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The flavonoid baicalin inhibits superantigen-induced inflammatory cytokines and chemokines

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Abstract Excessive release of proinflammatory cytokines mediates the toxic effect of superantigenic staphylococcal exotoxins (SE). Baicalin, a flavone isolated from the Chinese herb *Scutellaria baicalensis* Georgi and used in China to treat infectious diseases, inhibited SE-stimulated T-cell proliferation (by 98%) and production of interleukin 1β, interleukin 6, tumor necrosis factor, interferon γ, monocyte chemotactic protein 1, macrophage inflammatory protein (MIP)-1α, and MIP-1β mRNA and protein by human peripheral blood mononuclear cells. These data suggest that baicalin may be therapeutically useful for mitigating the pathogenic effects of SE by inhibiting the signaling pathways activated by superantigens.

Key words: Immunosuppression; Superantigen; Cytokine and chemokine; Baicalin; Flavonoid

1. Introduction

Staphylococcal toxic shock syndrome toxin 1 (TSST-1) and the distantly related enterotoxin A and B (SEA and SEB) are potent activators of the immune system and cause a variety of diseases in humans, ranging from food poisoning and toxic shock to autoimmune diseases [1–5]. These toxins bind to both the major histocompatibility complex class II molecules on antigen-presenting cells and specific Vβ regions of the T-cell antigen receptors [6–9]. These toxins are called superantigens because of their ability to polyclonally activate large populations of T-cells [6]. Their interactions with cells of the immune system result in a massive production of proinflammatory cytokines and chemokines [10–14]. The cytokines, tumor necrosis factor (TNFα), interleukin 1 (IL-1), IL-6 and interferon γ (IFNγ), are pivotal mediators in superantigen-induced toxic shock [10,14]. Both TNFα and IL-1 have potent immunostimulating activities and act synergistically with IFNγ to enhance immune reactions and promote tissue injury [15]. Consequently these cytokines are pathogenic at high concentrations in vivo and are responsible for fever and toxic shock induced by SE [4,5,13,16].

Baicalin (BA, 7-glucuronic acid, 5,6-dihydroxyflavone) is a flavone isolated from the Chinese medicinal herb *Scutellaria baicalensis* Georgi and has been used to treat various infectious and allergic diseases in China [17]. Its in vitro effects include the inhibition of reverse transcriptase activity in HIV-1-infected cells [18,19] and interference with the interaction of HIV-1 envelope proteins with chemokine receptors [20]. Recently, BA was shown to bind selectively to several chemokines (stromal cell-derived factor 1α, IL-8, macrophage inflammatory protein (MIP)-1β, monocyte chemotactic protein (MCP)-2, and lymphotactin) and interfere with the ability of these chemokines to bind to chemokine receptors [21]. It also blocked the proliferative effect of platelet-derived growth factor on vascular smooth muscle cells [22]. In vivo, BA suppressed carrageenin-induced paw edema in rats [23,24] and reduced chemokine-induced neutrophil infiltration in vivo [21]. This study was undertaken to determine the effect of BA on staphylococcal superantigen-induced T-cell activation and cytokine production from human peripheral blood mononuclear cells (PBMC).

2. Materials and methods

2.1. Materials

Purified SEB and TSST-1 were obtained from Toxin Technology (Sarasota, FL, USA). The endotoxin content of these preparations was <1 ng endotoxin/mg protein, as determined by the *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD, USA). Human (h) recombinant (r) TNFα, antibodies against hTNFα, peroxidase-conjugated anti-rabbit IgG, and peroxidase-conjugated anti-goat IgG were obtained from Boehringer-Mannheim (Indianapolis, IN, USA). Recombinant MCP-1, MIP-1α, MIP-1β; antibodies against hIL-1β, hIL-6, hMIP-1α, and MIP-1β were purchased from R&D Systems (Minneapolis, MN, USA). Human caspase 3 and caspase 8 assay kits were also obtained from R&D Systems. Human rIL-1β was kindly provided by Dr. J. Oppenheim (National Cancer Institute, Frederick, MD, USA). Human rIFNγ and rIL-6 were obtained from Collaborative Research (Boston, MA, USA). Antibodies against hIFNγ and MCP-1 were obtained from Pharmingen (San Diego, CA, USA). BA was isolated and prepared as described previously [19]. All other reagents were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Human PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from normal human donors. PBMC (10^6 cells/ml) were cultured at 37°C in 24-well plates contain-

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Abbreviations: TSST-1, staphylococcal toxic shock syndrome toxin 1; SEA and SEB, staphylococcal enterotoxin A and B; BA, baicalin; IL, interleukin; MCP-1, monocyte chemotactic protein 1; MIP-1, macrophage inflammatory protein 1; TNFα, tumor necrosis factor α; IFNγ, interferon γ; PBMC, peripheral blood mononuclear cells

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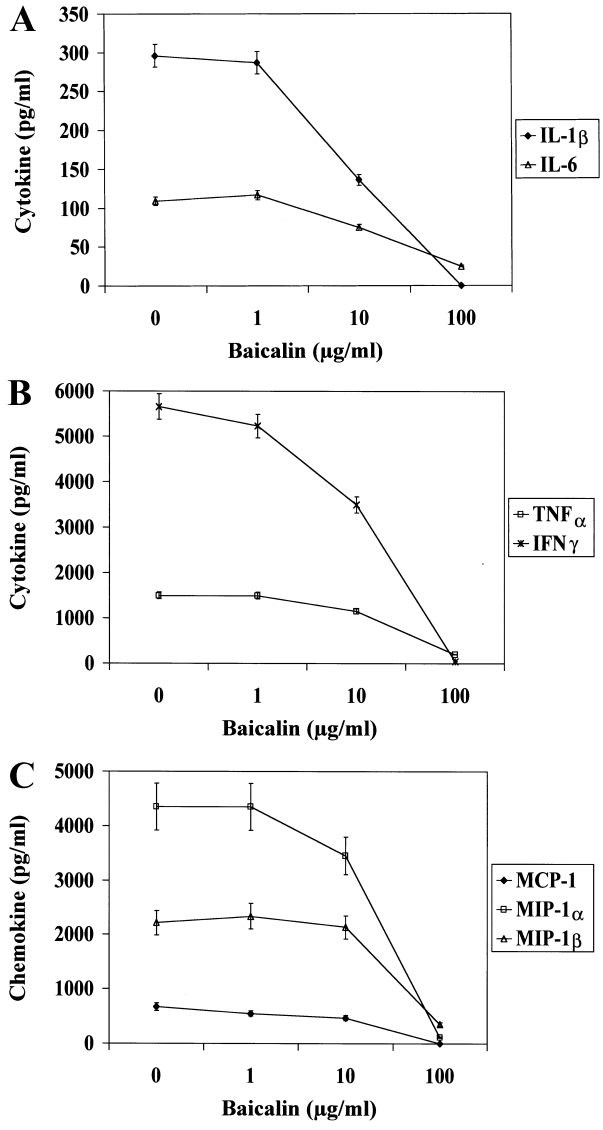

Fig. 1. Dose–response inhibition of (A) IL-1β and IL-6, (B) TNFα and IFNγ, and (C) MCP-1, MIP-1α, MIP-1β production by PBMC stimulated with 150 ng/ml of SEB in the presence of various concentrations of BA. Values represent the mean ± S.D. of duplicate samples from two experiments. Inhibition of all concentrations of BA, with the exception of 1 μg/ml, was statistically significant by comparisons to control SEB-stimulated cells ($P < 0.05$).

ing RPMI 1640 medium and 10% heat-inactivated fetal bovine serum. Cells were incubated with either SEB (150 ng/ml) or TSST-1 (150 ng/ml) and the supernatants were harvested and analyzed for IL-1β, TNFα, IL-6, IFNγ, MCP-1, MIP-1α, and MIP-1β. Cytokines and chemokines were measured by an enzyme-linked immunosorbent assay (ELISA) with cytokine- or chemokine-specific antibodies according to the manufacturer's instructions [11,25]. Human recombinant cytokines and chemokines (20–1000 pg/ml) were used as standards for calibration on each plate. The detection limit of each assay was 20 pg/ml. The cytokine and chemokine data were expressed as the mean reading ± S.D. of duplicate samples. BA, when present, was added simultaneously with SE. T-cell proliferation was assayed with PBMC (10^5 cells/well), plated in triplicate with SEB or TSST-1 (150 ng/ml), with or without BA, for 48 h at 37°C in 96-well microtiter plates. Cells were pulsed with 1 μCi/well of [^{3}H]thymidine (New England Nuclear, Boston, MA, USA) during the last 5 h of culture as described previously [25]. Cells were harvested onto glass fiber filters, and incorporated [^{3}H]thymidine was measured by liquid scintillation.

2.3. RNase protection assay

Total RNA was isolated 4 h after SE treatment from cells by using a guanidinium isothiocyanate/chloroform-based technique (Trizol, Gibco, Grand Island, NY, USA) as per the manufacturer's instructions. The RNase protection assay was performed as follows: total cellular RNA (5–10 μg) was hybridized with a [^{33}P]UTP-labeled RNA probe (mck-1, mck-2b, mck-3b, mck-5 utilizing the Pharmingen RiboQuant In Vitro Transcription kit, 1×10^6 cpm/RNA sample) using the Pharmingen hybridization buffer according to the manufacturer's directions (Pharmingen). After hybridization, the samples were treated with RNase A and T1 according to the procedure provided by Pharmingen, the RNase was inactivated and the protected RNA precipitated with a master cocktail containing 200 μl Ambion (Austin, TX, USA) RNase inactivation reagent, 50 μl ethanol, 5 μg yeast tRNA and 1 μl Ambion GycoBlue co-precipitate per RNA sample. The samples were mixed well, incubated at −70°C for 30 min and centrifuged at 14 000 rpm for 15 min at room temperature. The pellets were resuspended in 3 μl of Pharmingen sample buffer and subjected to polyacrylamide gel electrophoresis as recommended by the manufacturer (Pharmingen).

2.4. Statistical analysis

All data were analyzed for significant differences by the Student's *t*-test with Stata (Stata Corp., College Station, TX, USA). Differences between BA-treated and untreated control groups were considered significant if P was < 0.05.

3. Results and discussion

Based on the reports that BA blocked paw edema and reduced neutrophils infiltration in vivo [21,23,24], we tested the hypothesis that this natural flavonoid might have direct effects on the expression of proinflammatory cytokines. As shown in Fig. 1, BA, in a dose-dependent manner, blocked the production of cytokines IL-1β, IL-6, TNFα, and IFNγ; and chemokines MCP-1, MIP-1α and MIP-1β from PBMC

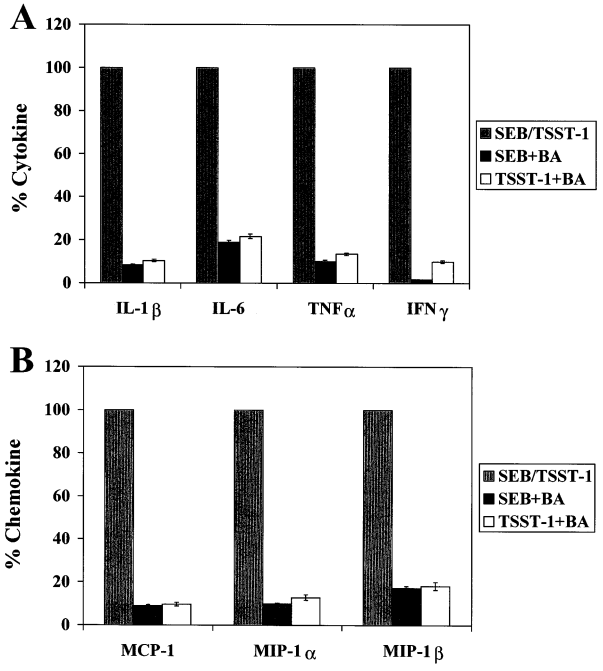

Fig. 2. Inhibition of (A) IL-1β, TNFα, IL-6, and IFNγ and (B) MCP-1, MIP-1α, and MIP-1β production by PBMC stimulated with SEB (150 ng/ml), or TSST-1 (150 ng/ml) in the presence of 100 μg/ml BA. Values represent the mean ± S.E.M. of PBMC cultures from six blood donors. Results are statistically significant ($P < 0.02$) between SE and SE plus BA samples.

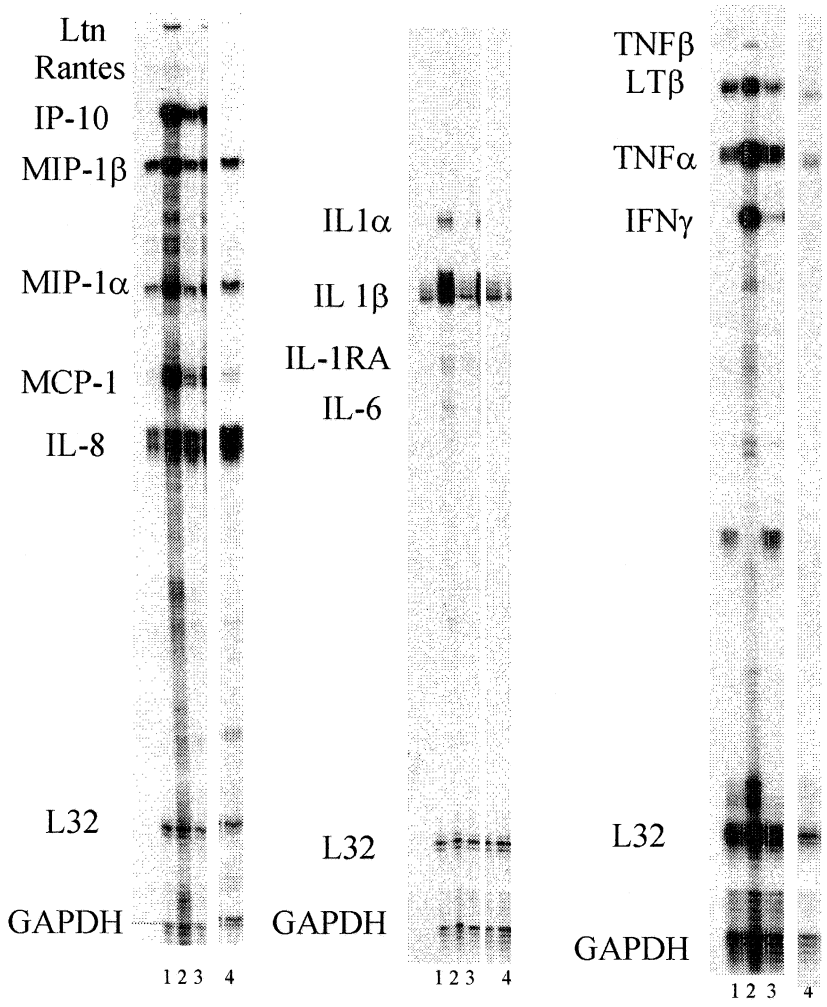

Fig. 3. Cytokine and chemokine mRNA analysis. Total RNA was extracted from human PBMC treated for 4 h with SEB in the presence or absence of BA. Multiprobe RNase protection analysis was performed as described in Section 2 using 5 μg of total RNA per lane. Lanes 1, 2, 3, and 4 represent cells in medium alone, SEB-stimulated cells, SEB-stimulated cells plus BA, and cells with BA, respectively. Data in each probe set represent experiments repeated at least three times.

incubated with SEB. We observed near complete inhibition of these cytokines and chemokines at 100 μg/ml of BA. Similar dose–response inhibition by BA was observed at lower concentrations of SEB (1 and 10 ng/ml). With 10 ng/ml of SEB, TNFα and MIP-1α were blocked 45% and 35%, respectively, by 10 μg/ml BA. More complete inhibition of these mediators was achieved at 100 μg/ml of BA. BA was not cytotoxic to PBMC at this concentration as measured by the exclusion of trypan blue and the lack of lactate dehydrogenase release from treated cells. Additionally, intracellular caspase 3 and caspase 8 were absent from BA-treated PBMC.

The effect of BA on cytokine and chemokine production by TSST-1 was compared with that of SEB-stimulated cells from six blood donors (Fig. 2). Production of IL-1β, IL-6, TNFα and IFNγ by TSST-1-stimulated cells was inhibited 90%, 79%, 87% and 90%, respectively, by 100 μg/ml BA. BA also decreased the levels of MCP-1, MIP-1α and MIP-1β 90%, 88% and 82%, respectively, in TSST-1-stimulated PBMC. Inhibition of cytokines and chemokines in SEB-stimulated cells was similar to that of TSST-1-stimulated cells.

Next we sought to determine if the inhibition of cytokine and chemokine expression by BA was observed at the mRNA level. Total RNA was extracted 4 h after treatment of the PBMC with superantigen in the presence or absence of BA or with BA alone. Gene expression was measured with a Multiprobe RNase protection assay. In this assay, L32 rRNA and GAPDH RNA are used as internal standards for RNA quantitation. As seen in Fig. 3, BA blocked SEB-induced increase in the RNA for MIP-1α, MIP-1β, lymphotactin, IP-10, MCP-1, IFNγ, TNFα and β, lymphotoxin β and IL-1α and β. Interestingly, IL-8 RNA expression was not inhibited, indicating there was some selectivity in the effects of BA on SEB-induced gene expression. Thus, SEB-induced IL-8 protein as measured by ELISA was inhibited by BA by only 37% (data not shown). Also, while BA alone did not alter cytokine/chemokine gene expression (Fig. 3, lane 4), levels of cytokine/chemokine RNA present in the unstimulated PBMC were also not affected by BA, indicating that BA directly blocked the superantigen signaling pathway that leads to new RNA synthesis.

As the ability of superantigens to induce T-cell proliferation is well known, the effect of BA on SE-induced T-cell proliferation was analyzed. Fig. 4 shows that BA dose-dependently inhibited SEB- and TSST-1-stimulated T-cell proliferation,

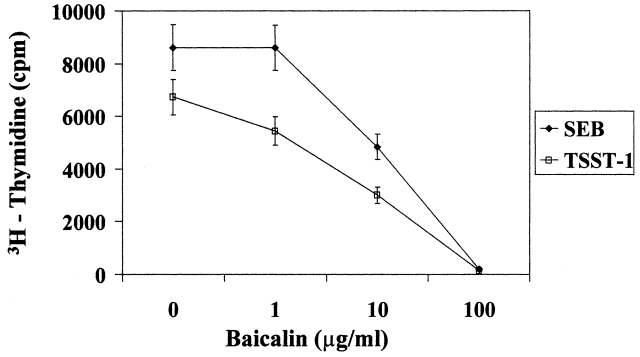

Fig. 4. Inhibition of T-cell proliferation in PBMC stimulated with 150 ng/ml of SEB or TSST-1 by varying concentrations of BA. Values are the mean counts ± S.E.M. of triplicate cultures and represent five experiments. Results are statistically significant ($P < 0.02$) between SE and SE plus BA samples at both 10 and 100 μg/ml of BA.

achieving 99% inhibition at 100 μg/ml of BA. Suppression of T-cell proliferation by BA was not due to the cytotoxic effect of BA as cells were 90% viable by the trypan blue exclusion test.

This study demonstrated that BA effectively inhibited superantigen-mediated production of cytokines and chemokines by human PBMC in vitro. Previous reports indicate that monocytes are the cell source of the cytokine IL-1β and the chemokines, MCP-1, MIP-1α and β [11,26]. In addition, T-cell proliferation induced by staphylococcal superantigens was also completely suppressed. Therefore, BA most likely exerts its effects on both monocytes and T-cells. Down-regulation of proinflammatory cytokines and chemokines by BA in SEB- and TSST-1-stimulated PBMC suggests that BA may affect the pathophysiology of toxic shock. In view of recent clinical studies showing the failure of TNF receptor:Fc protein [27] or IL-1 receptor antagonist [28] in the treatment of septic shock, novel small molecular weight inhibitors capable of suppressing multiple proinflammatory cytokines may prove more useful in the management of shock. Recently, an emerging neonatal exanthematous disease was found to be induced by TSST-1 produced by methicillin-resistant *Staphylococcus aureus* [29,30]. BA may also be useful in the treatment of this neonatal infection because hyperactivation of T-cells plays a prominent role in this disease. In conclusion, this study clearly indicates that BA down-regulates expression of proinflammatory cytokines and chemokines, thus suggesting its potential utility for treating superantigen-induced toxic shock.

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References

[1] Bergdoll, M.S. and Schlievert, P.M. (1984) Lancet ii, 691.
[2] Chesney, P.J., Davis, J.P., Purdy, W.P., Wand, P.J. and Chesney, R.W. (1981) J. Am. Med. Assoc. 246, 741–748.
[3] Friedman, S.M., Posnett, D.N., Tumang, J.R., Cole, B.C. and Crow, M.K. (1991) Arthritis Rheum. 34, 468–480.
[4] Kotzin, B.L., Leung, D.Y.M., Kappler, J. and Marrack, P.A. (1993) Adv. Immunol. 54, 99–166.
[5] Schlievert, P.M. (1993) J. Infect. Dis. 167, 997–1002.
[6] Choi, Y., Kotzin, B., Hernon, L., Callahan, J., Marrack, P. and Kappler, J. (1989) Proc. Natl. Acad. Sci. USA 86, 8941–8945.
[7] Fraser, J.D. (1989) Nature 339, 221–223.
[8] Gascoigne, N.R.J. and Ames, K.T. (1991) Proc. Natl. Acad. Sci. USA 88, 613–616.
[9] Scholl, P., Diez, A., Mourad, W., Parsonnet, J., Geha, R.S. and Chatila, T. (1989) Proc. Natl. Acad. Sci. USA 86, 4210–4214.
[10] Jupin, C., Anderson, S., Damais, C., Alouf, J.E. and Parant, M. (1988) J. Exp. Med. 167, 752–761.
[11] Krakauer, T. (1999) J. Leukocyte Biol. 66, 158–164.
[12] Miethke, T., Wahl, C., Heeg, K., Echtenacher, B., Krammer, P.B. and Wagner, H. (1992) J. Exp. Med. 175, 91–98.
[13] Miethke, T., Wahl, C., Regele, D., Gaus, H., Heeg, K. and Wagner, H. (1993) Immunbiology 189, 270–284.
[14] Parsonnet, J., Hickman, R.K., Eardley, D.D. and Pier, G.B. (1985) J. Infect. Dis. 151, 514–522.
[15] Krakauer, T., Vilcek, J. and Oppenheim, J.J. (1998) in: Fundamental Immunology (Paul, W., Ed.), pp. 775–811, Lippincott-Raven, Philadelphia, PA.
[16] Huang, W.T., Lin, M.T. and Won, S.J. (1997) Infect. Immun. 65, 2656–2662.
[17] Huang, X., Tu, C.H.L., Zhang, L.X. et al. (1987) in: Immunopharmacology (Huang, X., Ed.), pp. 159–160, Shanghai Science and Technology Press, Shanghai.
[18] Kitamura, K., Honda, M. and Yoshizaki, H. et al. (1998) Antiviral Res. 37, 131–140.
[19] Li, B.Q., Fu, T. and Yan, Y.D. et al. (1993) Cell. Mol. Biol. Res. 39, 119–124.
[20] Li, B.Q., Fu, T. and Yao, D. et al. (2000) Biochem. Biophys. Res. Commun. 276, 534–538.
[21] Li, B.Q., Fu, T. and Gong, W.H. et al. (2000) Immunopharmacology 49, 295–306.
[22] Huang, H.C., Wang, H.R. and Hsieh, L.M. (1994) Eur. J. Pharmacol. 251, 91–93.
[23] Kubo, M., Matsuda, H. and Tanaka, M. et al. (1984) Chem. Pharm. Bull. 32, 2724–2729.
[24] Lin, C.C. and Shieh, D.E. (1996) Am. J. Chin. Med. 24, 31–36.
[25] Krakauer, T. (1994) J. Infect. Dis. 172, 988–992.
[26] Carlsson, R., Fischer, H. and Sjogren, H.O. (1988) J. Immunol. 140, 2484–2488.
[27] Fisher Jr., C.J., Agosti, J.M. and Opal, S.M. et al. (1996) New Engl. J. Med. 334, 1697–1702.
[28] Fisher Jr., C.J., Dhainaut, J.F. and Opal, S.M. et al. (1994) J. Am. Med. Assoc. 271, 1836–1843.
[29] Takahashi, N., Nishida, H. and Kato, H. et al. (1998) Lancet 351, 1614–1619.
[30] Takahashi, N., Kato, H. and Imanishi, K. et al. (2000) J. Clin. Invest. 106, 1409–1415.