Microbial Growth Inhibition by Alternating Electric Fields in Mice with Pseudomonas aeruginosa Lung Infection

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High-frequency, low-intensity electric fields generated by insulated electrodes have previously been shown to inhibit bacterial growth in vitro. In the present study, we tested the effect of these antimicrobial fields (AMFields) on the development of lung infection caused by Pseudomonas aeruginosa in mice. We demonstrate that AMFields (10 MHz) significantly inhibit bacterial growth in vivo, both as a stand-alone treatment and in combination with ceftazidime. In addition, we show that peripheral (skin) heating of about 2°C can contribute to bacterial growth inhibition in the lungs of mice. We suggest that the combination of alternating electric fields, together with the heat produced during their application, may serve as a novel antibacterial treatment modality.

The 20th century was the golden era of the antibacterial agents, with millions of people owing their lives to the discovery of and treatment with the numerous antibiotic families used today. Surely, antibacterial agents will continue to play a major role in the battle against pathogenic bacteria in the 21st century; however, the extensive use of antibiotics holds a threat of a far less optimistic future due to the rapid rise of multidrug-resistant bacteria. Recently, the potential use of physical means as an aid to antibiotics in the battle against bacterial pathogens has been studied: photodynamic therapy (12, 21, 35), ultrasound wave therapy (7, 23, 25), thermotherapy (26), and weak electric currents (4, 6, 24, 32, 33) are all being tested as treatment modalities against pathogenic microorganisms. The major drawback of the methods mentioned above is their low therapeutic index due to the high levels of heating produced by ultrasonic waves, thermotherapy, and photodynamic therapy (36) and the activated oxygen generated by photodynamic therapy, both of which can damage the tissues in and around the target area (22). In addition, the use of conductive electrodes for the generation of electric currents is associated with the release of metal ions and free radicals at the electrode surface, all of which are toxic to living cells (18). Indeed, as of today, none of the above-mentioned means has matured into a treatment realistic.

In view of the above-described factors, the objective of the present study was to test the feasibility of using AMFields generated by insulated electrodes for the inhibition of P. aeruginosa proliferation in the infected lungs of mice.

MATERIALS AND METHODS

Reagents. Unless stated otherwise, all reagents were purchased from Sigma (Israel). Dehydrated culture medium was purchased from Difco (Difco Laboratories, Detroit, MI).

Test strains and growth conditions. Pseudomonas aeruginosa strain PAO1 was a generous gift from Shiri Navon-Venezia (Division of Infectious Diseases, Sourasky Medical Center, Tel Aviv, Israel). Bacteria were grown in LB medium (1.0% Bacto tryptone, 0.5% yeast extract, 1.0% NaCl [Frutarom, Haifa, Israel]). Broth cultures of freshly plated bacteria were grown in 250 ml of liquid medium at 37°C in an orbital shaker at 220 rpm (New Brunswick Scientific, NJ) up to logarithmic phase, centrifuged, and resuspended in saline containing 15% glycerol. Stocks were divided into aliquots of 50 μl each containing 2.17 × 10^7 CFU/ml and kept at −70°C until use.

Experimental animals. All experiments were conducted on 7- to 8-week-old (body weight, 26 to 35 g) ICR female mice obtained from Harlan Laboratories (Israel). Mice were housed under standard conditions of light and temperature and were fed standard laboratory chow and water ad libitum. The experiments were approved by the NovoCure Internal Animal Care and Use Committee in accordance with the Technion-Israel Institute of Technology guidelines for the care of laboratory animals.

AMFields generation system. AMFields were generated by 2 pairs of parallel, insulated electrodes, 15 mm long and 3 mm high (Fig. 1A) (8). Each electrode contained a thermistor placed within the epoxy directly on top of the ceramic in order to allow for constant monitoring of the electrode’s temperature (Fig. 1A).
In order to allow for AMFields generation in mice while maintaining the strict isolation required when working with animals infected with pathogens, the mice were held in an individually ventilated cage system (IVC) (TouchSLIM; Techniplast, Italy) that was modified as follows: each cage (GR900; Techniplast, Italy) was split by a polycarbonate partition into two units, each housing one mouse (Fig. 1B). Each mouse was treated with a separate AMFields generator consisting of a radiofrequency (RF) amplifier (75A250; AR Worldwide, Souderton, PA) activated by a sine wave generator (model 662; OR-X, Israel) (Fig. 1C). Six hermetically sealed, shielded electric connection sockets (SM3416 BNC; S.M. electronics, TX) were positioned at the front panel of the cage. Each socket allowed for the connection (by means of coaxial cables) of a connection box placed inside the cage to an exterior device—an RF amplifier or a temperature measuring system. The connection box served for transferring the electric currents to the mouse electrodes and the mouse temperature readout to the computer.

The entire IVC system, together with the AMFields generating system, was placed inside a Faraday cage in order to meet the guidelines for limiting personnel exposure to time-varying electric, magnetic, and electromagnetic fields (International Non-Ionizing Radiation Committee [INIRC]). To avoid field interference with temperature measurements, temperatures were measured periodically while the field was briefly turned off. The skin temperature of both control groups was constantly monitored as described above for the AMFields electrodes.

Two control groups were set in each experiment: a sham heat control and an untreated control (referred to as “control”). Control electrodes identical in size and shape to the AMFields electrodes were placed on mice of the two control groups. The sham heat electrodes produced temperature changes equal to those produced by the field electrodes by means of a heating resistor incorporated within them. Except for the differences in the electrodes, the control mice and the sham heat-treated mice were held in conditions identical to those of the AMFields-treated mice (Fig. 1C). The skin temperature of both control groups was constantly monitored as described above for the AMFields electrodes.

The electric field intensity was measured using a shielded coaxial cable and probe having two exposed tips positioned at a distance of 1 cm from each other. The probe was connected to a floating input oscilloscope (190B; Fluke, Netherlands). Field intensities within lungs were measured by making a 5-mm-long incision in the thoracic skin of an anesthetized mouse and inserting the measurement probe at an intercostal space to a depth of 5 mm into the thorax. The probe was oriented such that the line connecting the two measuring points was parallel to the lines of the electric field. Field intensities are expressed as peak-to-peak volts per centimeter of distance (V/cm).

**Development of P. aeruginosa pulmonary infection in ICR mice.** The pulmonary infection model of Takeda et al. (31) was used with minor modifications: mice were rendered neutropenic (neutrophil counts, <100/mm³), as described in Andes and Craig (2), by two intraperitoneal injections of cyclophosphamide, one four days before infection (150 mg/kg of body weight) and an additional injection one day before infection (100 mg/kg of body weight). On the day of infection, immediately before use, frozen bacterial cultures were thawed and diluted in saline to the desired concentration. Anesthetized ICR mice were challenged intranasally with 20 μl of saline containing various concentrations (1 × 10² to 1 × 10⁴ CFU/ml) of *P. aeruginosa* strain PA01. Mice were sacrificed at 0, 3, 6, 9, 24, and 48 h after infection in order to determine the CFU counts in their lungs. After skin disinfection with 70% alcohol, lungs from sacrificed mice were re-
moved aseptically, photographed, and weighed, and their CFU contents were determined.

Application of AMFields to mice with *P. aeruginosa* pulmonary infection. The application of AMFields to the mouse torso was carried out as follows. Four days before infection, the torso of the anesthetized mouse was shaved with electrical clippers and depilated using cold wax. On the day of infection, four insulated electrodes were placed around the mouse torso (Fig. 1D) so as to generate fields of two perpendicular directions through the lungs. Mice were placed inside the IVC cages, and the electrodes were connected to the connection box. The electrode wires were encapsulated within silicon cable protector and tied to the cage top in order to protect the wires from being damaged by the mice. Treatment with AMFields began 3 h after infection and was maintained for 48 h with 2 brief stops daily for monitoring the physical condition of the mice. After 48 h of treatment, the mice were sacrificed and the lungs were removed aseptically.

Combined treatment of *P. aeruginosa* pulmonary infection by AMFields and ceftazidime. The combined effect of AMFields and antibiotics was tested using ceftazidime, an agent recommended by the Infectious Diseases Society of America for the treatment of hospital-acquired pneumonia.

A preliminary experiment was conducted with the aim of determining the bacterial titer of the inoculum and the ceftazidime concentration that allow the maintenance of a persistent infection without fully eradicating it, thus allowing the combined effect of AMFields and the antibiotic to be measured.

Experiments were conducted as described above for AMFields treatment of *P. aeruginosa* pulmonary infection, with the following modifications. Mice were challenged intranasally with a predetermined bacterial titer. Ceftazidime (5 mg/kg of body weight) was administered intraperitoneally twice daily for 48 h starting 3 h after infection. Antibiotic administration was stopped at least 12 h before the termination of the experiment.

Bacterial counts and statistical analysis. Cold sterile saline was added to each lung sample tube, with the saline-to-tissue weight ratio maintained at 10:1. Tubes were placed in an ice bath, and the suspensions were homogenized using an Omni tissue homogenizer (Omni, GA) with Omni disaggregation tips. Approaches were placed in an ice bath, and the suspensions were homogenized using an Omni tissue homogenizer (Omni, GA) with Omni disaggregation tips. Approaches were placed in an ice bath, and the suspensions were homogenized using an Omni tissue homogenizer (Omni, GA) with Omni disaggregation tips. Approaches were placed in an ice bath, and the suspensions were homogenized using an Omni tissue homogenizer (Omni, GA) with Omni disaggregation tips.

Histological evaluation. Lungs were fixed and embedded in parafin wax. Histological sections, 5 μm thick, were stained with hematoxylin-eosin (HE) and examined by an independent expert. A semiquantitative grading scheme was used to evaluate the extent of the lesions in the sections as follows: minimal (grade 1) lesions involving less than 10% of the lung section; mild (grade 2), involving 11% to 40%; moderate (grade 3), involving 41% to 80%; and marked (grade 4), involving 81% to 100%.

**RESULTS**

Measurement of temperature and AMFields intensity in lungs of treated mice. In a preliminary set of experiments, field intensities and temperature measurements were done during AMFields treatment and sham heat application. The average AMFields intensity in the lungs was 12 ± 4 V/cm (mean ± standard deviation). Under these conditions, lung temperature was identical to or up to 1°C lower than the electrode surface temperatures, which were set to 37°C for both the AMFields and the sham heat control. The skin temperature of the control mice was in the range of 33 to 35°C.

Development of animal models. Pulmonary infection was induced by intranasal administration of different amounts of *P. aeruginosa* strain PAO1 to neutropenic ICR mice. Bacterial inocula of around 3.6 log CFU/mouse were found to induce a severe pulmonary infection which persisted for at least 48 h. Larger inocula resulted in a high mortality rate, while smaller ones resulted in spontaneous recovery.

Effect of AMFields on pulmonary infection caused by *P. aeruginosa*. AMFields treatment for 48 h of mice suffering from pulmonary infection resulted in a 3.4-log reduction in the CFU content of the lungs in treated mice compared to the CFU count in untreated control mice (Table 1). The sham heat control treatment led to a 2.5-log reduction compared to the CFU count in the control mice (Table 1). The average bacterial growth \[ \log(\text{CFU}_{T48}/\text{CFU}_{0}) \] in both AMFields-treated mice and sham heat-treated mice was significantly lower than in control mice (Fig. 2A). The CFU content of the AMFields-treated mice did not reach a significant level of decrease compared to the CFU count in the sham heat-treated mice, although a clear trend was seen \((P = 0.09)\). A significant reduction was observed in the weight of the lungs of AMFields-treated mice compared to the lung weights of both sham heat-treated control mice \((P = 0.02)\) and untreated control mice \((P < 0.001)\) (Fig. 2B). Representative photos of lungs from uninfected, AMFields-treated, and control group mice are shown in Fig. 2C, D, and E, respectively. Histopathological examination of the lung samples from AMFields-treated mice graded them as normal to grade 1 with focal acute inflammation. Lungs from both control mice and sham heat-treated mice were graded as grade 2 to 3, associated with acute necrotizing pneumonia and the presence of numerous bacterial colonies (Fig. 2F, G, and H).

Combined treatment with AMFields and ceftazidime of pulmonary infection caused by *P. aeruginosa*. The inoculum and ceftazidime concentration were determined experimentally so as to establish a persistent infection throughout the experiment without leading to a complete recovery or a significant death rate in the presence of the antibiotic. The inoculum that was found to induce a persistent pulmonary infection in the presence of ceftazidime (5 mg/kg of body weight twice daily) alone was approximately 4.6 log CFU/mouse. The results presented in Table 1 demonstrate that combined treatment with both AMFields and ceftazidime for 48 h resulted in a 1.5-log reduc-

<table>
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<th>Treatment</th>
<th>Without ceftazidime</th>
<th>With ceftazidime</th>
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<tr>
<td></td>
<td>Log inoculum ± SD</td>
<td>Log CFUₜ₄₈ ± SD</td>
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<tr>
<td>AMFields</td>
<td>3.6 ± 0.2</td>
<td>4.1 ± 1.5</td>
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<tr>
<td>Sham heat</td>
<td>3.6 ± 0.2</td>
<td>5.0 ± 2.5</td>
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<tr>
<td>Control</td>
<td>3.6 ± 0.2</td>
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*P* values are in comparison to the results for the control.
tion in the lung CFU content compared to the CFU count in the antibiotic-treated control. The combined treatment of sham heat and ceftazidime did not result in a significant CFU count reduction compared to the CFU count in the control (Table 1). The bacterial growth \[\log(CFU_{T48}/CFU_{T0})\] in the lungs of mice treated with both AMFields and ceftazidime was significantly lower than that in the control mice \((P = 0.004)\) (Fig. 3A). The average lung weight was significantly lower in both the ceftazidime/AMFields combination treatment group \((P < 0.001)\) and the ceftazidime/sham heat combination treatment group \((P = 0.047)\) than in the control group (Fig. 3B). The CFU content of the ceftazidime/AMFields-treated mice nearly reached a significant decrease compared to that of the ceftazidime/sham heat-treated mice \((P = 0.06)\). The lung weights of the ceftazidime/AMFields-treated mice were significantly lower than those observed for the ceftazidime/sham heat-treated mice \((P < 0.05)\).

FIG. 2. Effect of AMFields on mice with \(P.\ aeruginosa\) lung infection. (A) Relative bacterial growth compared to the inoculum size after 48 h of treatment. (B) Lung weight at the end of treatment. STDEV, standard deviation. \(P\) values versus results for control: **, \(P < 0.01\); ***, \(P < 0.001\). (C to E) Representative photos of a healthy lung from an uninfected mouse (C), a lung from an AMFields-treated mouse (D), and a lung from a control mouse (E). (F to H) Microscope slides, stained with HE, prepared from a lung of an uninfected mouse (F), a lung of an AMFields-treated mouse (G), and a lung of a control mouse (H). Scale bars, 100 \(\mu\)m.

FIG. 3. Combined treatment with AMFields and ceftazidime of mice with \(P.\ aeruginosa\) lung infection. (A) Relative bacterial growth compared to the inoculum size after 48 h of treatment. (B) Lung weight at the end of treatment. STDEV, standard deviation. \(P\) values versus results for control: *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
DISCUSSION

In the present study, we show that AMFields, previously shown to be an effective in vitro tool for inhibiting bacterial pathogen proliferation (8), are also effective in vivo. Specifically, it is demonstrated that AMFields, applied using noninvasive, insulated electrodes, have a significant inhibitory effect on the growth of a pulmonary bacterial infection in mice. The inhibitory effect was observed when AMFields were applied either as a monotherapy or as an adjuvant to antibiotic therapy.

One striking outcome of the AMFields application was the fact that the number of bacteria in the lungs of AMFields-treated mice increased by only a fraction of an order of magnitude compared to the number in the inoculums used for infection, while at the same time the number of bacteria in the lungs of the control group increased by 3.8 orders of magnitude. This observation could be the consequence of either a bacteriostatic or a bactericidal effect of AMFields. Histopathological analysis demonstrated that in AMFields-treated mice, the lung morphology was normal and without any evidence of prior acute infection. Thus, it is reasonable to assume that the AMFields treatment led to cell proliferation arrest rather than to the elimination of the bacteria after they had proliferated in the lungs. Supportive evidence for this conclusion can be found in the results of the experiment in which the combined AMFields/ceftazidime treatment was tested. Ceftazidime is an expanded-spectrum cephalosporin antibiotic with a broad bactericidal activity against Gram-positive and Gram-negative bacteria. Like other beta-lactam antibiotics, ceftazidime is most effective against dividing bacteria. Indeed, ceftazidime treatment (5 mg/kg of body weight twice daily) alone resulted in a growth reduction of close to 2 logs compared to the growth in untreated lungs (1.9 versus 3.8 CFU, respectively) (Fig. 2A and 3A). Yet, the combined treatment of AMFields and ceftazidime did not lead to a reduction in bacterial growth compared to the growth with the AMFields treatment alone (0.4 versus 0.5, respectively) (Fig. 2A and 3A), nor did it significantly reduce CFU counts in the lungs compared to the inoculums (Table 1) suggesting that bacterial growth was arrested.

Previously, we demonstrated that the application of AMFields in vitro led to a 20% reduction in the number of treated bacteria compared to the number of control bacteria—considerably less than the level of inhibition seen in the present study. One possible explanation for the higher efficacy in the in vivo experiments could be the fact that the AMFields inhibitory effect is field intensity dependent (8). While the AMFields intensity in the in vitro experiments was limited to 4 V/cm in order to avoid overheating of the culture, the AMFields intensity in vivo was significantly higher (~12 V/cm). The application of AMFields of higher intensities was a consequence of active heat removal from the electrodes by the mice’s circulation.

Another possible explanation for the higher efficacy of AMFields treatment in vivo may be related to the inhibitory effect on bacterial growth seen with heat treatment alone. Though the sham heat control effect was smaller than the AMFields effect, it was still significant. Bacterial growth inhibition due to elevated temperatures may result from either a direct effect of the heat on bacterial growth or from local and systemic host responses to heating. The former hypothesis was tested in vitro by comparing the growth rate of P. aeruginosa at various temperatures within the range of normal-to-febrile temperatures. As shown previously (20), we found that the growth rate of P. aeruginosa is positively correlated with increasing temperature in the range of 34 to 38°C. Thus, we can rule out the possibility that heating was directly responsible for the observed reduction in bacterial growth. The second possibility, i.e., that local and systemic host reactions to elevated temperatures decrease bacterial growth, is not new. In fact, historically, heat and fever were used as a tool against microbial pathogens for over a century (3, 10). There are numerous reports regarding the effects of elevated host temperatures on the growth of bacterial pathogens. For example, the induction of fever after inoculation with malaria can serve as a treatment against Treponema pallidum (34). It has also been shown that febrile core temperature is essential for optimal host defense against Klebsiella pneumoniae peritonitis in mice (11) and that fever in elderly patients with community-acquired pneumonia is positively correlated with high survival rates (1). Finally, maintaining a normal core body temperature (36.6 ± 0.5°C) after anesthesia has been shown to decrease the incidence of infectious complications in patients undergoing colorectal resection (16). Among the mechanisms suggested in the antibacterial effect of heating, augmentation of the innate immune function is probably the most intensively studied (9, 10, 17, 19, 27).

Though our results demonstrate that the application of AMFields and sham heat did not cause an increase in lung temperatures, these treatments did raise the temperature of the peripheral organs of the treated mice by at least 2 degrees (the normal skin temperature of mice is 34 to 35°C, while in the present study, the electrode temperature was set to 37°C). Hanson (9) demonstrated that the response of mature primary T cells is strongly regulated by temperature changes in the range of 29 to 37°C, which corresponds to the peripheral tissue temperature elevation during febrile episodes. This finding provides a possible explanation for the inhibitory effect of the sham heat-treatment reported in the present study. Since the application of AMFields is always associated with heat production, it is reasonable to assume that the enhanced efficacy of the fields reported in the present study compared to the findings in our previous report (8) is due, at least in part, to the elevated temperatures of peripheral tissues.

Still, AMFields-treated mice demonstrated reproducible reductions in total CFU content, bacterial growth in the lungs, and lung weights compared to these parameters in sham heat-treated mice. This reduction was statistically significant for lung weights and reached nearly significant values for the other parameters, indicating that the effect could not be attributed solely to the heat generated.

Previous experiments that utilized electric currents as an in vivo treatment against microbial infections applied weak DC currents generated using conductive electrodes (5, 6, 28, 32). Such use of DC currents for the treatment of infections has the following three main limitations. (i) DC currents may stimulate nerves and muscles, causing pain and muscular contractions. (ii) DC currents can only be generated by using conductive electrodes and are therefore always associated with electrolysis, the release of metal ions, and the generation of free radicals. (iii) When relatively small electrodes are used, electric...
fields and DC currents spread in the body volume such that their density decreases with distance from the electrodes. Thus, current density of sufficient intensity can only be applied to superficial lesions unless implanted electrodes are used to reach deeper lesions. The DC current densities used in the above-mentioned reports were too low to induce nerve stimulation. However, it is reasonable to assume that treatment of deeper tissues, such as lung infections, would require much higher currents, thus subjecting the patient to the risk of nerve stimulation, electrolysis, and the formation of toxic derivatives. Note also that in some studies, the currents were generated by silver electrodes and the inhibitory effect was observed only when the silver electrode served as the anode, implying the involvement of silver ions that may potentially be toxic. Unlike the fields in the above-mentioned reports, AMFields are not associated with electrolysis, the production of free radicals, toxic metal ions, etc. due to their unique properties. Indeed, during the AMFields treatment described above, no side effects were detected in the treated mice.

The mechanisms responsible for the inhibitory effect of AMFields can be deduced from the experiments conducted by Kirson et al. (13, 14) with tumor-treating fields (TTFields). TTFields are used to inhibit the growth of cancerous cells and are similar to AMFields in all aspects, with the exception of frequency (100 to 200 kHz for TTFields versus 5 to 30 MHz for AMFields). Kirson et al. demonstrated that the inhibitory effect of TTFields is related to the formation of inhomogeneous electric fields near the bridge separating the daughter cells during cytokinesis. Such inhomogeneous fields, of any frequency in the relevant ranges, exert unidirectional dielectrophoresis forces on charged and polar particles and molecules and, thus, may result in their movement toward the furrow. We hypothesized that the AMFields inhibitory effect is the result of a similar mechanism. Modeling of the AMFields distribution and the associated electric forces inside dividing bacteria during cytokinesis, using finite element mesh simulations, has shown that the magnitude of these forces in a bacterial cell is sufficient to induce particle and macromolecule distortion and movement (8). This mechanism is very different from the ones suggested to be involved with DC current (18, 29, 30).

The results presented herein serve as a proof of concept for the use of low-intensity alternating electric fields as a treatment against pulmonary infections caused by *P. aeruginosa*. Such a treatment would involve the placement of insulated electrodes on the skin surrounding the infected organs (e.g., on the chest) and the generation of the electric fields using an AMFields generator, similar to the treatment of patients suffering from brain glioblastoma and lung cancer using a TTField generator (13–15). In the near future, we intend to test the inhibitory effect of AMFields on additional bacterial species and other in vivo models of infection in order to test the generality of the observations reported above.

REFERENCES


