Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Research 342 (2007) 1133–1137
Carbohydrate RESEARCH

Note

Stimulation of multiple cytokine production in mice by alginate oligosaccharides following intraperitoneal administration

Yoshiko Yamamoto, Maki Kurachi, Kenichi Yamaguchi and Tatsuya Oda*

Division of Biochemistry, Faculty of Fisheries, Nagasaki University, Nagasaki 852-8521, Japan

Received 21 August 2006; received in revised form 13 January 2007; accepted 12 February 2007
Available online 17 February 2007

Abstract—We previously reported that alginate oligomers, prepared by specific enzymatic digestion of alginate polymer, induced cytokine secretion from mouse macrophage cell line RAW264.7. In the present study, we examined the cytokine levels in the mouse serum after intraperitoneal (ip) administration of a mixture of alginate oligomers. After ip injection of 700 mg/kg of oligomers, the serum level of G-CSF increased promptly and reached the maximum level after 2 h and this high level was sustained until 6 h, and then gradually decreased, whereas injection of 700 mg/kg of alginate polymer had no effect. The effect of alginate oligomer mixture was dose-dependent, and 70 mg/kg was sufficient to attain the maximum serum level of G-CSF. A Bio-Plex bead assay that can detect 23 cytokines at the same time revealed that ip administration of alginate oligomer mixture induced an increase in 20 cytokines in the serum at different levels and with different kinetics depending on the cytokine. Among the cytokines detected the level of G-CSF was the highest. The levels of monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6, keratinocyte-derived chemokine (KC), IL-12 (p40), and regulated upon activation normal T cell expressed and secreted (RANTES) were also relatively high and exceeded 5000 pg/mL serum at the peak point.

Keywords: Alginate oligomers; Cytokine; TNF-α; G-CSF; Intraperitoneal immuno-stimulation

Alginic acid is an acidic linear polysaccharide that is usually derived from several genera of brown seaweeds such as *Macrocystis pyrifera* and *Ascophyllum nodosum*. The polysaccharide is composed of two forms of uronic acids α-L-guluronate (G) and β-D-mannuronate (M) which form three types of polymer blocks: namely homopolymer of guluronate, homopolymer of mannuronate, or heteropolymer (a mixed sequence of these residues); these block structures are expressed as G-blocks, M-blocks, and MG-blocks, respectively.[1] The differences in the M/G ratio and the entire sequence are responsible for the diversity of physicochemical properties and function of alginates. Because of the variable properties, alginates are utilized for a wide range of commercial applications including thickening agents and dispersion stabilizers. Since alginates have gentle gelling properties in the presence of divalent cations such as calcium, alginates are also widely used for live cell encapsulation in vitro[2] and in vivo[3] and for several tissue engineering applications.[4,5] Moreover, previous in vivo and in vitro studies have demonstrated that alginates have a wide-variety of biological effects.[6–11] Alginate oligosaccharides produced by the enzymatic degradation of alginate polymers with relatively low molecular weight are also known to have several biological activities including suppression of fibroblast proliferation and collagen synthesis in human skin,[12] stimulation of endothelial cell growth and migration,[13] stimulation of human keratinocyte growth,[14] suppression of Th2 development and IgE secretion through

Abbreviations: ip, intraperitoneal; G, guluronate; M, mannuronate; TNF, tumor necrosis factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; RANTES, regulated upon activation normal T cell expressed and secreted; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; KC, keratinocyte-derived chemokine; FBS, fetal bovine serum; PBS, phosphate buffered saline; ELISA, enzyme-linked immunosorbent assay

* Corresponding author. Tel./fax: +81 95 819 2831; e-mail: t-oda@nagasaki-u.ac.jp

0008-6215/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2007.02.015

inducing IL-12 secretion.[15] In addition to these mammalian models, it has also been reported that enzymatically depolymerized alginates promotes the growth of bifidobacteria, while the original alginate had no effect.[16] Furthermore, alginate oligomers seem to have some biological effects on plants as well. Alginate oligomers prepared with bacterial alginate lyase increased shoot elongation of komatsuna (*Brassica rapa* var. *pervidis*) seeds[17] and promoted the elongation of barley roots.[18]

Our recent studies have demonstrated that alginate polymers induce TNF-α secretion from mouse macrophage cell line RAW264.7, and the activity was significantly influenced by the molecular size and M/G ratio.[19] Interestingly, a mixture of enzymatically depolymerized alginate oligomers showed more than 10-times higher activity in terms of TNF-α secretion from RAW264.7 cells as compared to original alginate polymers.[19] The mixture of alginate oligomers had fairly low viscosity in aqueous solution even at quite high concentration in contrast to the polymer, and had no gel-forming property in the presence of calcium. Gel-filtration analysis suggested that the mixture mainly contained trimer- to nanomer-oligosaccharides.[19] Increased cytokine-inducing activity and tractable physicochemical properties in aqueous solution of the alginate oligomer mixture prompted us to examine whether or not the alginate oligomer mixture is capable to induce cytokine production in vivo. In this study, we investigated the cytokine levels in mouse serum following intraperitoneal administration of the alginate oligomer mixture.

Since our preliminary experiments suggested that ip injection of alginate oligomer mixture induced the increase in the G-CSF with relatively higher levels than other cytokines including TNF-α, the time-course analysis was conducted focusing on serum G-CSF level after ip administration of alginate oligomer mixture. As shown in Figure 1, the G-CSF level in the serum rapidly increased and reached the maximum level at 2 h after injection of 700 mg/kg body weight. The high level of G-CSF was sustained until 6 h, and then gradually decreased and returned to baseline level at 24 h. On the other hand, no significant level of G-CSF was detected in the serum at least at the examined time points after ip injection of 700 mg/kg of original alginate polymer. These results suggest that the alginate oligomer mixture is superior cytokine inducer to the original polymer in vivo. In an in vivo system, high viscosity as well as gel-forming property of alginate polymer may prevent the efficient transfer or even dispersion of the alginate polymer from the injected area to circulation system to reach the target organs or cells that may be involved in cytokine secretion. Improvement of these drawbacks of alginate polymer by enzymatic oligomerization as well as the increased cytokine-inducing activity at cellular level may partly explain the reason for the potent cytokine-inducing activity of the alginate oligomer mixture in vivo.

A dose–response experiment was performed by ip injecting 0.07, 0.7, 7, 70, 700 mg/kg of the alginate oligomer mixture and examined G-CSF serum levels after 3 h. As shown in Figure 2, G-CSF level in the serum increased with increased dose of the mixture and reached a plateau level at 70 mg/kg, suggesting ip injection of 70 mg/kg of a mixture is sufficient to induce the maximum serum level of G-CSF. Based on the result, it is calculated to be 4.2 g for 60 kg body weight and this value is considered within an applicable range for human beings.

Although it is uncertain whether the cytokine-inducing activity is always responsible for the variety of bioactivities of alginates, our results obtained in this study may suggest that alginate oligomers even in mixture are useful as bioactive agents. Similar to our results, the effects of enzymatic digestion on the biological activities of alginate polymers have been reported.[16–18] Regarding the structure–activity relationships, our recent study demonstrated that both purified unsaturated

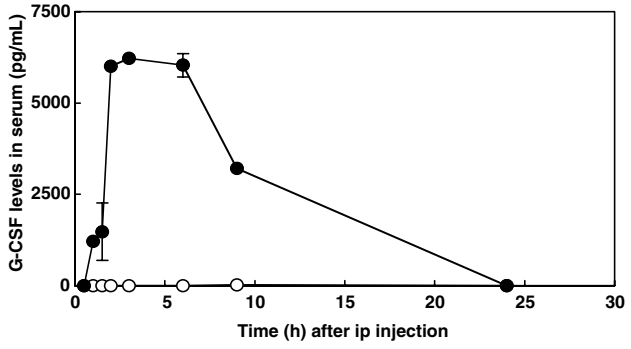

Figure 1. G-CSF levels in the serum after ip injection of alginate oligomer mixture or alginate polymer. Each point represents an average of triplicate measurements and each bar indicates the standard deviation.

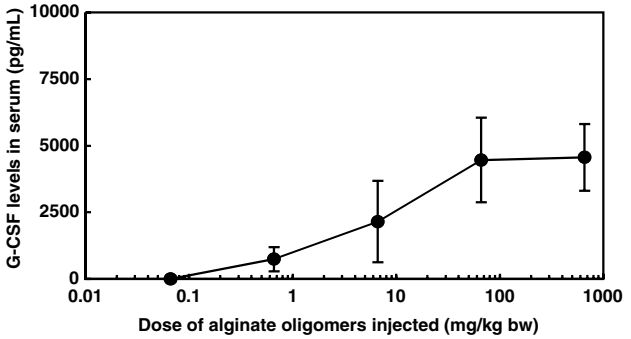

Figure 2. G-CSF levels in the serum after ip injection of varying doses of alginate oligomer mixture. Each point represents an average of triplicate measurements and each bar indicates the standard deviation.

mannuronate (M3–M9) and guluronate oligomers (G3–G9) with DP of 3–9 induced TNF-α secretion from mouse macrophage cell line RAW264.7 cells to a different extent depending on the structure. These results suggest that at least all these alginate oligomers are capable to induce cytokine secretion from RAW264.7 cells.[20] Furthermore, the enzymatically digested products derived from polymers with different molecular sizes and M/G ratios showed similar TNF-α inducing activities that were 20–480 times higher than those of original alginate polymers. Since the similar gel filtration profiles of those enzymatically digested products were obtained, oligomer compositions of the mixture derived from those polymers may also be analogous each other in terms of molecular size.[19] These findings suggest that enzymatic oligomerization may lead to an increase in TNF-α inducing activity regardless the structure of original polymers.[19] Such increased cytokine-inducing activity of alginate oligomer mixture in a cellular level may be attributable to the induction of high level of G-CSF in an in vivo system.

It has been reported that alginate polymer induces the secretion of several cytokines including TNF-α, IL-1, and IL-6 from human monocytes.[6] Our previous study demonstrated that alginate oligomers also induced the secretion of cytokines from RAW264.7 cells.[20] To investigate other cytokine levels in the mouse serum after ip injection of alginate oligomer mixture, we employed a Bio-Plex bead assay that can detect 23 cytokines simultaneously. As shown in Figure 3, ip administration of alginate oligomer mixture induced significant increase in 20 cytokines in the serum with different levels and different kinetics depending on the cytokines. G-CSF, MCP-1, IL-6, KC, RANTES, and IL-12 (p40) levels were relatively high with exceeded 5000 pg/mL serum level at the peak point. The serum level of TNF-α, interleukin (IL)-2, IL-3, IL-10, IL-12 (p70), IL-13, IL-17, GM-CSF, and IFN-γ clearly showed two peaks during 24 h interval, whereas G-CSF, granulocyte macrophage (GM)-CSF, monocyte chemoattractant protein (MCP)-1, IL-1α, RANTES, KC, eotaxin, and IL-1α tended to show one peak. As a possible reason for the biphasic behavior of some cytokines, resident cells in the intraperitoneal cavity may be responsible for the first peak of these cytokines, and then alginate oligomers transferred from the cavity to the circulation may stimulate second target cells that in turn respond to the second peak of the cytokines. Although several factors such as in vivo behavior of alginate oligomers and locations of sources of cytokines are considered to be involved in the kinetics of serum cytokine levels, further studies are required to clarify these points. Among the cytokines induced by alginate oligomer mixture, the level of G-CSF was higher than others. Although many compounds capable to induce CSFs in a variety of cells are known,[21–24] to our knowledge this is the first result

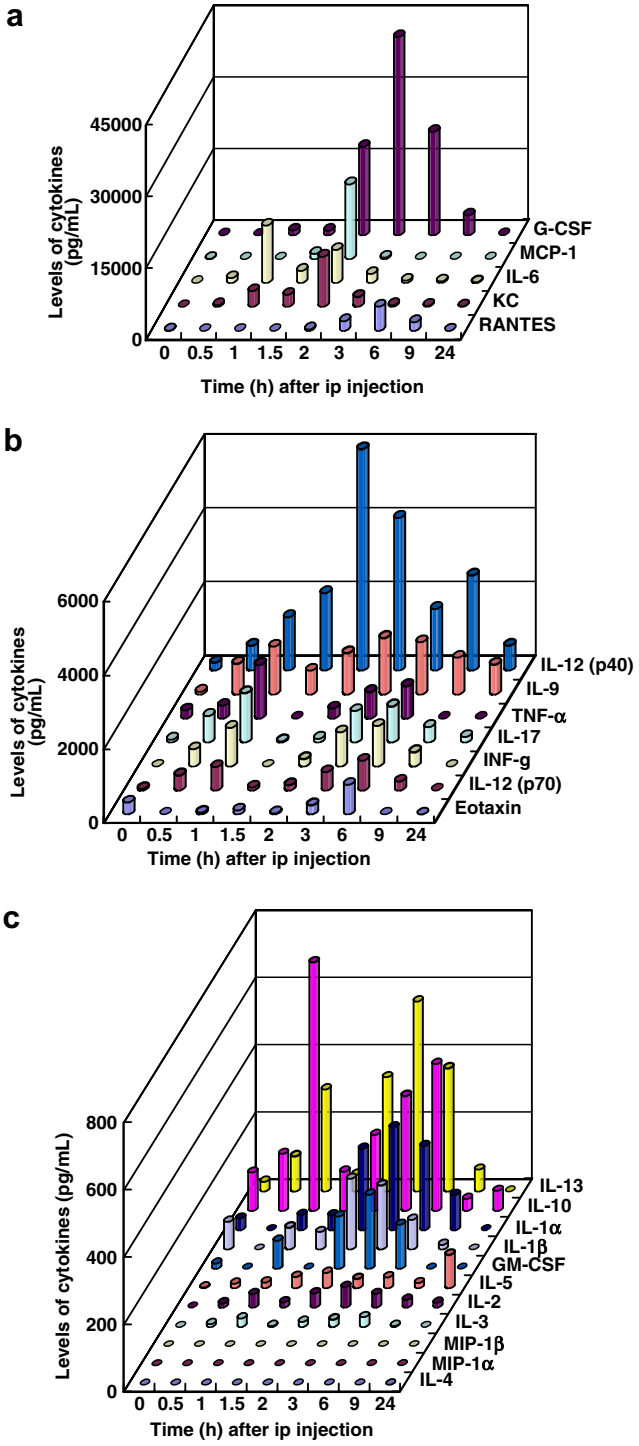

Figure 3. Levels of various cytokines in the serum after ip injection of alginate oligomer mixture. The cytokines detected in the serum were categorized into high (a), middle (b), and low (c) level. Each value represents an average of duplicate measurements. Differences between duplicate measurements for each value were within 5%.

indicating that alginate oligomer mixture have an ability to induce G-CSF in vivo. G-CSF is a hematopoietic factor that stimulates neutrophile production and release from bone marrow as well as activating mature neutro-

philic function.[25] Since neutrophils are primary effectors in host defense against invading pathogens, the use of G-CSF has been tested in the treatment of bacterial infectious diseases,[26] and antiviral effect of G-CSF on hepatitis virus has also been reported.[27] Thus the ability of alginate oligomer mixture to induce G-CSF may be a useful aspect that should be taken into consideration for the application of alginate oligomers. This ability of alginate oligomers to induce G-CSF may partly explain the previous findings that ip administration of alginate with high content of mannuronic acid enhanced survival of lethally irradiated mice and stimulated murine hematopoiesis in vitro.[28]

Similar to our results (Fig. 3b), Yoshida et al. have reported that ip injection of the mixture of alginate oligomers resulted in the suppression of both Th2 development and IgE production through the induction of IL-12 production.[15] Based on those results, they proposed the usefulness of alginate oligomers as a novel anti-allergic agent.

In conclusion, our results clearly indicate that alginate oligomer mixture induces multiple cytokines including G-CSF in vivo after ip administration. These findings may provide basic information of potential activity of alginate oligomers as a potent cytokine inducer. Behaviors of various cytokines obtained in this study may give a clue not only for the understanding the underlying mechanism of variety of biological activities of alginate oligomers reported so far but also for the future application study of alginate oligomers.

1. Experimental

1.1. Mice

Male ddy mice of 6 weeks old of age and approximately 30 g of weight were purchased from Kyudo (Tokyo, Japan) and used within a few days. Food and water was supplied ad libitum. Mice were intraperitoneally injected with either PBS or alginate oligomers (0.07–700 mg/kg) in 1 mL of PBS. For the time-course analysis, blood samples were collected at 0, 0.5, 1, 1.5, 2, 3, 6, 9, or 24 h after the administration of alginate oligomer mixture or alginate polymer. For the dose–response analysis, blood samples were collected at 3 h after the administration of varying doses of alginate oligomer mixture. Two mice were sacrificed at each time point or at each dose point. The serum prepared from each blood sample was subjected to triplicate measurements for the cytokine analysis by ELISA or a Bio-Plex assay.

1.2. Materials

Highly purified sodium alginate with 1000-cps (food and medical usage grade) was obtained from KIMIKA Co. (Tokyo, Japan) and used without further purification. Alginate lyase was purified from the culture medium of *Pseudoalteromonas* sp. strain No. 272, as described previously.[29] The purified enzyme, which can recognize both guluronate and mannuronate residues, produced both unsaturated guluronate and mannuronate oligomers with various degree of polymerization.[29] Enzymatic digestion of alginates were conducted by basically the same procedure as described previously.[19] In brief, 5% of alginate samples in aqueous solution was incubated with alginate lyase (final 1 μg/mL) at 40 °C for 3 days. No significant changes in the compositions of digestion products were observed after further enzymatic treatment.[19] The enzymatic reaction was stopped by heating the solution in boiling water for 10 min. Gel-filtration analysis suggested that the enzymatically digested product contained several oligomers with degree of polymerization (DP) of 3–9 (i.e., with molecular weight ranging in 529–1585).[19] Since the reproducible similar gel-filtration profiles were obtained by this enzymatic digestion even in the different alginate polymers with different molecular sizes and M/G ratios, it is considered that similar composition of alginate oligomer mixture can be prepared by this procedure in terms of molecular size.[19] Before use, all samples were filtered through an endotoxin-removing filter (Zetapor Dispo filter) purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). This filtration procedure could reduce even the bioactivity of 1 μg/mL of LPS to negligible level.[20]

1.3. ELISA for G-CSF

The concentration of G-CSF in mouse serum was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) similar to the assay for TNF-α detection as described previously.[20] In this ELISA, anti-mouse G-CSF monoclonal antibody (R&D Systems, MN, USA) and anti-mouse G-CSF polyclonal antibody (R&D Systems, MN, USA) were used.

1.4. A Bio-Plex

Bio-Plex mouse cytokine assay for simultaneous quantitation of tumor necrosis factor (TNF)-α, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, interferon (IFN)-γ, eotaxin, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated upon activation normal T cell expressed and secreted (RANTES), and monocyte chemoattractant protein (MCP)-1was employed according to the recommended procedure. In brief, the premixed standards were reconstituted in 0.5 mL of PBS, generating a stock

concentration of 50,000 pg/mL for each cytokine. The standard stock was serially diluted in PBS to generate 8 points for the standard curve. The assay was performed in a 96-well filtration plate supplied with the assay kit. Premixed beads (50 μL) coated with target capture antibodies were transferred to each well of the filter plate and washed twice with Bio-Plex wash buffer. Premixed standards or samples (50 μL) were added to each well containing washed beads. The plate was shaken for 30 s and then incubated at room temperature for 30 min with low-speed shaking. After incubation and washing, premixed detection antibodies (50 μL) were added to each well. The incubation was terminated after shaking for 10 min at room temperature. After 3 times washing, the beads were resuspended in 125 μL of Bio-Plex cytokine assay buffer. Beads were read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager™ software with 5PL curve fitting.

References

1. Haug, A. *Methods Carbohydr. Chem.* **1965**, *5*, 69–73.
2. Fremond, B.; Malandain, C.; Guyomard, C.; Chesne, C.; Guillouzo, A.; Campion, J.-P. *Cell Transplant.* **1993**, *2*, 453–460.
3. Chang, S. C. N.; Rowley, J. A.; Tobias, G.; Genes, N. G.; Roy, A. K.; Mooney, D. J.; Vacanti, C. A.; Bonassar, L. J. *J. Biomed. Mater. Res.* **2001**, *55*, 503–511.
4. Hauselmann, H. J.; Masumd, K.; Hunziker, E. B.; Neidhart, M.; Mok, S. S.; Michel, B. A.; Thonar, E. J.-M. A. *Am. J. Physiol.* **1996**, *271*, C742–C752.
5. Atala, A.; Kim, W.; Paige, K. T.; Vacanti, C. A.; Retik, A. B. *J. Urol.* **1994**, *152*, 641–643.
6. Otterlei, M.; Østgaard, K.; Skjåk-Bræk, G.; Smidsrød, O.; Soon-Shing, P.; Espevik, T. *J. Immunother.* **1991**, *10*, 286–291.
7. Kimura, Y.; Watanabe, K.; Okuda, H. *J. Ethnopharmacol.* **1996**, *54*, 47–54.
8. Logeart, D.; PrigentRichard, S.; Jozefonvicz, J.; Letourneur, D. *Eur. J. Cell Biol.* **1997**, *74*, 376–384.
9. Logeart, D.; PrigentRichard, S.; BoissonVidal, C.; Chaubert, F.; Durand, P.; Jozefonvicz, J.; Letourneur, D. *Eur. J. Cell Biol.* **1997**, *74*, 385–390.
10. Asada, M.; Sugie, M.; Inoue, M.; Nakagomi, K.; Hongo, S.; Murata, K.; Irie, S.; Takeuchi, T.; Tomizukaa, N.; Oka, S. *Biosci. Biotech. Biochem.* **1997**, *61*, 1030–1032.
11. Jeonh, H.-J.; Lee, S.-A.; Moon, P.-D.; Na, H.-J.; Park, R.-K.; Um, J.-Y.; Kim, H.-M.; Hong, S.-H. *Clinic. Exp. Allergy* **2006**, *36*, 785–794.
12. Tajima, S.; Inoue, H.; Kawada, A.; Ishibashi, A.; Takahara, H.; Hiura, N. *Arch. Dermatol. Res.* **1999**, *291*, 432–436.
13. Kawada, A.; Hiura, N.; Tajima, S.; Takahara, H. *Arch. Dermatol. Res.* **1999**, *291*, 542–547.
14. Kawada, A.; Hiura, N.; Shiraiwa, M.; Tajima, S.; Hiruma, M.; Hara, K.; Ishibashi, A.; Takahara, H. *FEBS Lett.* **1997**, *408*, 43–46.
15. Yoshida, T.; Hirano, A.; Wada, H.; Takahashi, K.; Hattori, M. *Int. Arch. Allergy Immunol.* **2004**, *133*, 239–247.
16. Akiyama, H.; Endo, T.; Nakakita, R.; Murata, K.; Yonemoto, Y.; Okayama, K. *Biosci. Biotechnol. Biochem.* **1992**, *56, 355–356.*
17. Yonemoto, Y.; Tanaka, H.; Yamashita, T.; Kitabatake, N.; Ishida, Y.; Kimura, A.; Murata, K. *J. Ferment. Bioeng.* **1993**, *75*, 68–70.
18. Tomoda, Y.; Umemura, K.; Adachi, T. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 202–203.
19. Kurachi, M.; Nakashima, T.; Miyajima, C.; Iwamoto, Y.; Muramatsu, T.; Yamaguchi, K.; Oda, T. *J. Infect. Chemother.* **2005**, *11*, 199–203.
20. Iwamoto, M.; Kurachi, M.; Nakashima, T.; Kim, D.; Yamaguchi, K.; Oda, T.; Iwamoto, Y.; Muramatsu, T. *FEBS Lett.* **2005**, *579*, 4423–4429.
21. Rennick, D.; Yang, G.; Gemmell, L.; Lee, F. *Blood* **1987**, *69*, 682–691.
22. Kohama, T.; Handa, H.; Harigaya, K. *Exp. Hematol.* **1988**, *16*, 603–608.
23. Zsebo, K. M.; Yuschenkoff, V. N.; Schiffer, S.; Chang, D.; McCall, E.; Dinarello, C. A.; Brown, M. A.; Altrock, B.; Bagby, G. C., Jr. *Blood* **1988**, *71*, 99–103.
24. Munker, R.; Gasson, J.; Ogawa, M.; Koeffler, H. P. *Nature* **1986**, *323*, 79–82.
25. Demetri, G. D.; Griffin, J. D. *Blood* **1991**, *78*, 2791–2808.
26. Dale, D. C.; Conrad Liles, W.; Summer, W. R.; Nelson, S. *J. Infect. Dis.* **1995**, *172*, 1061–1075.
27. Martin, J.; Navas, S.; Quiroga, J. A.; Carreno, V. *Cytokine* **1996**, *8*, 313–317.
28. Halaas, Ø.; Olsen, W. M.; Veiby, O. P.; LØvhaug, D.; Skjåk-Bræk, G.; Vik, R.; Espsvik, T. *Scand. J. Immunol.* **1997**, *46*, 358–365.
29. Iwamoto, Y.; Hidaka, H.; Oda, T.; Muramatsu, T. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 1990–1992.