Original Article

Systemic toxicity of non-cell corynebacterium parvum (CP) in monkeys

Bao-qiu Li¹, Xin Dong², Shi-hong Fang², Gui-qin Yang², Ji-you Gao², Jian-xin Zhang²,
Fang-min Gu² and Hua Zhao²

¹Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, 44, Wen Hua Xi Road, Jinan, Shandong 250012, China

²Shandong Hongli Laboratory Animal Experiment Co., Ltd., A502 No.750 Shun Hua Road, Jinan, Shandong 250101, China

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ABSTRACT — *Aim:* Non-cell corynebacterium parvum product (NCP) is a new preparation of corynebacterium parvum (CP), an immunomodulator that displays anticancer activities. It is prepared by nanotechnology and is intended to minimize the side effects of CP. The aim of the present study was to evaluate the immunogenicity and systemic toxicity of NCP compared with CP in animals. *Methods:* 30 monkeys were randomly divided into 5 groups and given CP (3 mg/monkey), three doses of NCP (9, 3, 1 mg/monkey) and 0.9% normal saline (NS, 4 ml/monkey) individually by intramuscular injection twice a week for 13 weeks. The immunogenicity and systemic toxicity of NCP and CP were compared. *Results:* NCP and CP caused histopathological changes in the liver, spleen and kidney, but pathologic changes in NCP-treated groups were slighter than that in the CP group. Only 9 mg/monkey of NCP caused the similar damage as the CP in intensity. Deposition of immune complexes in the glomerular basement membrane was observed only in the CP group. ELISA detection showed that the anti-CP antibody was at a high level, while the anti-NCP antibody was at low level and disappeared during the recovery period. *Conclusion:* Our study has led to the view that NCP is safer than CP.

Key words: NCP, CP, Immunogenicity, Systemic toxicity

INTRODUCTION

Corynebacterium parvum (CP), also known as propionibacterium acnes, is a gram-positive anaerobic bacillus, which is a member of normal flora of skin and gastrointestinal tract. In the past 40 years, CP has been widely studied with respect to its therapeutic effect and action mechanism, including inhibition of tumor growth, resistance against various infections and so on (Dye *et al.*, 1981; Raiford *et al.*, 1994; Tekin *et al.*, 2001). Among these therapeutic effects, the anti-tumor effect has received much attention, and has been examined both *in vitro* and *in vivo* (Koukalová *et al.*, 1992; Purnell *et al.*, 1979; El Kappany *et al.*, 1980; Collins *et al.*, 1977).

As previously reported, CP is a superior nonspecific immune stimulator. It can elevate the immune function via multifaceted actions, including activating macrophages, regulating T cell immune responses and promoting haematopoietic stem cells to differentiate into mono-

cytes (Thatcher *et al.*, 1977; Rossol *et al.*, 1990; Matsui *et al.*, 1997). Its therapeutic effect is comparable in some respects to BCG in mice and human (Lau *et al.*, 1986; Hart, 1987; Lipton *et al.*, 1991). However, many investigations have demonstrated that CP-treated mice could develop splenic intumescences and intrahepatic granulomas (Riveros-Moreno *et al.*, 1979; Tsuji *et al.*, 1999). In clinical trials, it is also observed that administration of killed CP can induces multiple adverse effects, such as fever, chills of considerable severity, nausea, "flu-like" syndrome and scleromes at the site of injection (Fisher *et al.*, 1976; Minton *et al.*, 1976). Such side effects are disagreeable and have restricted its clinical applications. Most of these side effects are attributed to its immunogenicity.

Evidence indicates that some fractions of CP have similar effects as CP (Mára *et al.*, 1994), which means that reconstruction of CP can retain its biological functions, and reducing its adverse effects by this way may be feasible. Non-cell corynebacterium parvum product (NCP) is

Correspondence: Bao-qiu Li (E-mail: liqiu@163169.net.)

prepared by breaking CP using nanometer technique. It is designed to be administrated by intramuscular injection. Previously, NCPP has been prepared and evaluated by our group with a specific focus on the general pharmacological traits of NCPP. Results showed that NCPP possesses multiple pharmacological actions, including anti-tumor activity, antiviral activity and anti-tuberculosis activity *in vitro* and *in vivo* (Gao *et al.*, 2003a, 2003b and 2004). It maintains strong anti-tumor effects of CP and can enhance the phagocytic capacity of macrophage (Gao *et al.*, 2007). *In vivo* experiment indicates that NCPP cause no scleromes at the site of injection when administrated by intramuscular injection. Liver injury caused by NCPP is slighter than that caused by CP (Gao *et al.*, 2007). To our knowledge, no data has been available on the systematic evaluation of its immunogenicity and systemic toxicity. The present study was designed to compare the immunogenicity and systemic toxicity of NCPP and CP in macaca fascicularis.

MATERIALS AND METHODS

Drugs

NCPP was supplied by National Institute for the Control of Pharmaceutical & Biological Products (Beijing, China). CP (77-1 strain) was obtained from Changchun Institute of Biological Products (Changchun, China).

Animals

Thirty acaca fascicularis (3-4 years, 3-5 kg) were purchased from animal centre of Chinese Academy of Military Medical Sciences (Beijing, China). The quarantine and acclimatization period was 3 weeks. Monkeys were bred under specific pathogen-free conditions, and had free access to food and water.

Drug administration

Monkeys were randomly divided into 5 groups (6 monkeys/group), and were given CP (3 mg/monkey), three doses of NCPP (9, 3, 1 mg/monkey) and 0.9% normal saline (NS, 4 ml/monkey) individually by intramuscular injection twice a week for the 13 weeks. Half of monkeys in each group were sacrificed under ketamin anesthesia 24hr after the last injection at the 13th week. After the recovery period (2 weeks), 2 monkeys in each group were sacrificed. Monkeys in NCPP groups were then given double dose of NCPP by intravenous bolus injection at the 20th week and were sacrificed 3 weeks later. Other animals in CP-treated group were sacrificed at the same time.

General observation and electrocardiogram examinations

The monkeys were observed twice daily (before and after the injection) to note the following features: behavior, food intake, salivation, diarrhea, depression, congested breathing and local reactions at the site of injection. The body weight, body temperature and the electrocardiogram of all monkeys were recorded weekly since the quarantine and acclimatization period.

Hematology and serum biochemistry tests

Hematology and serum biochemistry tests were performed 1 week before the treatment and 1, 2, 3, 4, 6, 8, 10, 13, 15 weeks after the first injection using the blood samples obtained from the upper limb vein.

The red blood cell count (RBC), white blood cell count (WBC), differential count of WBC, platelet count (PLC), hematocrit (HCT), mean platelet volume (MPV) and hemoglobin (Hb) were tested with a CA-500 automatic blood cell analyzer (Aitaike medical Co., Ltd., Japan). Reticulocyte count (RET) was performed manually by staining at 37°C for 20 min with brilliant cresyl blue (Shanghai Yope Biotech Inc., Shanghai, China) (Mythili *et al.*, 2004). Blood smears were observed under oil immersion lens of microscope (Jiangnan Novel Optics Co., Ltd, Nanjing, China).

Serum biochemical parameters including aspartate aminotransferase (AST), alanine aminotransferase (ALT), r-glutamate-transpeptidase (r-GT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), total protein (TP), albumin (ALB), glucose (GLU), total bilirubin (TBIL), creatinine (Crea), total cholesterol (T-CHOL), triglyceride (TG), lactic acid dehydrogenase (LDH), creatine kinase (CK), Albumin/Globulin Ratio(A/G), K⁺, Na⁺ and Cl⁻ were detected using a BTS-370 plus automatic biochemistry analyzer (BioSystems, Barcelona, Spain).

T-cell subset counts

CD₃, CD₄, CD₈, CD₄/CD₈, CD₃₄ T-cell subset counts were determined. Heparinized peripheral blood samples were stained with two-color fluorescence labeled monoclonal antibodies, including CD₃-F/CD₄-P, CD₃-F/CD₈-P, CD₄-F/CD₈-P, CD₃₄-F (Immunotech, Marseille, France). After incubation, the blood samples were processed in Multi-Q-Prep (Beckman Coulter, Inc., Fullerton, CA, USA) employing a 35-cycle program and then analyzed using an EPICS XL flow cytometer (Beckman Coulter, Inc.). The flow cytometric analysis was performed 1, 2, 3, 4 weeks after the first injection.

System autopsy, organ coefficient and pathological examination

Complete necropsy was performed on all sacrificed monkeys. Selected organs (brain, heart, lung, liver, kidney, adrenal glands, spleen, testicle and uterus) were weighed before fixation, and the organ coefficient was calculated as organ weight (g) $\times$ 100/body weight (g).

Organs and tissues, including brain, heart, liver, lung, kidney, stomach, spleen, colon, lymph nodes (mesentery), bladder, testis, epididymis, uterus, ovary, thymus, adrenal glands, pancreas, thyroid gland, esophagus, prostate, pituitary, optic nerve, spinal cord, sternum were collected, fixed with 10% neutral buffered formalin (testis and epididymis were fixed in Bouin's fluid), dehydrated through a graded ethanol series, embedded in paraffin, sectioned (7 μ m), stained with hematoxylin and eosin (H-E) and examined histopathologically using an Olympus IX70 microscope (Olympus Optical Co., Tokyo, Japan).

Immunohistochemical assay

Homolateral kidneys of 3 monkeys in every group were taken out when the monkeys were sacrificed at the 13th week. Routine paraffin section was prepared and stained using an ABC kit (Vector laboratories Inc, Burlingame, CA, USA) according to the manufacturer's instruction in order to check the immune complex in basement membrane. The goat anti-rabbit IgG (dilution 1:100, Sigma, St Louis, MO, USA) and phosphate-buffered saline (PBS, replacing the primary antibody in the negative control group) were used. Two physicians who were blind to the experiment designation were appointed to inspect and evaluate the result using an Olympus IX70 microscope (Olympus Optical Co.). More than five visual fields were evaluated in every slice. Buffy precipitate appearing in endochylema was regarded as positive reaction.

Antibody titration

The anti-NCPP antibody and anti-CP antibody were detected 3, 4, 6, 10, 13, 14 and 17 weeks after the first injection (Monkeys which were given double dose of NCPP were detected again 20, 21, 23 weeks after the first injection). NCPP and CP were diluted to 10 μ g/ml in coating buffer (0.1 M buffer bicarbonate, pH 9.5) and added to the 96-well plates (200 μ l/well). After coating overnight at 4°C, plates were rinsed two times with PBS (pH 7.0) containing 0.5% Tween-20, blocked for 2 hr at 37°C with 0.2 mol/l PBS (pH 7.0) containing 10% fetal bovine serum, and then rinsed two times. Serum samples (100 μ l) were serially diluted (1:10, 1:100, 1:1,000,

1:10,000, 1:100,000, 1:100,000), added to the plates in triplicate and incubated for 2 hr. horseradish peroxidase labeled goat anti-monkey total IgG (Sigma) was added to the plates (100 μ l/well) at a dilution of 1:1,000 and incubated for 1 hr. After rinsing the plates, 100 μ l substrate solution was added and incubated for 0.5 hr. The substrate solution was prepared by dissolving 20 mg of OPD (o-phenylenediamine dihydrochloride) in 50 ml 0.1 M phosphate-citrate buffer (pH 6.0) and 167 μ l of 3% H₂O₂. The reaction was stopped by adding an equal volume of 1 M H₂SO₄ (50 μ l/well). All incubations described herein were performed at 37°C.

The optical density (OD) was detected at 492 nm using a multiskan ascent 354 microplate reader (Thermo Labsystems, Helsinki, Finland). Samples were considered positive if the OD value was higher than or equal to cutoff value (2.1 $\times$ OD value of negative control group). Antibody titers were presented as the mean log₁₀ of the reciprocal highest serum dilution exhibiting a positive reaction. Negative samples (< 1:10) were assigned a value of one-half the lowest dilution assessed (1:5).

Statistical analysis

Values were expressed as mean $\pm$ S.D. Statistical significance in intergroup comparison was analyzed by Student's t-test. P-values below 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

CP is indicated for the treatment of malignant pleural effusions. It evidently promotes immunoreactions of organism and inhibits the growth of tumors, which has been proved by preclinical and clinical studies. However, lipoid in its cell wall shows obvious immune stimulation activity. General and local reactions often occur in clinical practice.

NCPP is prepared by disposing CP in an extra-high voltage jet crushing machine and making it into uniform nano-scale sized particles containing low levels of pyrogen. Can it really reduce the side effects of CP by this way? The aim of the present study was to evaluate the immunogenicity and systemic toxicity of NCPP, when administered to monkeys by intramuscular injection for a period of 13 consecutive weeks, so as to provide a rational base for the evaluation of its toxicological risk in clinical research. *Macaca fascicularis* are considered to be suitable animal models for the safety assessment of medicine because they share many common characteristics with humans. Based on the result of our preliminary studies, three doses of NCPP were selected and served as low

Fig. 1. Pathological sections (H-E, $\times 400$) of liver, spleen and kidney 13 weeks after the first injection. A: NCPP (9 mg/monkey); B: NCPP (3 mg/monkey); C: NCPP (1 mg/monkey); D: CP (3 mg/monkey). 1: liver; 2: spleen; 3: kidney. See text for details.

dose (1 mg/monkey), mid dose (3 mg/monkey) and high dose (9 mg/monkey) respectively. The dose of NCPP used in clinical practice was proposed to be about 8-30 $\mu\text{g/kg}$ twice a week. The relative overdose of the NCPP in the present study could ensure that most treatment-related side effects and toxicities would be intensified and therefore more likely to be detected. Drug administration route is considered to be one of the main factors that contribute to the side effects of CP in incidence, intensity and clinical manifestation. Therefore, intramuscular injection

was used for the administration of NCPP and CP. Administration frequency of NCPP in the present work was in accordance with the designed dose schedule that will be performed in clinical practice.

Significant local reactions are main side effects that accompany with the administration of CP (Fisher B *et al.*, 1976). In this study, 4 monkeys in the CP group showed loose stools 2-13 days after injection and obvious local reactions at the site of injection were found after injection. In contrast, local reactions at the injection sites such

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Fig. 2. Pathological sections (H-E, $\times 400$) of liver, spleen and kidney 15 weeks after the first injection. A: NCPP (9 mg/monkey); B: NCPP (3 mg/monkey); C: NCPP (1 mg/monkey); D: CP (3 mg/monkey). 1: liver; 2: spleen; 3: kidney. See text for details.

as swelling and sclerome were not found in NCPP groups. A cutaneous absorbability experiment has been conducted in rabbits by our group. NCPP or CP (0.5 mg/rabbit) was cutaneously injected into a rabbit's back. No obvious local swelling and sclerome were observed after the injection (data not shown). These results indicate that NCPP is easy to be absorbed and can minor local inflammatory reactions when compared with CP. No febrile response was observed in NCPP groups, indicating that pyrogen in NCPP was at low levels.

Hematology parameters, serum biochemistry parameters, T cell subsets and differential leukocyte counts were within the normal ranges and showed no significant differences (data not shown).

Gross examinations showed no obvious abnormali-

ties in all monkeys, however, obvious histopathological changes of liver were found in NCPP (9 mg/monkey group) 13 weeks after the first injection, including cloudy swelling of hepatocyte, extensive inflammatory cell infiltrate in portal areas, spotty necrosis. Karyopyknosis was observed in CP group 13 weeks after the first injection. Liver sections from monkeys received low dose or mid dose of NCPP showed slight cloudy swelling of hepatocyte and cytoplasmic rarefaction (Fig. 1, A1-D1). Results of pathological examination indicated that liver injury is in correlation with the dose of NCPP and is reversible. After the recovery period, histopathological changes in NCPP groups were significantly reduced (Fig. 2, A1-D1). Slight apoptosis were observed in NCPP (9 mg/monkey group) after the recovery period.

Fig. 3. Deposition of immune complexes in the glomerular basement membrane. Routine paraffin sections of kidneys were prepared and stained using an ABC kit. Original magnification: $\times 400$. Buffy precipitate appearing in endochylema was regarded as positive reaction. A: NCPP (9 mg/monkey); B: NCPP (3 mg/monkey); C: NCPP (1 mg/monkey); D: CP (3 mg/monkey). Obvious immune complexes (emphasized by ellipses) were found in CP group.

Fig. 4. Antibody titers over time in NCPP and CP groups. The optical density (OD) was detected at 492 nm. Samples were considered positive if the OD value was higher than or equal to cutoff value ($2.1 \times$ OD value of negative control group). Antibody titers were presented as the mean $\log_{10}$ of the reciprocal highest serum dilution exhibiting a positive reaction. Negative samples (dilution $< 1:10$) were assigned a dilution of 1:5. One monkey in each group was detected 17 weeks after the first injection. Monkeys which were given double dose of NCPP were detected again 20, 21, 23 weeks after the first injection. * $P < 0.05$ vs NS group, ** $P < 0.01$ vs NS group.

Study indicated that increasing of spleen coefficient had a remarkable positive correlation with anti-tumor effects of CP and NCPP (Gao *et al.*, 2007; Chare *et al.*, 1977). In the present study, no significant difference was found in spleen coefficient, although spleen coefficients of NCPP and CP groups were higher than that of NS group (data not shown). However, pathological examination showed that both NCPP and CP could increase the number of acinus lienalis and enlarge the splenic germinal centers (Fig. 1, A2-D2). It means that both NCPP and CP can stimulate the proliferation of germinal center B cell and are able to enhance the humoral immunity functions of organism.

Histopathological abnormalities including swelling of glomerula, cloudy swelling of the nephric tubules, effusion in the glomerular capsule were observed in the kidney sections of CP group 13 weeks after the first injection. No obvious histopathological changes were found in the kidney sections of NCPP groups (Fig. 1, A3-D3).

Deposition of immune complexes in the glomerular basement membrane was not observed in all NCPP groups. In contrast, immune complex in the CP group was obvious (Fig. 3).

Biotech drugs often contain heterogenic macromolecular compound and immunogenicity is one of the factors influencing their safety. Therefore, immunogenicity assessment is necessary for NCPP. In the present study, Anti-CP antibody was detected 3 weeks after the first injection and was at high level over the entire course of study. Anti-NCPP antibody was detected 4 weeks after the first injection and became antibody negative 14 weeks after the first injection. No anti-NCPP antibody was detected 20, 21 and 23 weeks after the first injection even double dose of NCPP was injected (Fig. 4). No other related symptoms were found in all NCPP groups. In another word, the elevated anti-NCPP antibody has no influence on the safety of NCPP.

In summary, we conclude that the immunogenicity and systemic toxicity of NCPP are lower than that of CP. However, much attention should be paid to the liver injury after administration of NCPP.

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REFERENCES

- Chare, M.J. and Baum, M. (1977): The effect of *Corynebacterium parvum* on the proliferation of monocyte precursors in the bone marrow of mice. *Dev. Biol. Stand.*, **38**, 195-200.
- Collins, A.L. and Song, C.W. (1977): Combined radiotherapy and *Corynebacterium parvum* treatment of a murine fibrosarcoma. *Radiology*, **125**, 235-241.
- Dye, E.S., North, R.J. and Mills, C.D. (1981): Mechanisms of anti-tumor action of *corynebacterium parvum* I. Potentiated tumor-specific immunity and its therapeutic limitations. *J. Exp. Med.*, **154**, 609-620.
- El Kappany, H., Chopra, C., Nigam, V.N., Brailovsky, C.A., and Elhilali, M. (1980): The antitumor activity of maltose tetrapalmitate compared with other immunoadjuvants, and its effectiveness after tumour surgery. *Br. J. Cancer*, **42**, 703-711.
- Fisher, B., Rubin, H., Sartiano, G., Ennis, L. and Wolmark, N. (1976): Observations following *Corynebacterium parvum* administration to patients with advanced malignancy. a phase I study. *Cancer*, **38**, 119-130.
- Gao, S., Song, J., Liu, X. *et al.* (2003): Anti-Tumor Effects and Mechanism of the Product from *Corynebacterium Parvum* at Nano Scale (NCPP). *Chin. J. Cancer Biother.*, **10**, 101-104. Chinese.
- Gao, S., Ma, H., Ma, S. *et al.* (2003): Anti-tuberculosis effects and mechanism of the nano-scale product from *corynebacterium parvum*. *Chin. J. Pharm. Anal.*, **23**, 323-328. Chinese.
- Gao, S., Li, B.-Q., Qu, S. *et al.* (2004): Study on anti-MuLV effects of NCPP. *Chin. J. Pharm. Anal.*, **24**, 193-197. Chinese.
- Gao, S., Liu, C., Qu, S., Song, J., Li, J., Zhang, P., Wang, Q., Guo, C., Gao, F. and Zhang, L. (2007): Non-cell *Corynebacterium parvum* generated by nanotechnology: a promising immunomodulator with less side effects. *Int. Immunopharmacol.*, **7**, 1334-1342.
- Hart, D.A. (1987): *Corynebacterium parvum*, but not BCG, induces elevations in plasma proteinase activity similar to those observed in tumor-bearing mice. *Haemostasis*, **17**, 79-88.
- Koukalová, D., Kod'ousek, R., Hájek, V. and Kolár, M. (1992): Experimental nonspecific immunostimulation by the Propionibacterium acnes vaccine. *Acta. Univ. Palack. Olomuc. Fac. Med.*, **133**, 19-23.
- Lau, B.H., Woolley, J.L., Marsh, C.L., Barker, G.R., Koobs, D.H. and Torrey, R.R. (1986): Superiority of intralesional immunotherapy with *Corynebacterium parvum* and *Allium sativum* in control of murine transitional cell carcinoma. *J. Urol.*, **136**, 701-705.
- Lipton, A., Harvey, H.A., Balch, C.M., Antle, C.E., Heckard, R. and Bartolucci, A.A. (1991): *Corynebacterium parvum* versus bacille Calmette-Guérin adjuvant immunotherapy of stage II malignant melanoma. *J. Clin. Oncol.*, **9**, 1151-1156.
- Mára, M., Julák, J., Bednár, M., Ocenásková, J., Miková, Z. and Dohnalová, A. (1994): The influence of Propionibacterium acnes (*Corynebacterium parvum*) fractions on immune response *in vivo*. *Zentralbl. Bakteriologie*, **281**, 549-555.
- Matsui, K., Yoshimoto, T., Tsutsui, H., Hyodo, Y., Hayashi, N., Hiroishi, K., Kawada, N., Okamura, H., Nakanishi, K. and Higashino, K. (1997): Propionibacterium acnes treatment diminishes CD4⁺ NK1.1⁺ T cells but induces type I T cells in the liver by induction of IL-12 and IL-18 production from Kupffer cells. *J. Immunol.*, **159**, 97-106.
- Mythili, M.D., Nair, S.C. and Gunasekaran, S. (2004): Effect of cyclophosphamide pretreatment on hematological indices of

- Indian Bonnet monkeys. *Indian J. Pharmacol.*, **36**, 175-176.
- Purnell, D.M., Bartlett, G.L., Kreider, J.W., Biro, T.G. and Kontra, J. (1979): Comparative antitumor effects of *Corynebacterium parvum*, *Bordetella pertussis*, *Bacillus Calmette-Guérin*, and levamisole alone or in combination with cyclophosphamide in the CaD2 murine mammary adenocarcinoma system. *Cancer Res.*, **39**, 4838-4842.
- Raiford, D.S. and Thigpen, M.C. (1994): Kupffer cell stimulation with *Corynebacterium parvum* reduces some cytochrome P450-dependent activities and diminishes acetaminophen and carbon tetrachloride-induced liver injury in the rat. *Toxicol. Appl. Pharmacol.*, **129**, 36-45.
- Riveros-Moreno, V. and Niblock, A. (1979): Chemical properties of the principle in *C. parvum* that produces splenomegaly in mice. *Immunology*, **36**, 495-499.
- Rossol, S., Voth, R., Brunner, S., Müller, W.E., Büttner, M., Gallati, H., Meyer zum Büschenfelde, K.H. and Hess, G. (1990): *Corynebacterium parvum* (*Propionibacterium acnes*): an inducer of tumor necrosis factor- α in human peripheral blood mononuclear cells and monocytes *in vitro*. *Eur. J. Immunol.*, **20**, 1761-1765.
- Tekin, E., Kocdor, M.A., Saydam, S., Bora, S. and Harmancioglu, O. (2001): Seroma prevention by using *corynebacterium parvum* in a rat mastectomy model. *Eur. Surg. Res.*, **33**, 245-248.
- Thatcher, N. and Crowther, D. (1977): Effects of BCG and *Corynebacterium parvum* on immune reactivity in melanoma patients. *Dev. Biol. Stand.*, **38**, 449-453.
- Tsuji, H., Mukaida, N., Harada, A., Kaneko, S., Matsushita, E., Nakanuma, Y., Tsutsui, H., Okamura, H., Nakanishi, K., Tagawa, Y., Iwakura, Y., Kobayashi, K. and Matsushima, K. (1999): Alleviation of lipopolysaccharide-induced acute liver injury in *Propionibacterium acnes*-primed IFN- γ -deficient mice by a concomitant reduction of TNF- α , IL-12, and IL-18 production. *J. Immunol.*, **162**, 1049-1055.