L. Herzenberg, Stanford University. AE6-5 (anti-rabbit IgG antibody-producing hybridoma) and DG8 (anti-rabbit IgG antibody producing-hybridoma) were produced by our laboratory. AE6-5 was formed by the fusion of the mouse myeloma line P3U1 with spleen cells immunized with rabbit IgG. We used spleen cells for the fusion of AE6-5 21 d after the first immunization. DG8 was formed by the fusion of the mouse myeloma line P3U1 with spleen cells unimmunized. The hybridomas and P3U1 (myeloma) were maintained by endotoxin-free RPMI 1640 plus 10% FCS or by GIT medium plus 2% FCS.

1) Short-Term Exposure of B Cell Hybridomas to Nitrite: Reagents sterile-filtrated through a 0.22-μm membrane were added to the treated cultures at a final concentration of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} M; the vehicle alone (GIT medium) was added to the control cultures. Cells were plated in 24-well culture plates (Corning 25010) in the above medium at a density of 1.0×10^5 cells/well. After 2 d, 2 μCi of [^{3}H]thymidine (specific activity 40—60 Ci/mmol) in 100 μl GIT medium were added to each well and incubated for 4 h.

2) Long-Term Exposure of B Cell Hybridomas to Nitrite: Balb/c thymocytes or spleen cells as feeder cells were plated in 6 cm dishes in RPMI-1640 plus 10% FCS at a density of 2.5×10^7 cells/dish. After overnight incubation, the culture media were removed and 2 ml of the RPMI-1640 medium with supplemented with 20% FCS and 0.25% agar. Thirty minutes later, DG8 (B cells hybridoma secreting IgM anti-rabbit IgG) was plated at 500 cells/dish in 1.7 ml of RPMI-1640 medium supplemented with 20% FCS and 0.08% agar. Finally, 2 ml of 10% FCS RPMI was added. The agar media and liquid media were supplemented with varying concentrations of sodium nitrite. These cultures were treated for 18 d, then cell numbers were determined by a hemocytometer, and concentrations of antibody by enzyme-linked immunosorbent assay (ELISA).

[^{3}H]Thymidine Incorporation Cells incubated with [^{3}H]thymidine were harvested on a glass fiber filter by washing with saline and 1% trichloroacetic acid. Two milliliters of scintillant were added to the dried glass filter, and the trichloroacetic acid (TCA)-insoluble radio-activity was determined by a liquid scintillation counter. Each sample consisted of 4 wells. Data is represented as means $\pm$ S.D.

Analysis of Antibody Immunoglobulin levels in the supernatants of the cultured cells were analyzed by ELISA in 96-well flat-bottomed polystylene microtiter plates (Falcon 3912). To assay IgG or IgM levels, wells were coated overnight with rabbit anti-mouse IgG or IgM antibody at 10 μg/ml (H + L chain specific) in 0.1 M $NaHCO_3$ buffer, pH 8.2. Wells were washed, blocked with Tris-buffered saline/0.1% Tween 20/1% bovine serum albumin (BSA), and filled with 100 μl of serial 10-fold dilutions of culture

supernatants. After overnight incubation at 4 °C, wells were washed and sequentially filled with 100-μl of aliquots of a 1:1000 dilution of a peroxidase conjugate goat anti-mouse IgG or IgM (heavy chain specific, Cappel Laboratories), and substrate (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) plus H_2O_2; Wako Junyaku Co.). After 15 min the reaction was stopped by the addition of 37 mM KCN, and wells were analyzed for 415 nm in an ELISA reader (Toso Co., Japan).

Results

Influences of NO_2^- on the Function in the B Cell Stimulated with LPS Mouse splenic B cells were stimulated with LPS and increasing concentrations of nitrite. [^{3}H]Thymidine incorporation and IgM concentration in the culture supernatant were measured on each of days 2—5. The addition of NO_2^- at 10^{-7} and 10^{-6} M caused a modest augmentation in [^{3}H]thymidine incorporations on day 2 and 3 (112—132% and 130—151%, respectively) (Fig. 1-A). However, [^{3}H]thymidine incorporations to the cells decreased at 10^{-3} M nitrite on all days (60.8±11.6%). On the other hand, IgM concentrations in the culture supernatant showed progressive decline at the NO_2^- concentration increased (Fig. 1-B). The greatest degree of suppression was observed with 10^{-3} M of nitrite (0% on day 4 and 25.5±1.5% on day 5).

In a previous report, we demonstrated that nitrite production in immune spleen cells continued for 10 d. As shown in Fig. 1, [^{3}H]thymidine incorporation of splenic B cells was highest on day 2. Therefore we studied the effect of nitrite on [^{3}H]thymidine incorporation and IgM production of total spleen cells stimulated with LPS to investigate the relationship between nitrite and cell growth on days 4 and 5 (Fig. 2). With increasing NO_2^- concentrations, IgM levels and [^{3}H]thymidine incorpora-

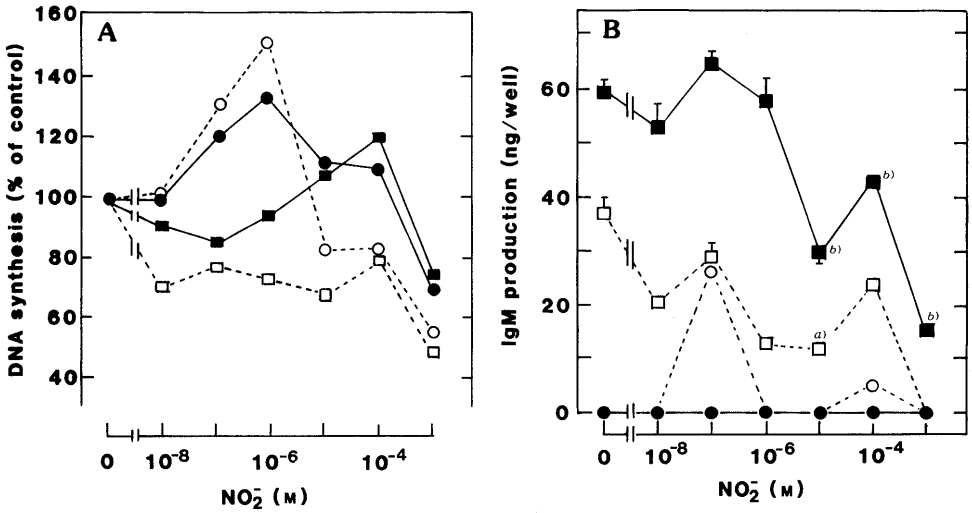

Fig. 1. Effect of NO_2^- on [^{3}H]Thymidine Incorporation (A) and IgM Production (B) of Mouse Splenic B Cells

Splenic B cells (1.0×10^6 cells/well) were cultured for 2 (○), 3 (●), 4 (□), and 5 (■) days with LPS (10 μg/ml) and various concentrations of nitrite. (A) during the last 15 h in the respective cultivation periods, 1 μCi/ml of [^{3}H]thymidine was added to each well. One hundred percent of [^{3}H]thymidine incorporation each day was 436±46 (day 2), 407±110 (day 3), 366±138 (day 4), and 361±75 dpm/well (day 5). (B) IgM production of each sample was measured by ELISA. The points are means±S.D. for quadruplet. *a*) $p<0.01$, *b*) $p<0.005$.

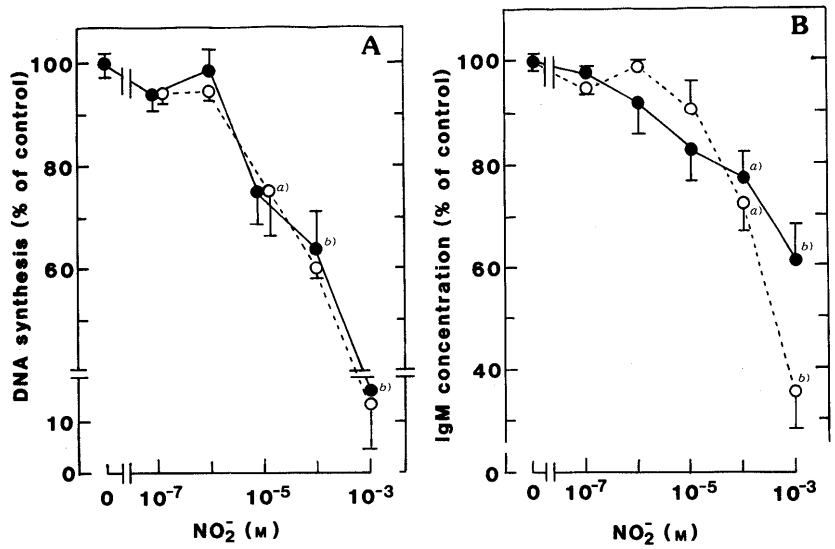

Fig. 2. Effect of NO_2^- on [^{3}H]Thymidine Incorporation (A) and IgM Production (B) of Mouse Spleen Cells

Spleen cells (1.0×10^6 cells/well) were cultured for 4 (○), and 5 (●) days with LPS (10 μg/ml) and various concentrations of nitrite. (A) during the last 15 h in the respective cultivation periods, 1 μCi/ml of [^{3}H]thymidine was added to each well. One hundred percent of [^{3}H]thymidine incorporation each day was 13189±904 (day 4) and 11453±164 dpm/well (days 5). (B) IgM production of each sample was measured by ELISA. One hundred of IgM concentration in the culture supernatant was 7.139±0.149 (day 4) and 7.484±0.366 μg/ml (day 5). The point are means±S.D. for quadruplet. *a*) $p<0.01$, *b*) $p<0.005$.

TABLE I. Effect of NO_2^- on [^{3}H]Thymidine Incorporation of B-Cell Hybridomas and Myeloma

NO_2^- (M)	[^{3}H]Thymidine incorporation (dpm × 10^{-4})[b]					
	P3U1[a]		30H12		AE6-5	
0	7.83±0.58	(100.0)[c]	9.98±0.73	(100.0)	7.87±0.57	(100.0)
10^{-7}	7.04±0.41	(91.9)	8.70±0.36	(87.2)[d]	12.80±0.72	(127.3)[e]
10^{-6}	6.87±0.44	(91.0)	9.71±0.63	(99.5)	12.16±0.40	(116.1)[e]
10^{-5}	6.41±0.25	(93.1)	9.83±0.16	(98.9)	12.67±1.48	(115.5)[e]
10^{-4}	7.36±0.43	(104.3)	10.23±0.29	(91.4)	13.37±0.43	(139.6)[e]
10^{-3}	8.54±0.90	(105.0)	6.89±0.90	(61.4)	18.72±2.09	(239.7)[e]

a) P3UI, 30H12, and AE6-5 (1×10^5 cells) were cultured for 48 h with various concentrations of nitrite. *b*) Thymidine incorporation during the final 4 h of culture. *c*) Results are expressed as the mean±S.D. for quadruplet. Numbers in parentheses are percent of control. *d*) $p<0.01$, *e*) $p<0.005$.

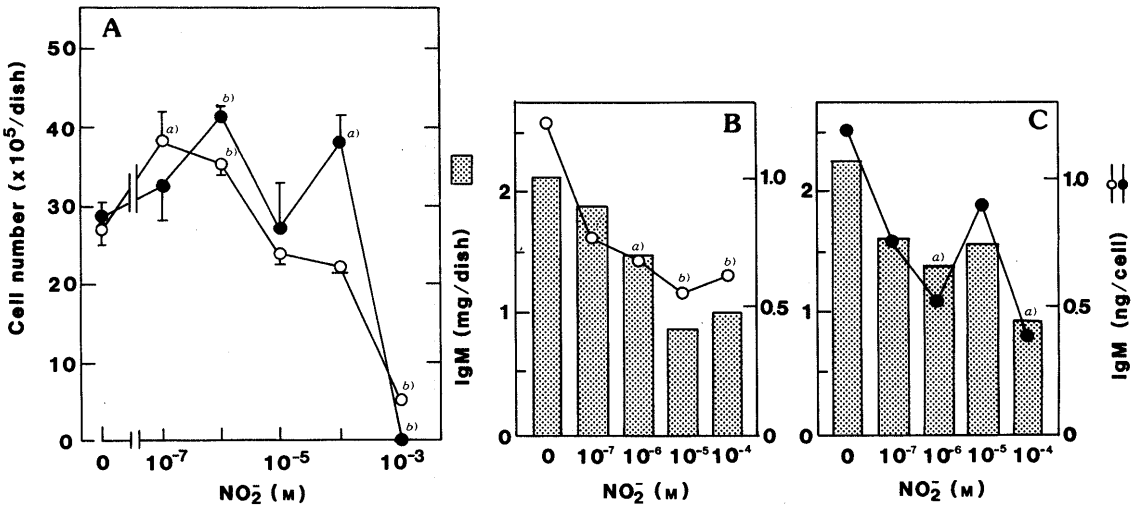

Fig. 3. Effect of NO_2^- on Cell Proliferation (A) and Antibody IgM Production (B, C) of B Cell Hybridoma in Long-Term Culture

B cell hybridoma (500 cells/dish) was cultured with various concentrations of nitrite as described in Materials and Methods. After culture for 18 d, the cell numbers were counted (A) and IgM concentrations in the culture supernatant were measured (B, C). Feeder cells were spleen cells (○, B) and thymocytes (●, C). Values are means for quadruplet. *a*) $p<0.01$, *b*) $p<0.005$.

tion progressively declined, reaching a nadir at 10^{-3} M (13—16% for [^{3}H]thymidine incorporation and 35—60% for IgM production).

Influences of Nitrite on Cell Growth and Antibody Production in B Cell Hybridomas Nitrite contradictorily influenced [^{3}H]thymidine incorporation of splenic B cells and spleen cells in the above data. Therefore we selected three B cell hybridoma clones on the basis of different immunization, and examined the effect of nitrite on the functions of the cells. As nitrate was contained in the culture suprenatants of activated macrophages, we first studied the effect of nitrate on B cell hybridomas. Short-term exposure of mouse myeloma and B cell hybridomas to nitrate did not cause obvious changes of [^{3}H]tymidine incorporation (data not shown). On the other hand, short-term exposure of the B cell hybridomas to nitrite produced marked changes of DNA synthesis (Table I). [^{3}H]Thymidine incorporation of a clone (30H12) was obviously reduced by the addition of 10^{-3} M nitrite. By contrast, another clone (AE6-5) was significantly and markedly enhanced by the addition of nitrite at 10^{-7}—10^{-3} M ($p<0.0005$). Antibody in the culture supernatants of both hybridomas was slightly reduced.

To examine the effects of nitrite on the production of antibody in B cell hybridomas, a B cell hybridoma was cultured for 18 d in soft agar with increasing concentrations of sodium nitrite. Cells numbers in this system reached a maximum at nitrite concentrations of between 10^{-7} and 10^{-6} M, and those levels were $41.2 \pm 1.2 \times 10^5$ cells/dish compared with $28.7 \pm 1.5 \times 10^5$ cells/dish in cells not treated with nitrite (Fig. 3-A). However, as the nitrite concentration increased further, to 10^{-3} M, cell numbers remarkably declined, reaching a nadir. As shown in Fig. 3-B, C, IgM production progressively and significantly declined in a NO_2^- concentration dependent manner (Fig. 3-B).

Discussion

Nitric oxide and nitrite are agents which had previously been shown to inhibit cell growth and DNA synthesis.[12] In the present study, we examined the effects of nitrite over a wide range of concentration. We actually observed several properties of nitrite in the regulation of cell proliferation and antibody production by LPS-stimulated B cells and B cell hybridomas. Among these observations are (1) a certain concentration of nitrite causes a marked and significant enhancement of cell growth and [^{3}H]thymidine incorporation in a B cell hybridoma; (2) increasing nitrite concentration causes a suppression of cell growth in a certain B cell hybridoma and in LPS-stimulated spleen cells; (3) the nitrite-induced decline in antibody production shows a dose–response relationship.

In the long-term exposure of a B cell hybridoma (DG8)

to nitrite, cell number increased at 10^{-7}—10^{-5} M. This result indicates that nitrite enhances not plating-efficiency, but cell proliferation, because a doubling time in the cell line is about 22 h. Furthermore, nitrite enhanced [^{3}H]thymidine incorporation of a B cell hybridoma (AE6-5). However, in LPS-stimulated total spleen cells and a B cell hybridoma (30H12), nitrite reduced the [^{3}H]thymidine incorporation of the cells. 30H12 was formed by the fusion of rat myeloma with mouse spleen cells immunized with mouse thymocyte and spleen cells (twice or thrice priming). Hybridomas are artificially formed by myeloma and splenic B cells. However, the observed pattern may be an intrinsic property of B cells. We previously reported that nitrite production in immune spleen cells changed cyclically and that [^{3}H]thymidine incorporation of antigen-prime spleen cells was progressively enhanced following the pattern of the increasing concentration of nitrite production. Furthermore, as shown in Figs. 1 and 2, [^{3}H]thymidine incorporation of splenic B cells and spleen cells on nitrite were different. From this data, we suppose that sensitivities of B cells on nitrite may vary, dependent on their characteristics.

In light of striking changes of antibody production in LPS-stimulated total spleen cells, it was important to determine whether these effects were due to a decline in cell number. Experiments of antibody production in B cell hybridomas indicate that inhibition of antibody production cannot be explained by any general effects on cell proliferation (Fig. 3). Billiar *et al.*[13] and Curran *et al.*[14] reported that nitric oxide inhibited protein synthesis of hepatocytes. This data suggests that nitric oxide and nitrite reduce protein synthesis in some cell types.

The physiological concentrations of nitrogen oxides in spleen and lymphoid tissues are unclear. The half-life time of nitric oxide is very short. Nitric oxide and nitrite are diffusible. In addition, nitrite binds to hemoglobin.[15] However, a high concentration of nitrate was analyzed in the urine of endotoxin-treated rat[16] and, in the *in vitro* antibody production system, splenic macrophage produced nitrite at approximately 10^{-8} M/10^6 speen cells.[11] From these findings, nitrige-produced macrophages may be elucidated to have a distinctive effect on B cells in the spleen cells.

In conclusion, we suppose that nitrite or nitric oxide formed from nitrite during culture regulates the function of B cells as like protein cytokines.

References

1) C. M. Snapper and W. E. Paul, *Science*, **236**, 236 (1987).
2) D. Y. Kunimoto, R. P. Nordan and W. Strober, *J. Immunol.*, **143**, 2230 (1989).
3) G. Thypheronitis, G. C. Tsokos, C. H. June, A. D. Levine and F. D. Finkelman, *Proc. Natl. Acad. Sci., U.S.A.*, 86, 5580 (1989).
4) E. Sonoda, R. Matsumoto, Y. Hitosi, T. Ishi, M. Sugimoto, S. Araki, A. Tominaga, N. Yamaguchi, and K. Takatsu, *J. Exp. Med.*, **170**, 1415 (1989).
5) D. J. Stuehr and M. A. Marletta, *J. Immunol.*, **139**, 518 (1987).
6) R. Iyengar, D. J. Stuehr and M. A. Marletta, *Proc. Natl. Acad. Sci., U.S.A.*, **84**, 6369 (1987).
7) K. Takagi, T. Hosaka and Y. Suketa, *J. Cell. Physiol.*, **147**, 864 (1991).
8) J. C. Drapier and J. B. Hibbs, *J. Immunol.*, **140**, 2829 (1988).
9) J. B. Hibbs, Z. Vavrin and R. R. Taintor, *J. Immunol.*, **138**, 550 (1987).
10) C. G. Uttam and A. Hassid, *Biochem. Biophys. Res. Comm.*, **171**, 474 (1990).
11) K. Takagi, H. Nakagami, T. Nakano and Y. Suketa, *Biochim. Biophys. Acta*, **1092**, 15 (1991).
12) D. J. Stuehr and C. F. Nathan, *J. Exp. Med.*, **169**, 1543 (1989).
13) T. M. Billiar, R. D. Curran, D. J. Stuehr, M. A. West, B. G. Bentz and R. L. Simmons, *J. Exp. Med.*, **169**, 146 (1989).
14) R. D. Currant, F. K. Ferrari, P. H. Kispert, J. Stadler, D. J. Stuehr, R. L. Simmons and T. R. Billiar, *FASED J.*, **5**, 2085 (1991).
15) M. P. Doyle, R. A. Pickering, T. M. DeWeert, J. W. Hoekstra and D. Pater, *J. Biol. Chem.*, **256**, 12393 (1981).
16) L. C. Green, K. R. Luzuriaga, D. A. Wagner, W. Rand, N. Istfan, V. R. Young, S. R. Tannenbaum, *Proc. Natl. Acad. Sci., U.S.A.*, **78**, 7764 (1981).