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Effect of Millimeter Waves on Natural Killer Cell Activation

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Millimeter wave therapy (MMWT) is being widely used for the treatment of many diseases in Russia and other East European countries. MMWT has been reported to reduce the toxic effects of chemotherapy on the immune system. The present study was undertaken to investigate whether millimeter waves (MMWs) can modulate the effect of cyclophosphamide (CPA), an anticancer drug, on natural killer (NK) cell activity. NK cells play an important role in the antitumor response. MMWs were produced with a Russian-made YAV-1 generator. The device produced modulated 42.2 ± 0.2 GHz radiation through a 10×20 mm rectangular output horn. Mice, restrained in plastic tubes, were irradiated on the nasal area. Peak SAR at the skin surface and peak incident power density were measured as 622 ± 100 W/kg and 31 ± 5 mW/cm^2, respectively. The maximum temperature elevation, measured at the end of 30 min, was 1 °C. The animals, restrained in plastic tubes, were irradiated on the nasal area. CPA injection (100 mg/kg) was given intraperitoneally on the second day of 3-days exposure to MMWs. All the irradiation procedures were performed in a blinded manner. NK cell activation and cytotoxicity were measured after 2, 5, and 7 days following CPA injection. Flow cytometry of NK cells showed that CPA treatment caused a marked enhancement in NK cell activation. The level of CD69 expression, which represents a functional triggering molecule on activated NK cells, was increased in the CPA group at all the time points tested as compared to untreated mice. However, the most enhancement in CD69 expression was observed on day 7. A significant increase in TNF-α level was also observed on day 7 following CPA administration. On the other hand, CPA caused a suppression of the cytolytic activity of NK cells. MMW irradiation of the CPA treated groups resulted in further enhancement of CD69 expression on NK cells, as well as in production of TNF-α. Furthermore, MMW irradiation restored CPA induced suppression of the cytolytic activity of NK cells. Our results show that MMW irradiation at 42.2 GHz can up-regulate NK cell functions. Bioelectromagnetics 26:10–19, 2005.

Key words: millimeter waves; NK cell cytotoxicity; CD69; TNF-α; cyclophosphamide

INTRODUCTION

Millimeter electromagnetic waves (MMWs) are being widely used for the treatment of many diseases in Russia and other East European countries [Betskii, 1993; Rojavin and Ziskin, 1998; Pakhomov and Murphy, 2000; Pletnev, 2000]. The three most common frequencies employed are 42.2, 53.6, and 61.2 GHz. Excellent clinical results have been reported in the treatment of various diseases, including peptic ulcers, bronchial asthma, infantile cerebral palsy, skin disorders, chronic alcoholism, diabetic angioneuropathies, and cancer. Based upon some estimates, there are more than 1000 medical facilities in Russia alone where millimeter wave therapy (MMWT) is being used, and the number of patients undergoing medical treatment exceeds 250 000 per year [reviewed by Betskii, 1993]. MMWs can be used as a monotherapy or in combination with other treatment methods. As an adjunct therapy, they are being widely used in the former Soviet Union to reduce the toxic effects of chemo- and radiation therapy

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in the treatment of cancer [for review, see Pletnev, 2000]. However, the molecular mechanisms involved in the combined MMW-chemo therapy are not well understood.

In a previous study in BALB/c mice we have shown that MMWs (42.2 GHz) can protect T cell functions from the toxicity of cyclophosphamide (CPA), a commonly used anticancer drug [Makar et al., 2003]. MMW irradiation restored the activation of $CD4^+$ T cells suppressed by CPA and enhanced IFN-γ production by these cells. More importantly, MMW exposure specifically shifted the immune response in the direction of Th1 type pathway, which plays an important role in the inhibition of tumor angiogenesis [Beatty and Paterson, 2001]. The present study was undertaken to investigate the effect of MMW irradiation (42.2 GHz) on natural killer (NK) cell functions of mice following CPA treatment.

NK cells are known to kill a wide variety of tumor cells while sparing normal cells [Smyth et al., 2002; Colucci et al., 2003]. NK cells represent a distinct population of lymphocytes in terms of both phenotype and function [Trinchieri, 1989]. They have large granular lymphocyte morphology and express characteristic cell surface receptors, such as the NK cell receptor protein-1 (termed NK1.1) [Trinchieri, 1989; Chambers and Brissette-Storkus, 1995]. NK cells are divided into two major populations, namely NK cells and NKT cells. NKT cells represent a minor T cell subset that possess phenotypic characteristics shared by conventional NK cells and T cells and express NK cell marker NK1.1, as well as TCR/CD3 complex [Sharif et al., 2002]. NK cells mediate resistance to viral infections and cancer development in vivo and exhibit cytotoxic activity, which is nonrestricted to MHC (major histocompatibility complex) [Trinchieri, 1989; Vujanovic et al., 1996; Biron, 1997]. Thus, NK cells represent major effector cells of innate immunity. In addition, NK cells possess a variety of other functions, including the ability to produce a number of cytokines, involved in the modulation of hematopoiesis, immune responses, and in the regulation of their own activities [Trinchieri, 1989; Djeu, 1992; Yu et al., 1992; Vitolo et al., 1993].

Based on flow cytometry of NK cells, we found that CPA treatment caused a marked enhancement in NK cell activation in terms of CD69 expression, as measured 2, 5, and 7 days following the drug administration. The level of CD69 expression, which represents a functional triggering molecule on activated NK cells, was increased in the CPA group as compared to the Nave group at all the specified time points. However, the most enhancement in CD69 expression was observed on day 7. A significant increase in TNF-α level was also observed on day 7 following CPA treatment. On the other hand, CPA caused a suppression of the cytolytic activity of NK cells. MMW irradiation of the CPA treated groups resulted in further enhancement of CD69 expression on NK cells as well as in production of TNF-α. Moreover, MMW irradiation restored CPA induced suppression of the cytolytic activity of NK cells. Our results show that MMW irradiation at 42.2 GHz can modulate CPA effect on NK Cell functions.

MATERIALS AND METHODS

Animals

Male BALB/c mice (20–25 g) were obtained from Taconic Co. (Germantown, NY). 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 American Association of Accreditation of Laboratory Animal Care. All animals were given Purina Chow and tap water ad libitum.

Millimeter Wave (MMW) Irradiation

MMWs were produced with a YAV-1 generator (Istok, Fryazino, Russia), a device commonly used in medical applications in the former Soviet Union. The device produced 42.2 ± 0.2 GHz (7.1 mm) modulated (60 Hz) radiation through a 10×20 mm rectangular output horn. The power output (31.5 mW) was measured using an Anritsu power meter model ML4803A (Anritsu Corp., Tokyo, Japan). The specific absorption rate (SAR) and distribution of absorbed energy was determined thermographically using an infrared camera (model AE-4256, Amber Engineering, Goleta, CA), as described in an earlier paper [Logani et al., 1999]. Peak SAR at the skin surface and peak incident power density were measured as 622 ± 100 W/kg and 31 ± 5 mW/cm^2, respectively. The maximum temperature elevation, measured at the end of 30 min, was 1 °C.

For irradiation, the animals were restrained in plastic tubes (3.5 cm diameter). An 11 mm hole was made in the front end of each tube and the restrained mouse was positioned in such a manner that its nose stuck out of the front hole (Fig. 1). The mice were irradiated on the nasal area as reported earlier [Rojavin et al., 1997; Radzievsky et al., 2000]. The nasal area was chosen for irradiation for the following reasons: (a) Since the absorption depth of MMWs in skin is less than 1 mm [Gandhi, 1983; Furia et al., 1986], the hair on other parts of the body may prevent sufficient penetration of MMWs into the skin. (b) Shaving or application of depilatories may cause local irritation of the skin and thus may affect the results. (c) The nasal skin of mice is

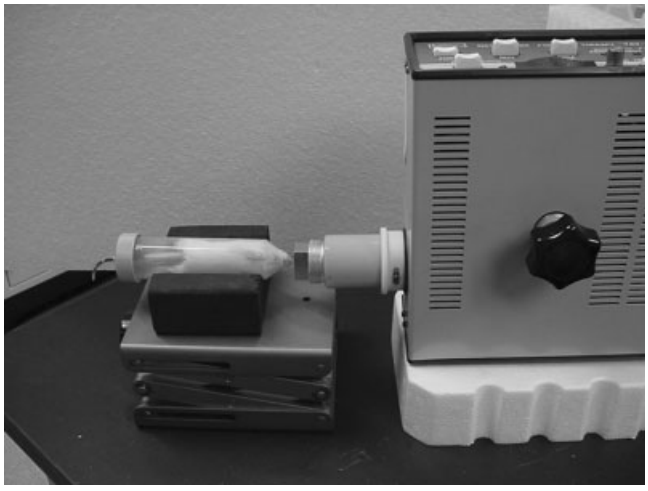

Fig. 1. Experimental apparatus for irradiation of mice. For irradiation the animals were restrained in plastic tubes (3.5 cm diameter) with a hole in the front. Each mouse was positioned in such a way that its nose was placed 5 mm from the horn of the Russian-made YAV-1 MMW generator.

naturally hairless and is particularly rich with neurons, which are hypothesized to be potential primary targets and have been reported to react to MMW exposure with low powers of 10–30 mW/cm^2 [Alekseev et al., 1997]. Furthermore, in a recent study on the hypoalgesic effect of MMWs, the nasal area was found to be more effective in comparison to other sites such as back or paw of mice [Radzievsky et al., 2000]. In order to focus energy in the nasal area, the central part of the horn of the YAV machine was kept at a distance of 5 mm from the nose during irradiation.

Four groups of animals, six mice per group, were used. 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 each day. The third group (CPA + Sham) served as a sham control; these animals were treated with CPA on day 2 and positioned in front of the YAV machines in a similar manner as the second group but the machines were not energized. The fourth group (CPA) was treated with CPA but not subjected to any restraint like the second and third groups. Animals were sacrificed by cervical dislocation 2, 5, and 7 days after CPA administration, and the spleens were removed aseptically. All the irradiation procedures were performed in a blinded manner.

Reagents

CPA and 3-(4-5-dimethylthiozol-2-yl) 2-5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Ficoll-Plaque™ PLUS for splenocyte isolation was obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). For purification of NK cell subset we used RosetteSep™ (StemCell Technologies, Vancouver, Canada). All antibodies used for flow cytometry were purchased from PharMingen (San Diego, CA). 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). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). For NK cell activation studies, lipopolysaccharide (LPS) from *Escherichia coli* and polyinosinic-poly cytidylic [poly(I:C)] were purchased from Sigma.

Preparation of Spleen Cells

Mice were sacrificed 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, 5×10^5 cells/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. LPS (serotype 0127:B8) (10 μg/ml) was used for NK cells stimulation in order to mimick the physiologic stimulation in vitro and to enhance the immune response. After culturing, cells were transferred to tubes, and two-step washing was performed.

Purification of NK Cells

After isolation of lymphocytes by density centrifugation, the NK cell subset was purified by negative selection using SpinSep from StemCell Technologies. In this technique, a cocktail of specific monoclonal antibodies to all other splenocytes except to the NK markers was added directly to a cell suspension. After 30 min of incubation on ice, dense microparticles that selectively bind unwanted cells were added. After 20 min of incubation at 4 °C, the cell suspension was centrifuged over the SpinSep DM-Murine density gradient medium for 10 min at $1200 \times g$ at room temperature. The enriched cells were collected, washed, counted, and resuspended in culture medium (RPMI + 5% FBS, HEPES, 2 mM glutamine, and Pen/Strep) at 10^6 cells/ml. The purity of NK enriched cells usually was 72%.

Cytotoxicity Test

NK cell cytotoxicity was determined by the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). CytoTox 96 Assay is a colorimetric alternative to ^{51}Cr release cytotoxicity assay [Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988]. The CytoTox Assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. The murine YAC-1 fibroblast cells were used as target cells. This cell line is sensitive to the cytotoxic activity of naturally occurring NK cells in mice [Hay et al., 1992]. Briefly, the target cells (10000 cells/well in complete RPMI media) were incubated with effector cells at four different E:T ratios in U-bottom 96 well plates (Corning Costar, Corning, NY). After 4 h incubation at 37 °C, 50 μl of supernatants was removed and transferred to an enzymatic assay plate. Released LDH in the supernatant was measured using a coupled enzymatic assay, which results in the conversion of a tetrazolium salt into a red formazan product. The amount of color formed is proportional to the number of lysed cells and was measured by an ELISA reader (VERSAmax™ Tunable Microplate Reader, Molecular Devices Corporation) at 490 nm. Spontaneous release and maximum release were determined by incubating target cells without effector cells in the medium alone or in 5% Triton X-10, respectively. The assay was performed in quadruplicate. The percentage of specific lysis was determined according to the formula:

$$\%\ \text{specific lysis} = \frac{\text{experimental - effector spontaneous - target spontaneous}}{\text{target maximum - target spontaneous}} \times 100$$

Flow Cytometry of NK Cells

Freshly isolated splenocytes 5×10^5 cells/0.5 ml/well were incubated for 24 h in 24 well tissue culture plates at 37 °C in humidified atmosphere air containing 5% CO_2. LPS (Sigma) (10 μg/ml) was used for NK cells stimulation. After culturing, cells were transferred to tubes, and two-step washing was performed.

To define NK subsets, splenocytes were stained with fluorescein isothianate (FITC)-conjugated rat anti-mouse CD49b/Pan-NK cells monoclonal antibody (Clone DX5) [Arase et al., 2001], R-phycoerythrin (R-PE) conjugated rat anti-mouse CD3 monoclonal antibody, and PerCP-CY5.5-conjugated hamster anti-mouse CD69 (very early activation antigen) monoclonal antibody. $CD3^-$ $DX5^+$ cells were considered as NK and assessed for CD69 expression, the earliest activation marker on NK cells. The samples were analyzed on a FACSCalibur instrument (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson). A total of 100 000 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.

As a positive control for NK activation study we used poly(I:C) injection. Poly(I:C) is a known activator of NK cells. The animals were treated with poly(I:C) (10 μg/mouse, intraperitoneally in a volume of 0.5 ml of saline solution) and NK cell activity in splenocytes was determined 48 h after administration.

Cytokine Measurement

After 24 h incubation, the supernatants from 0.5×10^6 purified NK cells were collected and TNF-α ELISA was performed using a standard kit from PharMingen. The PharMingen OptEIA 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. The same kind of test was used for IFN-γ measurement.

Statistical Analysis

Statistical analysis was performed using the Student *t*-test or the nonparametric Wilcoxon signed-rank test for paired samples comparison [Larsen and Marx, 1990]. P values < 0.05 were considered significant for all tests.

RESULTS

CD69 Expression in $CD3^-DX5^+$ NK Cells

To study the effect of CPA on NK cells activation male BALB/c mice were given a single injection of CPA

(100 mg/kg body weight) intraperitoneally. Then, 2, 5, and 7 days after the injection, mice were sacrificed and spleens cells were isolated. After 24 h culture with or without LPS, a nonspecific stimulator of NK cells, three-color flow cytometry was performed.

To analyze subsets of NK cells, spleen cells were labeled with DX5 mAb, a novel NK phenotype marker for BALB/c mice [Arase et al., 2001] as well as with CD3 mAb for distinguishing NK cells from NKT cells. $DX5^{+}CD3^{-}$ cells were quantified by flow cytometry for CD69 expression. CD69 is expressed shortly after activation on NK cells and therefore is a useful determinant in NK cell analysis. Flow cytometric analysis demonstrated strong up-regulation of CD69 expression on NK cells in CPA treated mice as compared to Nave control (Fig. 2). The most up-regulation of CD69 expression was observed on day 7 following CPA injection ($192.8 \pm 13.6\%$ as compared to Nave control level which was considered as 100%).

The foregoing results show that CPA treatment of mice leads to up-regulation of CD69 activation marker expression in NK cells at the specified time points. These results are based on flow cytometry data of four independent experiments. As a positive control, we also treated mice with poly(I:C), which is known to induce strong CD69 up-regulation in multiple cell types [Dow et al., 1999].

CPA Induced Activation of NK Cells

Since it has been demonstrated that MMW irradiation posseses immune stimulatory properties, we examined the combined effect of MW and CPA on NK cell activation. As shown in Figure 3A, MMW significantly enhanced CD69 activation marker expression in NK cells on day 7 following CPA treatment ($222 \pm 20\%$) as compared to the Sham group ($198 \pm 16\%$) after LPS stimulation. No differences were observed on 2nd or 5th day following CPA injection. These data demonstrate that MMWs potentiate the effect of CPA on NK cell activation. Interestingly, the MMW exposure by itself did not cause any changes in CD69 expression at the time points tested (data not shown).

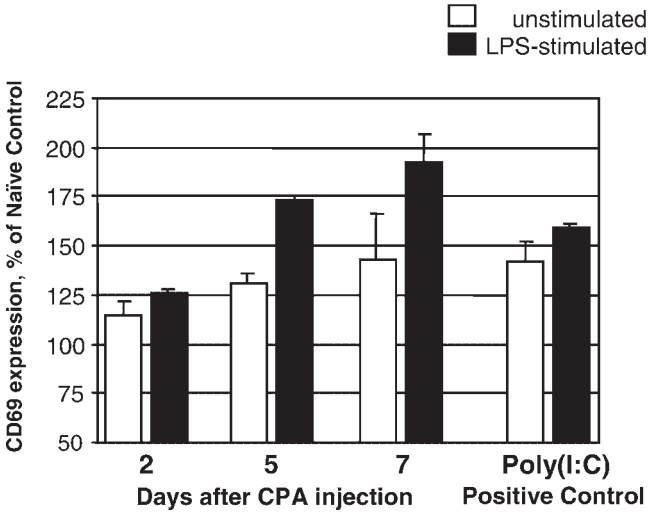

Fig. 2. Up-regulation of CD69 expression: 2, 5, and 7 days after CPA injection. The values reported are the mean ± SE of three separate experiments for each time point (three mice per group). As a positive control, mice were injected intraperitoneally with 10 μg poly(I:C). The CPA treated group at each time point showed a significant difference ($P < 0.05$) from the Naïve control group.

CPA Induced TNF-α Production

It is known that CD69 expression has a particular significance in NK cell functions. Once expressed on cell membrane, CD69 acts as a co-stimulatory molecule leading to cytokine secretion and/or cytotoxicity. Among the cytokines produced by NK cells, TNF-α and IFN-γ are thought to be particularly important. Therefore, we next examined the release of TNF-α and IFN-γ in purified NK cells. The enriched NK cell population contained >70% NK cells and <0.5% CD8+ T cells as determined by flow cytometry (data not shown). The purified NK cells were cultured for 24 h and then release of TNF-α and IFN-γ was quantified by ELISA.

As shown in Figure 4, CPA treatment significantly enhanced TNF-α production in purified NK cells as measured on days 5 and 7-post injection. MMW exposure further upregulated the level of TNF-α secretion by NK cells. The level of TNF-α on day 5 in the CPA+MMW group (0.57 ± 0.04 ng/ml) was significantly higher as compared to CPA+Sham group (0.32 ± 0.04 ng/ml). More pronounced effect was observed on day 7 post injection. MMW irradiation up regulated the level of TNF-α by more than twofold (2.68 ± 0.22 ng/ml) as compared to Sham group (1.26 ± 0.08 ng/ml). The data presented above clearly show that MMW irradiation potentiates the effect of CPA on TNF-α production by NK cells. Conversely, no significant level of IFN-γ was detected in CPA or CPA+MMW groups at the time points tested.

Combined Effect of MMWs and CPA on NK Cell Cytotoxicity

To determine the NK cytotoxic activity, spleen cells were harvested from the mice at the specified time points after CPA injection and were examined for the cytolytic activity by Non-Radioactive CytoTox 96 Assay. YAC-1 transformed cells, which are highly sensitive to mouse NK cells, were used as target cells. As shown in Figure 5, CPA treated group showed significantly less specific lysis on day 2 ($19 \pm 1\%$) and

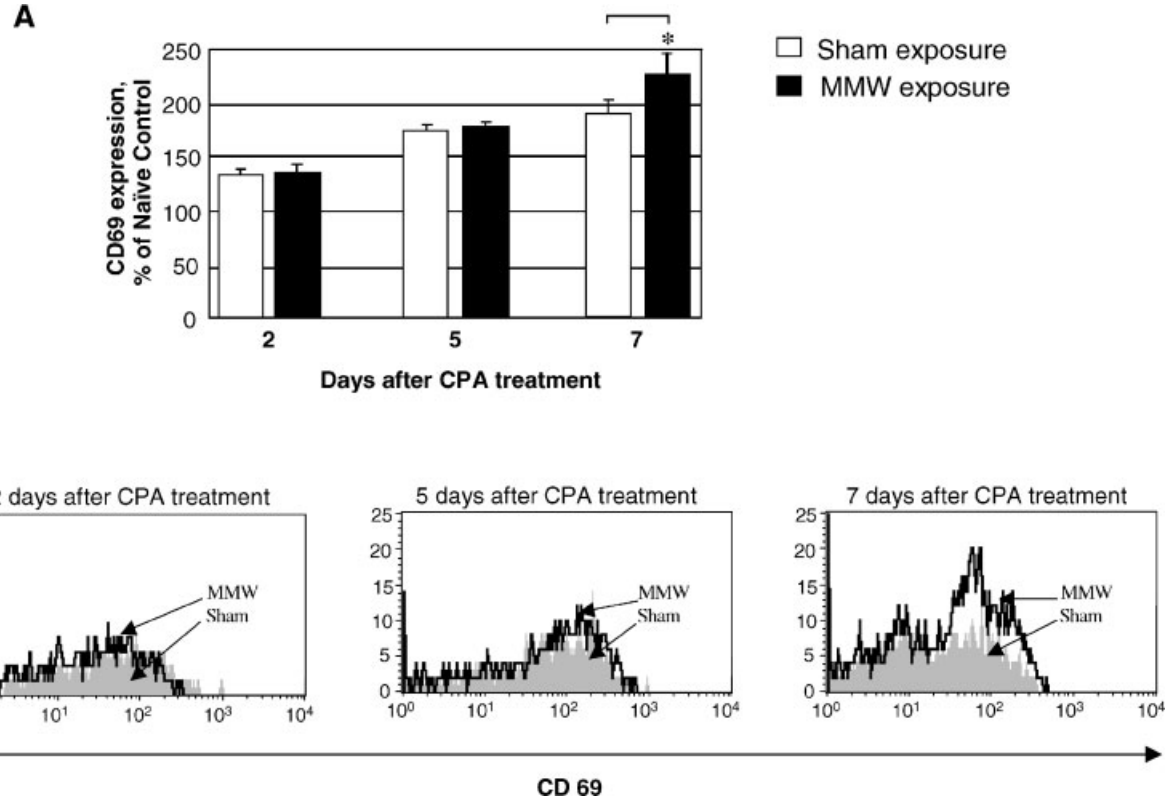

Fig. 3. Effect of MMWs on CD69 activation marker expression in NK cells 2, 5, and 7 days after CPA injection. **A**: Pooled flow cytometry data showing percentage of CD69 expression on $CD3^{-}DX5^{+}$ gated spleen cells. Asterisk indicates the group significantly different ($P < 0.05$) from Sham groups ($n = 8$). Bars depict mean $\pm$ SE. **B**: Representative flow cytometry data from one separate experiment. The histograms were generated by gating on $CD3^{-}DX5^{+}$ and display fluorescence intensity (log) of CD69 expression.

day 5 ($15.6 \pm 0.4\%$) as compared to Nave control ($34 \pm 1\%$ and $38 \pm 2\%$ on days 2 and 5, respectively). The cytolytic activity almost returned to control level on day 7. When the CPA treated mice were also irradiated with MMWs, a significant restoration of NK cell activity was observed on days 5 and 7. It was interesting to note that on day 7 MMW + CPA group showed even higher activity than Nave control. These results demonstrate that MMW irradiation accelerates recovery of NK cell activity after suppression with CPA.

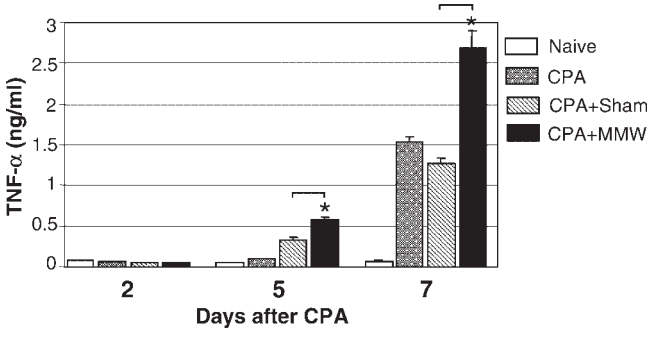

Fig. 4. Pooled ELISA data showing effect of MMW on TNF-α secretion by purified NK cells (0.2×10^6/0.5 ml/well) from mice spleen 2, 5, and 7 days post injection. Asterisk indicates the group statistically different ($P < .05$) from the CPA and CPA + Sham groups ($n = 8$). Bars depict mean $\pm$ SE.

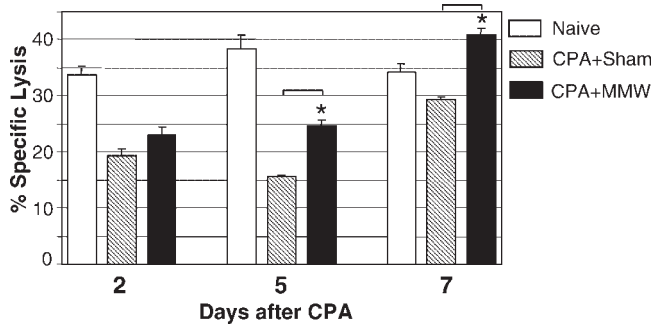

Fig. 5. Effect of MMWs on NK cell cytotoxicity. Cytotoxic activity of splenocytes was tested against YAC-1 cell line at the different effector:target (E:T) cell ratios. Representative data at the E:T ratio of 100:1 is shown. A similar pattern was seen at 50:1 and 25:1 (not shown). The data are presented as the mean percent of cytotoxicity $\pm$ SE (quadruplicate measurement for each time points). Similar results were obtained in four independent experiments. Asterisk indicates the group statistically different ($P < 0.05$) from the CPA + Sham groups.

DISCUSSION

Immunomodulatory effects of MMWs have been described previously. It was shown that MMWs increased delayed-type sensitivity reaction [Logani et al., 1999], phagocytic activity of macrophages [Khomenko et al., 1995; Rojavin et al., 1997], enhanced proliferation, 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, MMWT has been used in combination with chemo- and radiotherapy to increase immunity and to reduce the toxic effects of above therapies [Sevastyanova, 1979; Kabisov, 1992, 1997; Pletnev, 2000].

How MMWs protect the immune system in chemotherapy remains to be understood. We believe that MMWs restore immune functions by normalizing cytokine profiles modulated by chemotherapeutic drugs. In support of our hypothesis, we have shown previously that MMWs can restore T cell functions after suppression with CPA. It was further demonstrated that MMW irradiation facilitated T cell recovery by the up-regulation of pro-inflammatory cytokine production such as IFN-γ and TNF-α [Makar et al., 2003].

The present study was designed to investigate the effect of MMW irradiation (42.2 GHz) on activation and effector functions (cytokine secretion and cytotoxicity) of NK cells of mice after CPA treatment. We found that a moderate dose (100 mg/kg) of CPA caused multiple effects on NK cell functions. On one hand, CPA induced strong up-regulation of CD69 expression and TNF-α production; but on the other hand, it down regulated the cytolytic activity of NK cells (Fig. 2). Our results suggest that enhancement in CD69 expression and TNF-α level may not necessarily lead to increase in cytotoxicity of NK cells.

In recent years, mechanisms of NK cytotoxicity have been studied extensively. NK cells have the ability to destroy tumor cells by two main cytotoxic pathways, the well-known perforin/granzyme-mediated killing and the newly defined perforin-independent/TNF family ligand-mediated apoptotic killing, via FasL, TRAIL, and TNF-α pathways [Vujanovic et al., 1996]. It has been previously shown that perforin is a key mediator of cytolysis in YAC-1 lymphoma cells [van den Broek et al., 1995], which were used as target cells in our experiment. Perhaps CPA inhibits formation of IL-2 that is a potent inducer of perforin and granzymes [Smyth et al., 1990; Salcedo et al., 1993; Zhang et al., 1999].

Although cytotoxicity is the main function of NK cells, the evidence accumulated in the past few years has indicated that NK cells play an important role in regulating immune responses and homeostasis via release of cytokines. The cytokines produced by NK cells, TNF-α in particular, affect functions of other cell types, affect their survival, or mediate cytotoxic functions [Ostensen et al., 1987; Beutler and Cerami, 1988; Vilcek and Lee, 1991]. It was demonstrated that TNF-α stimulate macrophage cytotoxicity and augment humoral and cellular immune responses in mice [Talmadge et al., 1988]. It has also been shown that TNF-α, is capable of increasing Fas expression in certain solid tumor, potentially sensitizing tumor cells to Fas/mediated lysis, thus limiting the growth and metastases of these tumors [Sayers et al., 1998]. In the present study, we have demonstrated that MMW significantly augment TNF-α production by NK cells after CPA administration. This observation supports the idea that the immunomodulating effect of MMW irradiation could be beneficial when used in combination with chemotherapy.

In addition to TNF-α, several other cytokines have been implicated in the induction and regulation of NK cytotoxicity. For example IFN-γ, particularly in combination with TNF-α, is known to be a potent inducer of NK cytotoxicity [Currier and Miller, 2001]. Interestingly, we were unable to detect any changes in the level of IFN-γ secretion in CPA treated animals, whereas poly(I:C), which is a known inducer of IFN-γ [Dalton et al., 1993; Lee et al., 2000] up-regulated the level of this cytokine. One explanation is that NK cells produce IFN-γ under the physiologic condition where other co stimulatory signals are provided. For instance, it was shown that the production of IFN-γ is regulated by variety of other cytokines, and IL-12, IL-15, and IL-18 are the most characterized positive regulators [Lee et al., 1998; Salkowski et al., 2000; Nguyen et al., 2002]. It has been reported that NK cells become potent producers of IFN-γ only when other co stimulatory cytokines such as TNF-α are produced together with IL-12 [Wherry et al., 1991].

Our results show that MMW irradiation of CPA treated groups resulted in: (1) potentiation of CD69 expression and TNF-α release induced by CPA and (2) restoration of cytolytic activity suppressed by CPA. CPA is a widely used anticancer drug. The mechanisms of its action have been studied extensively but still are not fully understood. The primary mechanism involves alkylating of DNA resulting in the formation of cross-links in DNA, ultimately leading to cell death. The principal activation occurs in the liver and is mediated through hydroxylation by cytochrome P450 enzymes. It is possible that MMWs may interfere with the activation

of CPA or alkylation of DNA. However, we believe that MMWs accelerate the recovery of NK cells through stimulation of proinflammatory cytokines as is evident by increased production of TNF-α in CPA + MMW irradiated groups as compared to Sham group (Fig. 4).

It is important to understand whether the observed effects are specifically induced by MMWs or simply due to subtle warming of the nasal area. The maximum temperature elevation measured at the skin surface in our experiments was 1 °C. It is therefore, unlikely that thermal mechanisms play an important role under our experimental conditions. However, the formation of localized hot spots by MW absorption in skin has been reported [Khizhnyak and Ziskin, 1994]. The temperature in these spots may rise several degrees and therefore the participation of thermal effect in our studies cannot be completely ruled out.

Based on previous results, we believe that biological effects of MMWs (penetration depth less than 1 mm in skin) are initiated by activation of free nerve endings in the skin [Alekseev et al., 1997; Pakhomov et al., 1997, 1998, 2000; Alekseev and Ziskin, 1999, 2003]. Then the signal is conveyed to the central neural 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, 1997; Rojavin and Ziskin, 1998; Radzievsky et al., 2000, 2001]. Evidence for the involvement of the endogenous opioids in the regulation of immune functions is continuing to accumulate. The target of the effect of endogenous opioids could be NK cells [Boyadjieva et al., 2001], cytolytic functions of cytotoxic T cells, and the balance of Th1 and Th2 type cytokines [Besedovsky and del Rey, 1996; Sacerdote et al., 2000]. The involvement of endogenous opioids in modulation of NK cell functions by MMWs is under further investigation.

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