Bioelectromagnetics 27:458–466 (2006)

Effect of Cyclophosphamide and 61.22 GHz Millimeter Waves on T-Cell, B-Cell, and Macrophage Functions

V.R. Makar, M.K. Logani,* A. Bhanushali, S.I. Alekseev, and M.C. Ziskin

Center for Biomedical Physics, Temple University School of Medicine, Philadelphia, Pennsylvania, USA

The present study was undertaken to investigate whether millimeter waves (MMWs) at 61.22 GHz can modulate the effect of cyclophosphamide (CPA), an anti-cancer drug, on the immune functions of mice. During the exposure each mouse's nose was placed in front of the center of the antenna aperture (1.5×1.5 cm) of MMW generator. The device produced 61.22 ± 0.2 GHz wave radiation. Spatial peak Specific Absorption Rate (SAR) at the skin surface and spatial peak incident power density were measured as 885 ± 100 W/kg and 31 ± 5 mW/cm^2, respectively. Duration of the exposure was 30 min each day for 3 consecutive days. The maximum temperature elevation at the tip of the nose, measured at the end of 30 min, was 1 °C. CPA injection (100 mg/kg) was given intraperitoneally on the second day of exposure to MMWs. The animals were sacrificed 2, 5, and 7 days after CPA administration. MMW exposure caused upregulation in tumor necrosis factor-alpha (TNF-α) production in peritoneal macrophages suppressed by CPA administration. MMWs also caused a significant increase in interferon-gamma (IFN-γ) production by splenocytes and enhanced proliferative activity of T-cells. Conversely, no changes were observed in interleukin-10 (IL-10) level and B-cell proliferation. These results suggest that MMWs accelerate the recovery process selectively through a T-cell-mediated immune response. Bioelectromagnetics 27:458–466, 2006. © 2006 Wiley-Liss, Inc.

Key words: millimeter waves; TNF-α; IFN-γ; CD25

INTRODUCTION

During the past two decades, the effects of millimeter wave (MMW) irradiation on the immune system and its components have been extensively studied. There is promising new research showing that MMWs can beneficially modulate immune responses. Three commonly used frequencies, approved by the Russian Ministry of Health, are 61.22, 53.57, and 42.25 GHz [Rojavin and Ziskin, 1998]. Efficacy of MMWs to enhance immune responses has been confirmed in many studies. It was shown that MMWs increased phagocytic activity of macrophages [Khomenko et al., 1995; Rojavin et al., 1997a], enhanced proliferation of T-cells, and normalization of the ratio of CD4$^+$/CD8$^+$ T-lymphocytes [Postovit, 1989; Kutsenok, 1994; Ostrovsky and Nikolaeva, 1995; Zaporozhan et al., 1997], and increased amount of B lymphocytes and normalized production of immunoglobulins [Postovit, 1989; Babak and Goncharova, 1995; Bakalyuk, 1997]. In cancer treatment, MW therapy has been used in combination with chemo- and radiotherapy to increase immunity, and to reduce the toxic effects of the above therapies [Sevastyanova, 1979; Kabisov, 1997; Pletnev, 2000].

In a previous study using BALB/c mice we have shown that MMWs with 42.2 GHz can protect T-cell functions from the toxicity of cyclophosphamide (CPA), a commonly used anti-cancer drug [Makar et al., 2003]. MMWs enhanced T-cell recovery through upregulatuion in activation and effector functions of CD4$^+$ T-cells. We have also shown that MMWs at 42.2 GHz can upregulate cytolitic activity of NK cells [Makar et al., 2005]. In the present study, we have examined the effect of another therapeutic frequency 61.22 GHz to determine the effect of MMW irradiation

Grant sponsor: National Center for Complementary and Alternative Medicine; Grant numbers: RO1AT000492, PO1AT002025.

*Correspondence to: Dr. M.K. Logani, Center for Biomedical Physics, Temple University School of Medicine, 3420 North Broad Street, Philadelphia, PA 19140.
E-mail: mlogani@temple.edu

Received for review 11 April 2005; Final revision received 27 January 2006

DOI 10.1002/bem.20230
Published online 18 April 2006 in Wiley InterScience (www.interscience.wiley.com).

© 2006 Wiley-Liss, Inc.

on CPA-induced suppression of immune functions of macrophages, B-cells, and T-cells.

Macrophages are large mononuclear phagocytic cells, which play an important role both in innate and adaptive immunity. The adaptive immunity is comprised of cell-mediated immunity and humoral immune response. In cell-mediated immunity, T-cells are known to play a major role, whereas B-cells mediate humoral immunity. These two cell types come from a common lymphoid precursor cell but differentiate along different development lines. One line matures in the thymus and is referred to as T-cell; the other matures in the bone marrow and is referred to as B-cell. CPA was chosen as a representative anti-cancer drug because it is known to have an adverse effect on hematopoietic and lymphoid tissues [Hill, 1975; Angulo et al., 2000].

Macrophages play a critical role in host defense since they are known to be effector cells in both humoral and cell-mediated immunity. Macrophage activation is important in controlling many key processes of the immune system. When activated, macrophages are known to produce tumor necrosis factor-alpha (TNF-α). TNF-α was first described as an endotoxin-induced and macrophage-secreted factor that caused hemorrhagic necrosis of tumor cells. TNF-α is now recognized as a critical cytokine orchestrating cell differentiation, activation, proliferation, and survival [Armitage, 1994; Gruss and Dower, 1995; Chan et al., 2000; Screaton and Xu, 2000; Locksley et al., 2001].

The results of our present study demonstrate that MMWs can strongly increase the ability of macrophages to produce TNF-α as measured 2, 5, and 7 days following CPA administration in both stimulated and unstimulated macrophages. Upregulation of TNF-α was accompanied by a significant increase in interferon-gamma (IFN-γ) production by anti-CD3 stimulated splenocytes and enhanced proliferation of T-cells. On the other hand, no changes were observed in B-cell proliferation. These results suggest that MMWs accelerate the recovery process selectively through a cell-mediated immune response, where T-cells play a central role. Flow cytometry analysis showed that both CD4 and CD8 T-cells contribute to the recovery induced by MMWs.

MATERIALS AND METHODS

Animals

Male BALB/c mice (20–25 g) were obtained from Taconic Co. (Germantown, NY, USA). The animals were housed in the Central Animal Facility at the Temple University School of Medicine, under controlled temperature and 12 h light/dark cycle conditions. The facility is accredited by the Accreditation of the Association for the Assessment of the Laboratory Animal Care (AAALAC). All animals were given Purina Chow and tap water ad libitum. Protocols were approved by the Institutional Animal Care and Use Committee.

Millimeter Wave Irradiation

MMWs were produced with a Russian-made "Istok" (Model G4-142) generator (Istok, Fryazino, Russia). To prevent the influence of environmental electromagnetic "noise," MMW irradiation was conducted in a shielded chamber made of 0.5-inch thick (1.25 cm) low carbon steel sheets. The MMW generator together with the power meter ML 4803A (Anritsu Corp., Tokyo, Japan) and the spectrum analyzer (Hewlett Packard 8565B, Palo Alto, CA, USA) were located outside the shielded area. The device produced 61.22 ± 0.2 GHz (mean $\pm$ SD) wave radiation, which was introduced into the shielded chamber with a wave guide. The spatial peak Specific Absorption Rate (SAR) at the tip of the nose and spatial peak incident power density were measured as 885 ± 100 W/kg and 31 ± 5 mW/cm^2 (mean $\pm$ SD), respectively [Logani et al., 2004]. Duration of the exposure was 30 min.

Each mouse was restrained in a plastic tube (3.5-cm diameter) with the nose uncovered for breathing and irradiation (Fig. 1). During the exposure each mouse's nose was located in the center of the antenna aperture (1.5 × 1.5 cm; Antenna Gain: 19 dB) at a distance of 0–1 mm from its front edge as reported earlier

Fig. 1. Experimental set-up for irradiation of mice. For irradiation the animals were restrained in plastic tubes (3.5 cm diameter) with the nose uncovered for breathing and exposure purposes. Each mouse was positioned in such a way that its nose was placed in the center of the antenna aperture at a distance of 0–1 mm from its front edge.

[Radzievsky et al., 2004]. Additional experiments were performed to characterize the MMW exposure. An Amber-4256 IR camera (Amber Engineering, Inc., Goleta, GA, USA) with spatial resolution of 256×256 pixels per frame and temperature sensitivity of 0.02 K was used to measure the temperature distribution. Temperature elevation, measured at the end of 30 min, was maximal at the tip of the nose (1.05 ± 0.15 °C). Remote areas of the mouse head were heated to a lesser degree, except for the eyes that were heated a little more (0.44 °C). The temperature elevation at the nose reached its steady-state level about 5 min following the onset of the exposure.

Three groups of animals, three mice per group, were used in an experiment. The experiment was repeated three times for each time point studied and the data from nine animals for each group at each time were pooled. The first group (Naïve) was not given any treatment. The second group (CPA + MMW) was irradiated with MMWs on days 1–3 for 30 min each day. On day 2, 0.5 ml CPA solution in physiological saline was administered intraperitoneally (100 mg/kg) just before irradiating the animals. The animals were irradiated in the morning at the same time every day. The third group (CPA + Sham) served as a Sham control where the animals were treated with CPA and restrained in a similar manner as the second group. The Sham group was placed far enough from the horn antenna to protect them from any exposure to MMWs. The animals were sacrificed by cervical dislocation 2, 5, and 7 days after CPA administration, and the spleens were removed aseptically.

Reagents

CPA and 3-(4-5-dimethylthiozol-2-yl)2-5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ficoll-Plaque™ PLUS for splenocyte isolation was obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). All antibodies used for flow cytometry were purchased from PharMingen (San Diego, CA, USA). For cytokines measurement we used PharMingen OptEIA™ kit. The culture medium (RPMI-1640) and supplements (HEPES, Pen/Strep), phosphate buffered saline (PBS), and Hanks' balanced salts solution (HBSS) were purchased from Cellgro (Bridgeport, NJ, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). For T-cell activation anti-CD3 mAbs (hamster anti-mouse CD3, CD3ϵ chain, monoclonal antibodies) were purchased from PharMingen. For macrophages and B-cells stimulation, lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma.

Preparation of Spleen Cells and Macrophages

Mice were sacrificed by cervical dislocation, and the spleen from each mouse was removed aseptically. Splenocyte suspensions were prepared by homogenization of the spleen in tissue culture medium (RPMI-1640) supplemented with 5% FBS and 2 mM L-glutamine. The homogenate was centrifuged on a density gradient ($500 \times g$, 20 min), and mononuclear leukocytes were collected and washed three times in RPMI-1640 and resuspended to 1×10^6 viable cells/ml in complete RPMI (RPMI + 10% FBS, HEPES, 2 mM glutamine and Pen/Strep). For flow cytometry assays, 5×10^5cells/0.5 ml/well were incubated for 48 h in 24-well tissue culture plates at 37 °C in humidified atmosphere air containing 5% CO_2. Anti-CD3 monoclonal antibody was used as an antigen for T-cell stimulation in order to mimick the physiologic stimulation in vitro and to expand the immune response. To induce an optimal immune response for T-cell receptor stimulation assays, plates were prebound with hamster anti-mouse CD3 monoclonal antibodies (mAbs) (0.1 μg/ml/well). After culturing, cells were transferred to tubes, and two-step washing was performed. For macrophage preparation, peritoneal exudate cells were harvested by flushing peritoneal cavities with PBS. Cells were plated in a 24-well plate at an initial density of 1×10^6 cells/ml in a 500 μl volume of RPMI-1640, supplemented with 10% FBS. Two hours later, the wells were washed to remove all non-adherent cells and 500 μl of fresh medium was added together with LPS (1.0 μg/ml/well).

Flow Cytometry

The following mAbs were used to stain surface antigens: R-phycoerythrin (R-PE)-conjugated hamster anti-mouse CD3 (CD3ϵ chain) mAbs for total T-cell population, CY-Crome-conjugated rat anti-mouse CD4 mAbs or CY-Crome-conjugated rat anti-mouse CD8 mAbs for T-cell subsets, and fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD25 (IL-2 receptor α chain mAbs). The appropriate dilution for each mAb was determined by titration. After surface staining by conventional techniques, cells were washed in HBSS with 3% FBS and fixed in 1% paraformaldehyde solution. A total of 30 000 events per sample were collected. Dead cells and tissue debris were excluded according to forward and side-scatter properties in order to gate only live lymphocyte populations.

Cytokine Measurement

Macrophages (0.5×10^6/0.5 ml/well) were incubated as described for 24 h. Then, supernatants were collected and TNF-α ELISA was performed using

a standard kit from PharMingen. The PharMingen OptEIA^{TM} test is a solid phase sandwich ELISA, which utilizes a monoclonal antibody specific for mouse TNF-α coated on a 96-well plate. Standards and test samples were added to the wells and incubated for 2 h at room temperature. After washing with PBS/Tween, a mixture of biotinylated anti-mouse TNF-α together with avidin-horseradish peroxidaze was added. Peroxidase reaction was developed by the addition of 50 μl/well TMB (3,3′,5,5-tetramethylbenzidine) substrate and the reaction was terminated by addition of 50 μl/well 1 M phosphoric acid. Absorbance was determined at 450 nm. Standards and samples were assayed in triplicate. For measurement of IFN-γ and interleukin-10 (IL-10) in T-cells, splenocytes (0.5×10^6 cells/0.5 ml) from each mouse were cultured with anti-CD3 antibody for 48 h and the level of IFN-γ and IL-10 was determined by ELISA.

Proliferation Assays

Cell proliferation was examined by the MTT assay. In this assay, the tetrazolium salt MTT is metabolized by NAD-dependent dehydrogenase, an intracellular enzyme, to form a colored reaction product, and the amount of dye formed directly correlates with the number of viable cells. Briefly, splenocytes (2×10^5 cells) were grown in microplates in a final volume of 200 μl complete RPMI-1640 at 37 °C in humidified atmosphere air containing 5% CO_2. Splenocytes refer to all spleen cells except erythrocytes. After 72 h incubation, 10 μl of MTT (5 mg/ml) was added, and the cells were further incubated for 6 h at the same conditions. After solubilization in acid-isopropanol (0.04 N HCl in isopropanol) the formazan dyes were quantified using an ELISA plate reader at the wavelength of 590 nm, as recommended by the manufacturer. For T-cell proliferation anti-CD3 mAbs (0.1 μg/ml/well) was used for stimulation. For B-cell proliferation, cells were stimulated with LPS (1.0 μg/ml/well). The stimulation was given at the time of culturing cells in microplates as described previously [Makar et al., 2003].

Statistical Analysis

Because the statistical distributions failed the normality test, the non-parametric Kruskal ANOVA test was used when comparing more than two groups at a time. If statistical significance was found, each pair in the study was tested for significance using the Student's *t*-test or the Wilcoxin signed rank test [Larsen and Marx, 1990]. *P*-values $<.05$ were considered significant for all tests. All data are presented as mean ± SD.

RESULTS

MMWs Induced Upregulation in TNF-α Production Suppressed by CPA

CPA treatment caused a strong decrease in spontaneous release of TNF-α by peritoneal macrophages at all estimated time points. As shown in Figure 2A, the level of TNF-α production was significantly diminished on day 2 post-injection in CPA + Sham group (0.12 ± 0.01 ng/ml), on day 5 (0.35 ± 0.02 ng/ml), and on day 7 (0.35 ± 0.03 ng/ml) as compared to Naïve group (1.22 ± 0.32 ng/ml). When the MMW treatment was applied, a significant increase in TNF-α was observed at all estimated time points. It is important to note that on day 5 the TNF-α secretion capacity of CPA + MMW group reached to 80% (0.98 ± 0.18 ng/ml) and on day 7

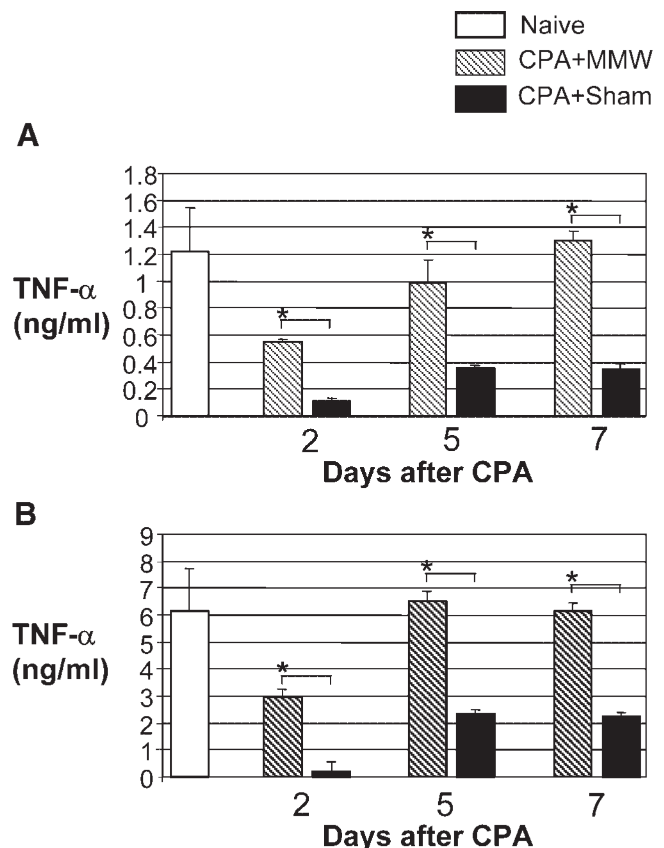

Fig. 2. MMW exposure restores TNF-α production by peritoneal macrophages as measured 2, 5, and 7 days after CPA treatment. **A**: Pooled ELISA data showing TNF-α secretion by peritoneal macrophages without LPS stimulation. **B**: Pooled ELISA data showing TNF-α secretion by peritoneal macrophages with LPS stimulation. Asterisk indicates the irradiated group statistically different ($P<.05$) from the corresponding CPA + Sham group ($n=9$, pooled data from three separate experiments, three animals per group). Assays were performed in quadruplicate. Bars depict mean ± SD. Naive control represents average of three time points ($n=27$).

to 106% (1.30 ± 0.07 ng/ml) of the mean Naïve control value (1.22 ± 0.32 ng/ml). Statistically, no significant differences were found among Naïve control and CPA + MMW groups after 5 or 7 days. A similar pattern was observed when LPS was used to stimulate macrophages (Fig. 2B). These results clearly demonstrate that MMW irradiation accelerates recovery of TNF-α production suppressed by CPA administration.

Effect of MMWs on IFN-γ and IL-10 Production by Splenocytes

It is known that, macrophages are activated by membrane-bound signals delivered by activated T-cells as well as by the potent macrophage-activating cytokine IFN-γ, which is secreted by activated T-cells. As shown in Figure 3A, MMW treatment significantly enhanced the level of IFN-γ production on day 5 (2.21 ± 0.42 ng/ml) and day 7 (1.50 ± 0.13 ng/ml) as compared to Sham exposure (1.03 ± 0.13 ng/ml and 0.14 ± 0.04 ng/ml on days 2 and 5, respectively). We also examined the level of IL-10 production. No changes were observed in IL-10 level after MMW exposure as compared to Sham exposed animals. The foregoing results suggest that the MMW irradiation enhance the ability of T-cells to produce IFN-γ in immunosuppressed mice, but has no effect on IL-10 secretion.

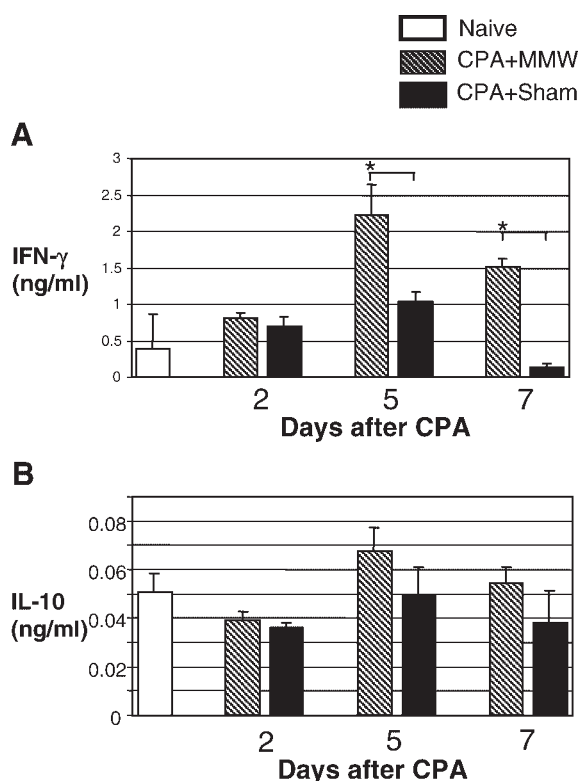

Fig. 3. MMW exposure upregulates IFN-γ secretion in anti-CD3 activated T-cells from spleen, but has no effect on IL-10 secretion. **A**: Pooled ELISA data showing IFN-γ secretion by T-cells (1×10^6 ml/well) from mice spleen. Asterisk indicates the irradiated group statistically different ($P < .05$) from the CPA + Sham group at each time point ($n = 9$). Assays were performed in quadruplicate. **B**: Pooled Elisa data showing IL-10 secretion by T-cells from mice spleen ($n = 9$). Bars depict mean ± SD. Naive control represents average of three time points ($n = 27$).

MMW Effect on Proliferative Activity of Splenocytes

Next, we examined whether the upregulation of IFN-γ by MMWs or TNF-α has any impact on proliferative activity of splenocytes. To determine cell proliferative activity, anti-CD3 antibodies were used for stimulation. No effect of MMWs was observed on T-cell proliferation as measured 2 days after CPA. We found that MMW irradiation significantly enhanced the proliferative activity of T-cells as measured 5 (73%) and 7 (279%) days after CPA (Fig. 4). On the other hand, no differences were observed in B-cell proliferative activity between CPA + Sham and CPA + MMW groups (Fig. 5). These results suggest that MMWs selectively accelerate recovery process associated with T-cell-mediated immune response.

Both CD4 and CD8 T-Cells Participate in the Recovery Process Induced by MMWs

Flow cytometric analysis shows that both CD4 and CD8 T-cells contribute to the recovery induced by MMWs (Figs. 6 and 7). The expression of CD25 (IL-2Rα) was determined in $CD3^+CD4^+$ or $CD3^+CD8^+$ cell populations induced by anti-CD3 stimulation. CD25 are known to be an important marker in determination of the T-cell activation process. Although CPA treatment did not effect significantly $CD4^+$ T-cells activation as measured on day 2 and day 5

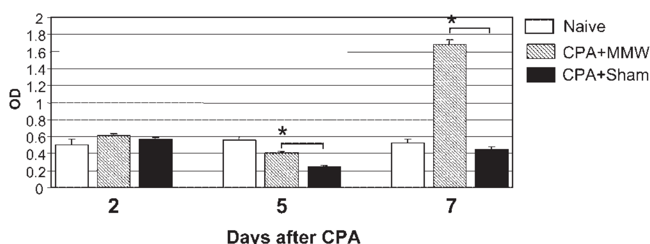

Fig. 4. Effect of MMWs on proliferative activity of T-cells 2, 5, and 7 days CPA post-injection. The values reported as optical densities (ODs) are the mean ± SD of nine mice in each group. (Pooled data from three separate experiments, three animals per group). Each sample was analyzed in quadruplicate. An asterisk indicates the irradiated group statistically different ($P < .05$) from the corresponding CPA + Sham group.

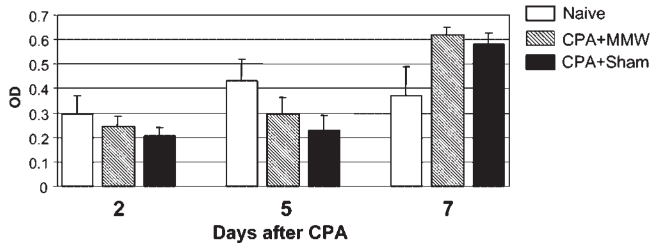

Fig. 5. Effect of MMW exposure on B-cell proliferation. The values reported as ODs are the mean $\pm$ SD of nine mice in each group (pooled data from three separate experiments, three animals per group). Each sample was analyzed in quadruplicate.

after CPA injection, on day 7 CD25 expression was downregulated from 41.4 $\pm$ 7.9% in the Naïve group to 27.05 $\pm$ 3.9% in the CPA + Sham group. A significant upregulation of CD25 expression was observed after CPA + MMW irradiation on days 2, 5, and 7 relative to CPA + Sham (Fig. 6). We next measured CD25 expression on $CD8^+$ T-cells (Fig. 7). The results indicate that $CD3^+CD8^+$ cells upregulate CD25 expression only at a later stage of CPA recovery process. An increased percentage of $CD8^+$ T-cell upregulated CD25 surface expression was observed in the CPA + MMW group (41.9 $\pm$ 2.8) as compared to CPA + Sham group (23.1 $\pm$ 5.3). There were no significant differences observed on day 2 or 5 following CPA injection.

DISCUSSION

CPA is a widely used chemotherapeutic agent. It is an anti-neoplasic drug, employed alone or in combination with other products or therapies. Used as anti-cancer drug, treatment with CPA severely injures hematopoietic and lymphoid tissues [Hill, 1975; Angulo et al., 2000]. Therefore, among the problems

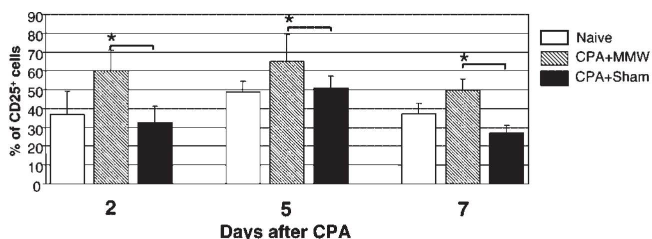

Fig. 6. MMWs enhance CD25 activation marker expression on $CD4^+$ T-cells as measured 2, 5, and 7 days after CPA injection. Pooled flow cytometry data showing percentage of CD25 expression on $CD3^+CD4^+$ gated cells. CD25 expression was assessed after 24 h of T-cell incubation. Anti-CD3 mAbs (0.1 µg/ml/well) were used for T-cell stimulation. An asterisk indicates the irradiated group statistically different ($P < .05$) from the CPA + Sham group at each time point. The values reported are the mean $\pm$ SD ($n = 9$, pooled data from three separate experiments, three animal per group).

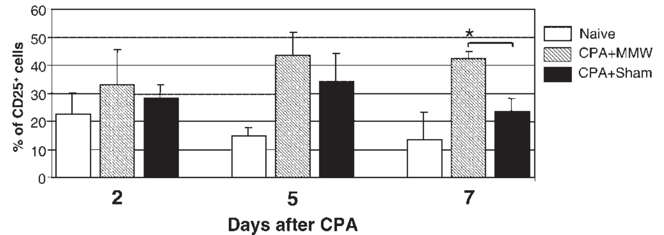

Fig. 7. MMWs effect on CD25 expression on $CD8^+$ T-cells as measured 2, 5, and 7 days after CPA injection. Pooled flow cytometry data showing percentage of CD25 expression on $CD3^+CD8^+$ gated cells. Anti-CD3 mAbs (0.1 µg/ml/well) were used for T-cells stimulation. An asterisk indicates the irradiated group statistically different ($P < .05$) from the CPA + Sham group. The values reported are the mean $\pm$ SD ($n = 9$, pooled data from three separate experiments, three animal per group).

associated with intensive chemotherapy, restoration of immunocompetence is a critical issue. It has been shown previously that MMWs protect the immune system from the adverse effect of chemotherapy [Sevastyanova, 1979; Kabisov, 1997; Pletnev, 2000]. For example, it has been reported that in cancer patients T-cell populations and functions, compromised by chemotherapy, were normalized by MMW therapy. However, the mechanisms involved in this protection are not known. Our results suggest that restoration of lymphocyte and macrophage functions by MMWs is mediated through modulation of cytokine production.

The present study has demonstrated that MMW irradiation at 61.22 GHz can restore TNF-α production by peritoneal macrophages suppressed by CPA treatment. This observation is of clinical relevance since TNF-α is a critical cytokine, orchestrating cell differentiation, activation, proliferation, and survival of most of the immunocompetent cells. TNF-α plays an important roles in host defense against infections and tumors and in mediating inflammatory and immune responses [reviewed by Trinchieri, 1992]. It triggers the local expression of chemokines and cytokines and promotes the adhesion, attraction, and activation of leukocytes at the site of infection [Vilcek and Lee, 1991]. Furthermore, it was shown that TNF-α could increase the therapeutic efficiency of chemotherapeutic agents [Lejeune, 2002; Senzer et al., 2004].

We found that upregulation of TNF-α production by MMWs was accompanied by a significant increase in IFN-γ production and enhanced proliferation rate of T-cells. IFN-γ is known to be the main cytokine through which T-cells activate macrophages to produce TNF-α. IFN-γ is a homodimeric glycoprotein produced by both $CD4^+$ and $CD8^+$ T-cells [Young and Hardy, 1990]. In the case of $CD4^+$ T-cells, IFN-γ is primarily produced by the Th1 subset and exhibits a number of classical macrophage-activated properties.

$CD4^+$ cells are divided into two major subsets Th1 and Th2 that are characterized by the patterns of cytokines they produce. Th1 cells produce IFN-γ, TNF-β, TNF-α, IL-2, whereas Th2 cells produce IL-4, IL-5, and IL-10, etc. [Mosmann and Coffman, 1989; Abbas et al., 1996; Constant and Bottomly, 1997]. Th1 cells are mostly involved in T-cell-mediated reactions, while the Th2 cytokines are commonly found in association with B-cell activation, differentiation, and are responsible for initiating B-cell proliferation and immunoglobulins production. Moreover, the characteristic cytokine products of Th1 and Th2 cells are inhibitory for the differentiation and effector function of the opposite subset.

Numerous studies have shown that IFN-γ markedly increases TNF-α production in monocytes and macrophages by increasing both TNF-α transcription and stability of TNF-α mRNA [Hayes et al., 1995]. One of the factors that may serve to limit TNF-α expression is IL-10. IL-10 was originally defined as a factor produced by activated Th2 type T-cells that inhibit IFN-γ in Th1 type T-cells [Fiorentino et al., 1989]. It was shown that IL-10 produced by monocytes feedback inhibits production of Th1 type cytokines, including TNF-α [de Waal Malefyt et al., 1991]. It has been demonstrated that IL-10 exerts a variety of effect on B-cells, such as proliferation and differentiation of B-cells [Fluckiger et al., 1993; Itoh et al., 1994; Donnelly et al., 1995] and plays an important role in determining the outcome of humoral immune responses [Itoh and Hirohata, 1995].

In our experiments, no changes were observed in IL-10 level and B-cell proliferation. Therefore, our results suggest that MMWs accelerate the recovery process selectively associated with T-cell-mediated immune response, but have no significant effect on B-cell-mediated immunity. In order to understand whether both CD4 and CD8 T-cells participate in the recovery process, we further analyzed T-cells by Flow cytometry. It was interesting to see that both $CD4^+$ and $CD8^+$ T-cells contribute to the recovery induced by MMWs. However, a significant increase in CD25, which is an activation marker for T-cells, was observed at all evaluated time points in $CD3^+CD4^+$ cells (Fig. 6). On the other hand, $CD3^+CD8^+$ cells showed an upregulation of CD25 expression on day 7 indicating that $CD3^+CD8^+$ cells participate in recovery process only at a later stage (Fig. 7).

In summary, our data strongly suggest that 61.22 GHz MMWs accelerate recovery selectively through the activation of cell-mediated immunity, where both $CD4^+$ and $CD8^+$ T-cells play a significant role. Furthermore, our results indicate that TNF-α and IFN-γ are important cytokines mediating this process. These results are similar to those obtained previously at 42.2 GHz frequency [Makar et al., 2003] indicating that the effect of two therapeutic frequencies (42.2 and 61.2 GHz) are similar on the immune functions studied under our experimental conditions. However, it is not clear whether similarity in our results at two different frequencies is due to an equal increase in temperature at the skin surface (1 °C) or due to a specific MMW effect.

Based on our previous results and results of others, we think that biological effects of MMWs (penetration depth less than 1 mm in the skin) are initiated by activation of free nerve endings in the skin [Alekseev et al., 1997; Pakhomov et al., 1997, 1998; Alekseev and Ziskin, 1999; Pakhomov and Murphy, 2000]. Then the signal is conveyed to the central nervous system where it modulates neural activity resulting in the development of various biological effects. Most probably, endogenous opioids and opioid receptors are involved in this chain of events [Rojavin and Ziskin, 1997b, 1998; Radzievsky et al., 2000, 2001, 2004]. In recent years, sufficient evidence has been accumulated for the involvement of endogenous opioids in regulation of immune functions. Endogenous opioids may upregulate release of immunostimulatory cytokines [Besedovsky and del Rey, 1996; Sacerdote et al., 2000] from T-cells and macrophages resulting in protection of their functions from the adverse effects of anti-cancer agents, such as CPA that was used in our study. Further studies with the aim of understanding different steps involved in the systemic effect of MMWs on the immune system are in progress.

ACKNOWLEDGMENTS

The study was supported by the National Center for Complementary and Alternative Medicine: Contract grant numbers RO1AT000492 and PO1AT002025. The authors are thankful to Dr. Alexander Radzievsky for his advice, Dr. Oleg Gordiienko for providing technical assistance, and Elaine Spencer for administrative assistance.

REFERENCES

Abbas AK, Murphy KM, Sher A. 1996. Functional diversity of helper T lymphocytes. Nature 383:787–793.

Alekseev SI, Ziskin MC. 1999. Effects of millimeter waves on ionic currents of Lymnaea neurons. Bioelectromagnetics 20: 24–33.

Alekseev SI, Ziskin MC, Kochetkova NV, Bolshakov MA. 1997. Millimeter waves thermally alter the firing rate of the Lymnaea pacemaker neuron. Bioelectromagnetics 18:89–98.

Angulo I, de las Heras FG, Garcia-Bustos JF, Gargallo D, Munoz-Fernandez MA, Fresno M. 2000. Nitric oxide-producing

CD11b(+)Ly-6G(Gr-1)(+)CD31(ER-MP12)(+) cells in the spleen of cyclophosphamide-treated mice: Implications for T-cell responses in immunosuppressed mice. Blood 95:212–220.

Armitage RJ. 1994. Tumor necrosis factor receptor superfamily members and their ligands. Curr Opin Immunol 6:407–413. Review.

Babak O, Goncharova L. 1995. Microwave therapy of patients with duodenal ulcer who participated in the elimination of the effects of Chernobyl accident. Lik Sprava 7:51–53 (in Ukrainian).

Bakalyuk TG. 1997. Mechanism of action and clinical efficacy of the microwave resonance therapy in primary osteoarthritis. Visnyk Naukovykh Doslidzhen. 1-5 (in Ukrainian).

Besedovsky HO, del Rey A. 1996. Immune-neuro-endocrine interactions: Facts and hypotheses. Endocr Rev 17:64–102. Review.

Chan KF, Siegel MR, Lenardo JM. 2000. Signaling by the TNF receptor superfamily and T-cell homeostasis. Immunity 13:419–422. Review.

Constant SL, Bottomly K. 1997. Induction of Th1 and Th2 CD4+ T-cell responses: The alternative approaches. Annu Rev Immunol 15:297–322.

de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, de Vries JE. 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T-cell proliferation by diminishing the antigen-presenting capacity of monocytes via down-regulation of class II major histocompatibility complex expression. J Exp Med 174:915–924.

Donnelly RP, Freeman SL, Hayes MP. 1995. Inhibition of IL-10 expression by IFN-gamma up-regulates transcription of TNF-alpha in human monocytes. J Immunol 155:1420–1427.

Fiorentino DF, Bond MW, Mosmann TR. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 170:2081–2095.

Fluckiger AC, Garrone P, Durand I, Galizzi JP, Banchereau J. 1993. Interleukin 10 (IL-10) upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes. J Exp Med 178:473–1481.

Gruss HJ, Dower SK. 1995. Tumor necrosis factor ligand superfamily: Involvement in the pathology of malignant lymphomas. Blood 85:3378–3404. Review.

Hayes MP, Freeman SL, Donnelly RP. 1995. IFN-gamma priming of monocytes enhances LPS-induced TNF production by augmenting both transcription and MRNA stability. Cytokine 7:427–435.

Hill DL. 1975. A review of cyclophosphamide. Springfield, IL: Charles C. Thomas.

Itoh K, Hirohata S. 1995. The role of IL-10 in human B-cell activation, proliferation, and differentiation. J Immunol 154:4341–4350.

Itoh K, Inoue T, Ito K, Hirohata S. 1994. The interplay of interleukin-10 (IL-10) and interleukin-2 (IL-2) in humoral immune responses: IL-10 synergizes with IL-2 to enhance responses of human B lymphocytes in a mechanism which is different from upregulation of CD25 expression. Cell Immunol 157:478–488.

Kabisov R. 1997. Millimeter waves in the rehabilitation of oncologic patients. 11th Russian Symposium ''Millimeter Waves in Medicine and Biology'', Zvenigorod, Russia, pp 13–14 (in Russian).

Khomenko A, Novikova L, Kaminskaya G, Efimova L, Panasek I, Golant M, Balakireva L, Gedymin L. 1995. Evaluation of functional state of blood phagocytes in the choice of optimal regime of EHF therapy in pulmonary tuberculosis. 10th Russian Symposium ''Millimeter Waves in Medicine and Biology'', Moscow, Russia, pp 13–15 (in Russian).

Kutsenok VA. 1994. The effect of electromagnetic radiation of millimeter range on the immune status of peptic ulcer patients. Likarska Sprava 10-12:14–20 (in Ukrainian).

Larsen RJ, Marx ML. 1990. Statistics. Prentice Hall, NJ: Engwood Cliffs.

Lejeune FJ. 2002. Clinical use of TNF revisited: Improving penetration of anti-cancer agents by increasing vascular permeability. J Clin Invest 110:433–435.

Locksley RM, Killeen N, Lenardo MJ. 2001. The TNF and TNF receptor superfamilies: Integrating mammalian biology. Cell 104:487–501. Review.

Logani MK, Bhanushali A, Anga A, Majmundar A, Szabo I, Ziskin MC. 2004. Combined millimeter wave and cyclophosphamide therapy of an experimental murine melanoma. Bioelectromagnetics 25:516–523.

Makar VR, Logani MK, Szabo I, Ziskin MC. 2003. Effect of millimeter waves on cyclophosphamide-induced suppression of T-cell functions. Bioelectromagnetics 24:356–365.

Makar VR, Logani MK, Bhanushali A, Kataoka M, Ziskin MC. 2005. Effect of millimeter waves on natural killer cells activation. Bioelectromegnetics 26:10–19.

Mosmann TR, Coffman RL. 1989. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7:145–173. Review.

Ostrovsky AB, Nikolaeva OV. 1995. Peculiarities of the immunomodulating effect of the EHF-therapy. 10th Russian Symposium ''Millimeter Waves In Medicine And Biology'', Moscow, Russia, pp 66–67 (in Russian).

Pakhomov AG, Murphy MR. 2000. Low-intensity millimeter waves as a novel therapeutic modality. IEEE Trans Plasma Sci 28(1):34–40.

Pakhomov AG, Prol HK, Mathur SP, Akyel Y, Campbell CB. 1997. Search for frequency-specific effects of millimeter-wave radiation on isolated nerve function. Bioelectromagnetics 18(4):324–334.

Pakhomov AG, Akyel Y, Pakhomova ON, Stuck BE, Murphy MR. 1998. Current state and implication of research on biological effects of millimeter waves: A review of literature. Bioelectromagnetics 19:393–413.

Pletnev SD. 2000. The use of millimeter band electromagnetic waves in clinical oncology. Crit Rev Biomed Eng 28:573–587.

Postovit NV. 1989. Mechanism of therapeutic effect of MWT in peptic ulcer. In: Sitko PS, editor. Fundamental and applied aspects of the use of millimeter electromagnetic radiation in medicine. Ukraine: Kiev, pp 199–200.

Radzievsky AA, Rojavin MA, Cowan A, Alekseev SI, Ziskin MC. 2000. Hypoalgesic effect of millimeter waves in mice: Dependence of the site exposure. Life Sci 66(21):2101–2111.

Radzievsky AA, Rojavin MA, Cowan A, Alekseev SI, Radzievsky AA Jr, Ziskin MC. 2001. Peripheral neural system involvement in hypoalgesic effect of electromagnetic millimeter waves. Life Sci 68(10):1143–1151.

Radzievsky AA, Gordiienko OV, Szabo SI, Alekseev SI, Ziskin MC. 2004. Millimeter wave-induced suppression of B16F10 melanoma growth in mice: Involvement of endogenous opioids. Bioelectromegnetics 25:466–473.

Rojavin MA, Ziskin MC. 1997b. Electromagnetic millimeter waves increase the duration of anesthesia caused by ketamine and chloral hydrate in mice. Int J Radiat Biol 72(4):475–480.

Rojavin MA, Ziskin MC. 1998. Medical application of millimeter waves. Q J Med 91:57–66.

Rojavin MA, Tsygankov AY, Ziskin MC. 1997a. In vivo effect of millimeter waves on cellular immunity of cyclophosphamide-treated mice. Electro Magnetobiology 16:281–292.

Sacerdote P, Gaspani L, Panerai AE. 2000. The opioid antagonist naloxone induces a shift from type 2 to type 1 cytokine pattern in normal and skin-grafted mice. Ann NY Acad Sci 917:755–763.

Screaton G, Xu XN. 2000. T-cell life and death signalling via TNF-receptor family members. Curr Opin Immunol 12:316–322. Review.

Senzer N, Mani S, Rosemurgy A, Nemunaitis J, Cunningham C, Guha C, Bayol N, Gillen M, Chu K, Rasmussen C, Rasmussen H, Kufe D, Weichselbaum R, Hanna N. 2004. TNFerade biologic, an adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene: A phase I study in patients with solid tumors. J Clin Oncol 22:592–601.

Sevastyanova LA. 1979. Peculiarities of the biological effects of millimeter range radiowaves and a possibility of their use in medicine. Vestn Akad Med Nauk SSSR 2:65–68 (in Russian).

Trinchieri G. 1992. Effect of TNF and Lymphotoxin on the hematopoietic system. Immunol Ser 56:289–313.

Vilcek J, Lee TH. 1991. Tumor necrosis factor: New insights into the molecular mechanisms of its multiple actions. J Biol Chem 266:7313–7316.

Young HA, Hardy KJ. 1990. Interferon-gamma: Producer cells, activation stimuli, and molecular genetic regulation. Pharmacol Ther 45:137–151. Review.

Zaporozhan V, Bespoyasnaya V, Sobolev R. 1997. Effect of mm-wave radiation on the state of encrine, immune and proteolytic systems of patients after the surgical removal of benign ovarian tumors. 11th Russian Symposium with international participation ''Millimeter Waves in Medicine and Biology'', Zvenigorod, Russia, pp 36–38 (in Russian).