EFFECT OF ELF ELECTROSTIMULATION ON FUNCTION OF MACROPHAGE

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Abstract: To clarify the effects of ELF field exposure on immune cells, the effects of ELF electrostimulation on macrophages were investigated. The endocytosis of peritoneal exudate macrophages and the free-radical production of macrophage-like cell line (J774.1) were studied *in vitro*. The capacitively coupled stimulation (0.35 A/m^2, 30Hz, sinusoidal wave-form) was applied to the sample container with insulated electrodes. As for the endocytosis, significant effect was observed in stimulated macrophages. The effect continued for at least several tens of minutes after turning off the stimulation. This suggested that the stimulated cells might secrete some substance to suppress the endocytosis. The oxidative product in J774.1 was also reduced by the electrostimulation. There is a possibility that the electrostimulation suppresses the free-radical production. The results show that ELF electrostimulation can modify the function of macrophages. Through this study, the feasibility of the immunomodulation by ELF field exposure is verified.

Key words: ELF electrostimulation, peritoneal exudate macrophages, J774.1, endocytosis, free-radical production.

1. Introduction

Effects of electric or electromagnetic fields on cells, organs, animals and human beings have been reported in the last several decades. Especially, those of extremely low frequency (ELF) electromagnetic fields, for example 50 or 60 Hz frequency, have attracted intense attention. Some studies suggest the ELF electromagnetic field exposure increase the potential health risks, such as childhood leukemia [1]. On the other hand, electromagnetic fields have been used in clinical treatment such as bone fraction rehealing [2]. In Japan, the apparatus for the electric field therapy was approved by the Japanese Ministry of Health and Welfare in 1964 [3].

We have been interested in the effects of ELF electro-stimulation on immune cells and possibility of the immunomodulation with ELF electric field. Up to now, the functional changes of macrophages and mast cells by the stimulation with 50 Hz capacitively coupled electric field have been investigated. The results showed that ELF electrostimulation can modulate the function of these cells [4-6]. In this paper, we report on the effects of ELF electro-stimulation on macrophages particulary on the phagocytosys and the free-radical production.

2. Material and methods

2.1 Macrophages and macrophage-like cell line

For endocytosis assay, peritoneal exudate macrophages were prepared. A normal mouse (Std:ddy, female, 6-11weeks) was intra-peritoneally injected with thioglycollate medium (4.05%, 2 ml). After 3 or 4 days, the cells were harvested from the mouse. The cells were isolated and washed 3 times with Hanks' solution by centrifugation (1500rpm, 5min). The cells were then suspended at a density of 10^6 - 5 x 10^6 cells/ml in RPMI-1640 medium containing 10% fetal bovine serum (FBS, INC Biomedicals).

For the assay of free-radical production, mouse macrophage-like cell line J774.1 (RIKEN) was used. The cells were cultured in RPMI-1640 with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2. In the experiment, the cells were resuspended at a density of 1 x 10^6 cells/ml in the medium.

2.2 Electrostimulation system

Figure 1 shows the structure of the sample container for ELF electrostimulation. The container was made of acrylic resin with two copper electrodes. The electrode was insulated with a thin plate of glass for capacitively coupled electric field exposure.

The outline of the electrostimulation system is shown in Fig.2. Two electrodes were connected to a high-voltage power source through a noise filter. As the power source, the power supply of a commercial apparatus for the electric field therapy was used (Healthtron , Hakuju Inst. Health Science, maximum supply voltage 10kV). A sinusoidal AC signal of 50Hz was supplied to the apparatus with a waveform generator to make the high-voltage with the sinusoidal waveform of 50Hz.

2.3 Endocytosis assay

Fluorescent latex beads (F8813, Molecular Probes) were used as the particles for ingestion. The diameter of the beads was 500 nm. The wavelengths for excitation and fluorescence were 505 nm and 515 nm, respectively. The beads suspension was diluted with the RPMI-1640 before mixing with the suspension of the cells.

In this paper, exp eriments based on three protocols are described. They are some different in the order of mixing the beads and stimulating the suspension.

(1) The suspension of the macrophages and the suspension of fluorescent latex beads were mixed into the sample container. Then the mixed sample was electrostimulated for 30min. In this experiments, the macrophages and beads were exposed simultaneously.

(2) The suspension of the macrophages was electrostimulated for 30min without the fluorescent latex beads. Then, the stimulated cell suspension was mixed with the suspension of fluorescent latex beads, and the ingestion was performed for 30min without electrostimulation. In this study, only macrophages were stimulated. During the ingestion by the macrophages, there was no electrostimulation.

(3) The suspension of the macrophages was electrostimulated for 30min without the fluorescent latex beads (same as (2)). Then, supernatant and cells were separated by centrifugation. The supernatant and intact macrophages, that were stored without electrostimulation, were mixed. The cells were allowed to ingest the beads for 30min without electrostimulation. In this experiment, the macrophages that injected the beads were not stimulated by the ELF field directly.

The current density supplied was fixed at 0.35 A/m^2. The sample container was placed in a shaking water bath at 37 °C during the experiment. After the ingestion, the cells were fixed with phosphate-buffered saline containing 1% grutaraldehyde at 4°C for 24 hours. Amount of the ingested beads in a cell was measured and analized by a flow cytometer (CTTRON HEF-100, ORTHO).

2.4 Free-radical production assay

The oxidative production in J774.1 cell line (RIKEN) was evaluated by DCFH method [7]. In this method, 2',7'-Dichlorofluorescein diacetate (Lambda Probes & Diagnostics), DCFH-DA, was used as a fluorescent probe. DCFH-DA was dissolved in Dimethyl sulfoxide (Sigma). As the stimulant, opsonized zymosan, OZ, was adopted. OZ was obtained by opsonizing the zymosan (Saccharomyces cerevisiae) with Normal Mouse Serum (Biodesign International)[8]. Opsonized zymozan was suspended in Hanks' solution (Sigma).

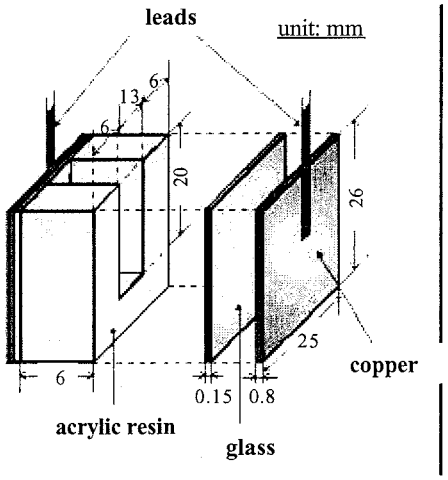

Fig.1 Sample container.

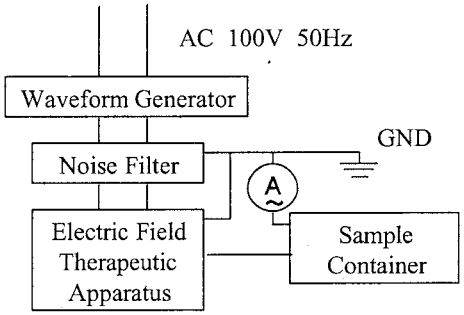

Fig.2 Schematic diagram of electrostimulation system.

J.774.1 suspension and the solution of DCFH-DA and OZ were mixed into the sample container. The solution was electrostimulated instantly. The elecrostimulation system and the condition of electrostimulation was the same as that used in the endocytosis experiments. The current density 0.35 A/m^2 was applied for 30min at 37°C in a water bath.

After the stimulation, the cells were isolated and washed 3 times with Hanks' solution by centrifugation (1500rpm, 5min, 4℃). The cells were resuspended in Hanks' solution. To break the cell membrane, the suspension was frozen with cooled ethanol (-80℃) and unfrozen. This procedure was repeated twice.

The fluorescence intensity of the suspension was measured by a fluorescence spectroscope (FP-6500, JASCO Co.). The wavelengths of excitation and fluorescent were 490nm and 515nm, respectively.

3. Results and Discussion

3.1 Results of endocytosis assay

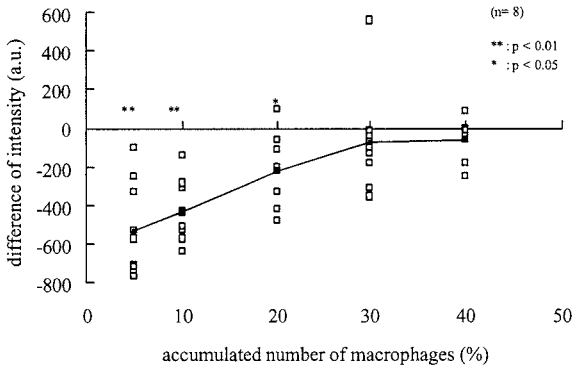

Fig.3 Effect on endocytosis when the cells and beads suspension was stimulated.

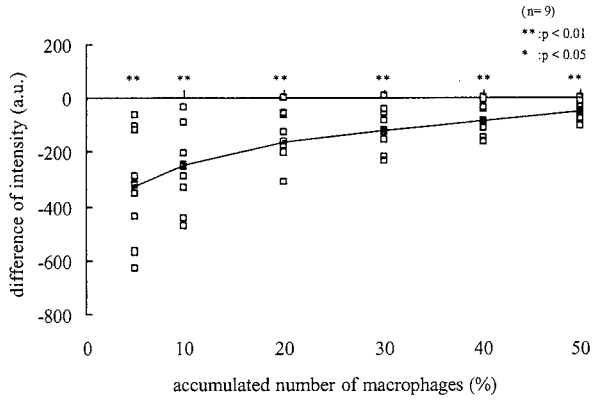

Fig.4 Effect on endocytosis when the beads were given after electrostimulation.

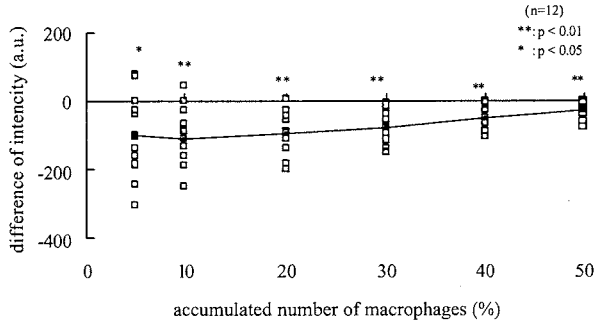

Fig.5 Effect on endocytosis with the supernatant after stimulation

In the flow cytometry, the number of the cells that have the same fluorescent intensity was obtained. The intensity represents the amount of the ingested beads in the cell. To evaluate the endo cytosis, the relation between the accumulated number of the cells and the corresponding threshold value of fluorescent intensity was analyzed. For example, if 10% rate of the cells has the threshold intensity of 100 (a.u.: arbitrary unit), it means that 10% of the total cells showed the fluorescent intensity more than 100 a.u.

Figure 3 shows the effect of electrostimulation in the experiment (1), the cells were electrostimulated during the injecting of the beads. The abscissa of the graph shows the accumulated rate of the cells. The ordinate shows the difference between the threshold intensities of the electrostimulated specimen and the sham specimen. "?" in the figure shows the result of the each specimen, and "|" shows the mean value of eight specimens at the same accumulated number of the cells. If the difference of the intensity is positive, it means that endocytosis was enhanced by the ELF electrostimulation. On the contrary, if the difference is negative, the electro-stimulation suppressed the endocytosis. The results shown in Fig.3 suggested that the given ELF electrostimulation suppressed the endocytosis of macrophages. Five, 10, and 20% of the cells showed the statistically significant reduction of the intensity based on the paired-t test.

Fig.4 shows the result of the experiment (2), the cells were electrostimulated before the ingestion of the beads. In this experiment, ELF electrostimulation significantly reduced the endocytosis of the macrophages as well as the result of experiment (1).

Based on the comparison between the experimental

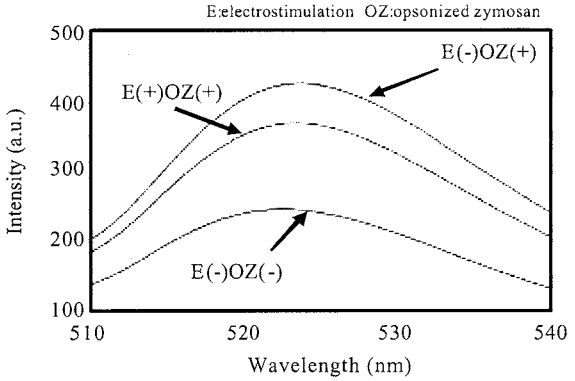

Fig.6 Example of the oxidative product measurement by fluorescence spectroscopy.

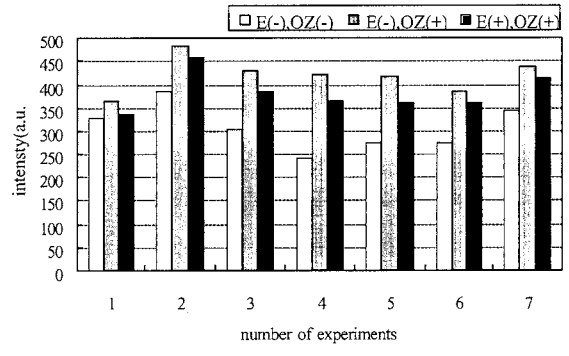

Fig.7 Effect on the oxidative product formation.

conditions of (1) and (2), it is suggested that the suppression was caused by the stimulation on the macrophages, not by the change of beads by electrostimulation. This effect continued after turning off the stimulation at least several tens of minutes.

Figure 5 shows the result of the experiment (3), the effect of the electostimulation of the supernatant. In this experiment, the macrophages that ingested the beads were not electrostimulated. The result shows that supernatant could significantly suppress the endocytosys.

In various cellular systems, including immune cells, many effects of ELF electromagnetic field have been reported, and different mechanisms have been proposed [9]. There is, however, no well-established mechanism to explain the effects. Based on our results, it has been strongly suggested that the electrostimulated cells secrete some substances that can modulate the endocytosys. The identification of the substance is the next objective of our study. The specimen contained some cells in addition to the macrophages. The identification of the cell that secrete the substance is required as well.

3.2 Results of free radical production assay

Figure 6 shows an example of the data in the fluorescence spectroscopy. The fluorescent intensity of the specimen with zymozan and with electro-stimulation (E(+)OZ(+)) was larger than that of the specimen without stimulations (E(-)OZ(-)), and was smaller than that of the sample with zymozan but without electrostimulation (E(-)OZ(+)). Figure 7 shows the result of the repeated experiments. Based on the paired-t test, there was statistically significant difference between E(+)OZ(+) and E(-)OZ(+). Although the effect of the electro-stimulation would not be enough to suppress the effect of zymozan, this suggested that electrostimu lation can suppress the production of oxidative substance, that is, the possibility of the suppression of free-radical production. The differentiation of this effect between the supernatant and the cell has not been tested yet.

It is well known that functions of macrophages play significant role in the chronic inflammation and autoimmune diseases. Especially, free-radicals cause many kinds of problems, such as arteriosclerosis, atopic dermatitis, etc. If we can suppress the functions of macrophages by field exposure, it will provide a potentially useful tool in clinical practices.

4. Conclusion

Effects of ELF electrostimulation on macrophages were investigated *in vitro*. The endocytosis of peritoneal exudate macrophages from a mouse and the free-radical production of macrophage-like cell line (J774.1) were investigated. The electro-stimulation (current density 0.35 A/m^2, 50Hz, sinusoidal wave-form) was applied to the sample container with insulated electrodes. As for endocytosis, significant differences were observed in stimulated macrophages. The effect continued for at least several tens of minutes after turning-off the stimulation. It is suggested that the stimulated cells may secrete some substances that suppress the endocytosis of the macrophages. The oxidative production in J774.1 was also reduced by the electrostimulation. It is possible that the electro-stimulation suppresses the free-radical production. Although the mechanism of the effect is still unknown, the results show that ELF electro-stimulation can modify the function of macrophages. The possibility of the immuno-modulation by ELF electric field exposure is suggested.

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