Jpn. J. Cancer Res. **89**, 775–782, July 1998

# Antitumor Immunity Induction by Intracellular Hyperthermia Using Magnetite Cationic Liposomes

Mitsugu Yanase,[1] Masashige Shinkai,[1] Hiroyuki Honda,[1] Toshihiko Wakabayashi,[2] Jun Yoshida[2] and Takeshi Kobayashi[1, 3]

*[1]Department of Biotechnology, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603 and [2]Department of Neurosurgery, School of Medicine, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550*

**Induction of antitumor immunity to T-9 rat glioma by intracellular hyperthermia using functional magnetic particles was investigated. Magnetite cationic liposomes (MCLs), which have a positive surface charge, were used as heating mediators for intracellular hyperthermia. Solid T-9 glioma tissues were formed subcutaneously on both femurs of female F344 rats, and MCLs were injected via a needle only into the left solid tumors (treatment side). The rats were then divided into two groups, which received no irradiation, or irradiation for 30 min given three times at 24-h intervals with an alternating magnetic field (118 kHz, 384 Oe). On the treatment side, the tumor tissue disappeared completely in many rats exposed to the magnetic field. The tumor tissue on the opposite side also disappeared completely, even though MCLs were not injected into the right solid tumors. To examine whether a long-lasting and tumor-specific immunity could be generated, the rats that had been cured by the hyperthermia treatment were rechallenged with T-9 cells 3 months later. After a period of transient growth, all tumors disappeared. Furthermore, immunocytochemical assay revealed that the immune response induced by the hyperthermia treatment was mediated by both $CD8^+$ and $CD4^+$ T cells and accompanied by a marked augmentation of tumor-selective cytotoxic T lymphocyte activity. These results suggest that our magnetic particles are potentially effective tools for hyperthermic treatment of solid tumors, because in addition to killing of the tumor cells by heat, a host immune response is induced.**

Key words: Antitumor immunity induction — Magnetite cationic liposomes — Magnetite — Intracellular hyperthermia — Glioma cells

Hyperthermia is a promising tool for cancer therapy.[1)] Various methods have been employed, such as whole body hyperthermia,[2)] radiofrequency hyperthermia,[3)] and inductive hyperthermia using microwave antenna[4)] or implantable needles.[5)] However, it is always difficult to achieve uniform heating of the tumor region to the desired temperature without damaging normal tissue. Therefore, some researchers have proposed intracellular hyperthermia and developed submicron magnetic particles for this purpose.[6–8)] These magnetic particles are easily incorporated into cells and generate heat under an alternating magnetic field through hysteresis loss.[9)] We have also developed 'magnetite cationic liposomes' (MCLs) for intracellular hyperthermia.[10, 11)] MCLs were developed to improve adsorption and accumulation into the tumor cells and show ten-fold higher affinity for tumor cells than neutrally charged magnetoliposomes,[10)] owing to electrostatic interaction with the negatively charged cell membrane.[12, 13)]

The hyperthermic effect of the MCLs was examined *in vivo*.[14)] MCLs were injected into solid tumors formed subcutaneously in F344 rats and the rats were irradiated three times for 30 min with an alternating magnetic field. Histological observations were carried out just after the irradiation and showed that some tumor cells survived, especially in the peripheral area. However, complete tumor regression was observed one month after the irradiation. We were interested in the possibility that antitumor immunity had been induced by the hyperthermic treatment using MCLs.

In the present paper, it is demonstrated that our hyperthermia system can induce an antitumor immune response and the acquired immunity is long-lasting.

## MATERIALS AND METHODS

**Materials** Dilauroylphosphatidylcholine and dioleoylphosphatidylethanolamine were purchased from Sigma Chemical Co. (St. Louis, MO), and *N*-(α-trimethylammonioacetyl)didodecyl-D-glutamate chloride was from Sogo Pharmaceutical Co., Ltd. (Tokyo). Mouse anti-rat monoclonal antibody and goat anti-mouse monoclonal antibody were purchased from Dainippon Pharmaceutical Co., Ltd. (Suita). All other chemicals were obtained from Wako Pure Chemical Industries Co., Ltd. (Osaka).

**Rats and cell culture** Female Fischer 344 (F344) rats,

[3] To whom requests for reprints should be addressed.

6–7 weeks old, were purchased from Japan SLC, Inc. (Shizuoka). These rats were housed in a specific pathogen-free animal facility in our institute.

A rat glioma cell line T-9, derived from an inbred F344 rat, was used. The cells were grown at 37°C in a 5% $CO_2$ atmosphere in Eagle's minimum essential medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 5 m*M* nonessential amino acids, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin.

**Preparation of the MCLs** Magnetite ($Fe_3O_4$; average particle size: 10 nm), used as the core of the MCLs, was a kind gift from Toda Kogyo Co., Ltd. (Hiroshima). MCLs were prepared by the sonication method as described previously.[14] All MCL concentration values are expressed as the net magnetite concentration.

**Preparation of tumor-bearing rats** Rat glioma T-9 cells were suspended at approximately $1\times10^7$ cells in 100 $\mu$l of phosphate buffer (0.05 *M* Na phosphate and 0.15 *M* NaCl, pH 7.4). To prepare tumor-bearing rats, the suspension was transplanted subcutaneously into the left femoral region of F344 rats under short-term anesthesia by intraperitoneal injection of Nembutal. On the 9th day after transplantation into the left side, another aliquot of T-9 cell suspension (approximately $1\times10^7$ cells) was transplanted subcutaneously into the right femoral region. The rats were then separated into two groups. Rats in group I, the control group, were not subjected to magnetic field irradiation, while magnetic field irradiation was applied three times for 30 min each to rats in group II. Group I consisted of 8 rats, and group II consisted of 21 rats. Five or eighteen animals in each group were used for monitoring tumor growth, respectively; the others were employed for the histological observation. Tumor sizes were measured every 2 days. The volume was determined by use of the following formula[15]:

$$\text{tumor volume} = 0.5\times(\text{length}\times\text{width}^2)$$

where the unit of length and width is the millimeter.

***In vivo* hyperthermia** On the 11th day after the first transplantation into the left side, when ellipsoidal tumors tissue 13–18 mm in length had formed, the needle (needle size: 25 G) of a syringe containing MCL solution was inserted into the left tumor tissue in the longitudinal direction subcutaneously from the tumor edge. Then 400 $\mu$l of MCL solution (net magnetite weight: 3mg) was injected at the center of the tumor using an infusion pump (SP100i; World Precision Instruments Inc., Sarasota, FL) for 30 min. Twenty-four hours after the injection, rats in group II were anesthetized again and subjected to the first hyperthermic treatment. A magnetic field was created by using a horizontal coil (inner diameter: 7 cm; length: 7 cm) with a transistor inverter (LTG-100-05; Dai-ichi High Frequency Co., Ltd., Tokyo).[16] Anesthetized rats were laid inside the coil such that the tumor region was at the center. The magnetic field frequency and intensity were 118 kHz and 30.6 kA/m (384 Oe), respectively. Treatment was carried out for 30 min and repeated three times at 24-h intervals. Tumor and rectum temperatures were measured by an optical fiber probe (FX-9020; Anritsu Meter Co., Ltd., Tokyo).

**Preparation of specimens for immunohistochemical staining** On the 14th day after the MCL injection, tumors of the two groups were removed and specimens for immunohistochemical staining were prepared as follows. Blood was flushed out with phosphate buffer, then the tumor tissues were extracted, and fixed with Tissue-Tek O.C.T compound (Sakura Finetechnical Co., Ltd., Tokyo) at −20°C. The frozen tumor tissues were sectioned at 10 $\mu$m thickness. Tissue sections were air-dried for 30 min and fixed with cold acetone for 15 min. These sections were incubated with 5% normal goat serum and 1% skim milk at 37°C for 30 min to block background staining. They were then incubated at 37°C for 60 min with mouse anti-rat CD3 (1F4), anti-rat CD4 (W3/25), anti-rat CD8 (MRC OX-8) or anti-rat NK cell (3.2.3) monoclonal antibody (Serotec Ltd., Oxford, UK) at a dilution of 1:200, and at 37°C for 60 min with goat anti-mouse monoclonal antibody-conjugated horseradish peroxidase (Caltag Laboratories, Burlingame, CA) at a dilution of 1:200. Each step was followed by washing with the phosphate buffer. Peroxidase activity was visualized by treatment at room temperature for 10 min with 0.02% diaminobenzidine

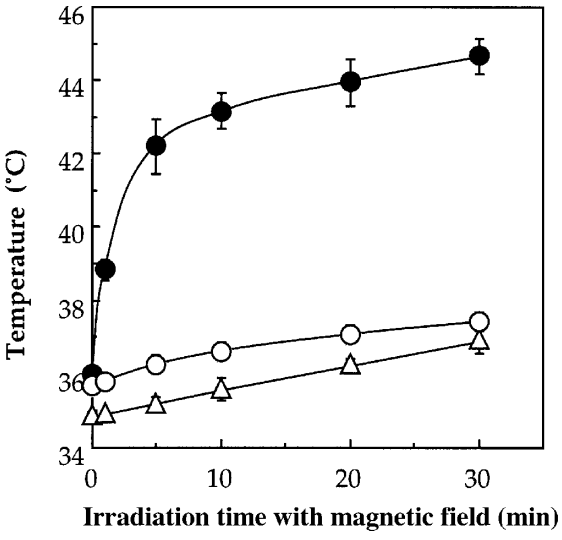


Fig. 1. Temperature increase at the surface of the skin over the tumor and in the rectum during magnetic field irradiation. Symbols: ● the tumor in the left femoral region, ○ the tumor in the right femoral region, △ rectum. Data points and bars are the means and SDs of 5 independent experiments.

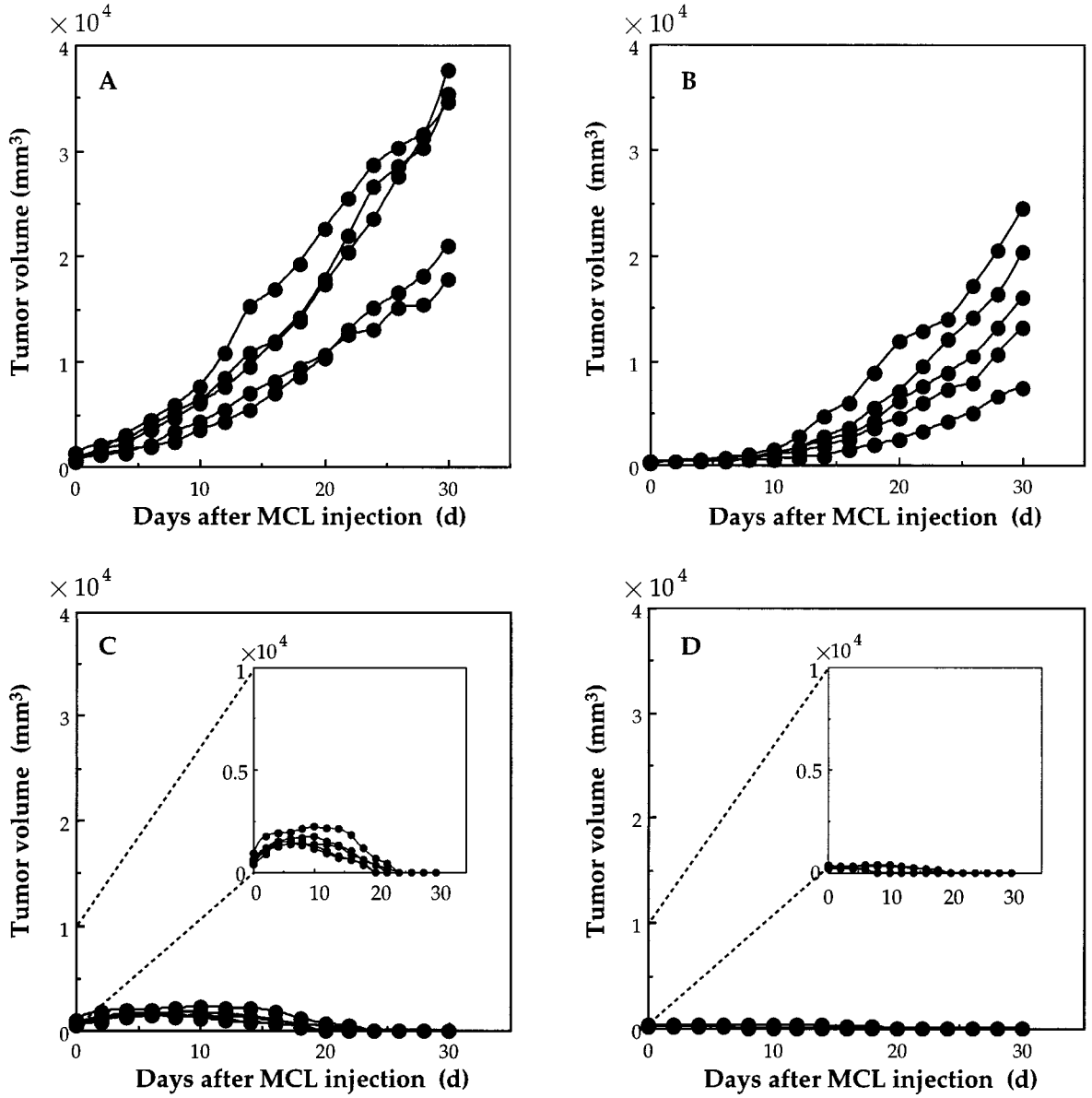


Fig. 2. Time courses of tumor growth after the MCL injection. (A) and (B) indicate tumor volumes in the left and right femoral regions of the rats in group I (control), respectively; (C) and (D) indicate tumor volumes in the left and right femoral regions of the rats in group II (irradiated 3 times at 24-h intervals), respectively. No tumor regrowth was seen within 3 months in group II rats that achieved complete regression.

tetrahydrochloride solution containing 0.005% hydrogen peroxide. All sections were also stained with hematoxylin.

**Rechallenge with T-9 tumor cells in rats cured by hyperthermia** The rats that had been cured by the hyperthermic treatment were challenged with T-9 or control cells at 3 months after the treatment. A malignant fibrous histiocytoma (MFH) cell line, derived from an inbred F344 rat, was used as a control. These cells (approximately $2\times10^7$ cells) were transplanted subcutaneously into the right femoral region as mentioned above. T-9 or MFH cells (approximately $2\times10^7$ cells) were also transplanted into naive rats that had been born at almost the same time as the cured rats, as a control. The growth of these transplanted cells was observed.

***In vitro* cytotoxicity assay** *In vitro* cytotoxicity assay was done at 3 months after the treatment. Naive rats that

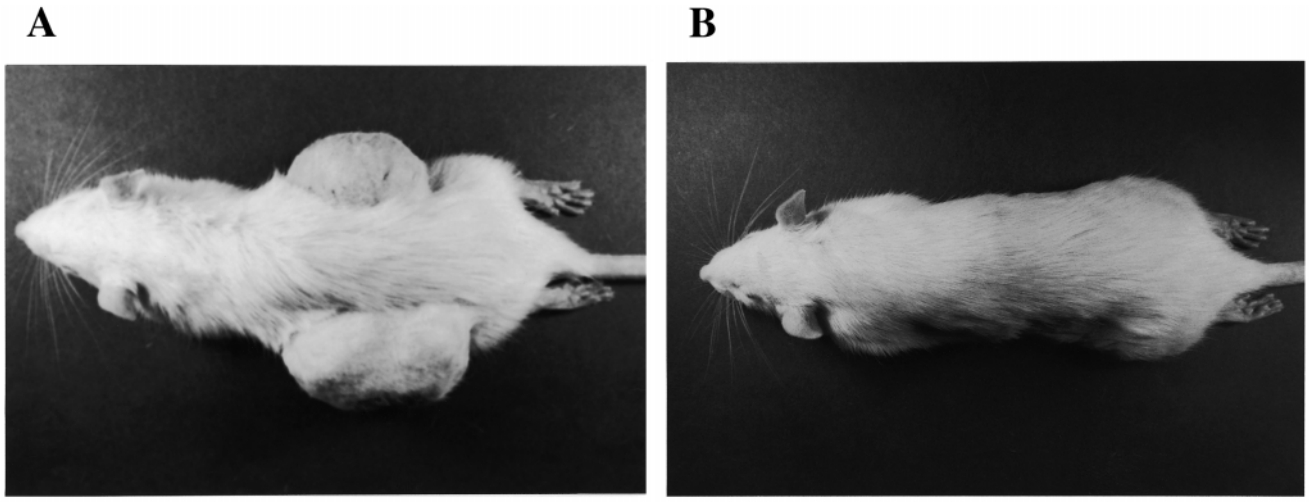


Fig. 3. Rats photographed on the 28th day after MCL injection. (A) A rat in group I; (B) a rat in group II.

Table I. Hyperthermic Effect on Solid Glioma Tissue Formed Subcutaneously 30 Days after the MCL Injection

| Group | Treatment | Tumor take (left) | Complete regression (%) | Tumor take (right) | Remote effect (%) |
|---|---|---|---|---|---|
| I | no irradiation | 5/5 | 0 | 5/5 | 0 |
| II | irradiated three times | 2/18 | 88.9 | 2/18 (0/16)[a)] | 88.9 (100)[a)] |

*a*) Excluding rats that were not cured.

had been born at almost the same time as the cured rats were used as a control. Spleen cells were harvested from the rats cured by the hyperthermic treatment or from the naive rats. Five rat tumor cell lines (other glioma cell lines GA1 and C6,[17] osteosarcoma cell line COS, MFH and T-9) were used as target cells.

The cytotoxicity was determined by means of a long-term cytotoxicity assay.[18] Briefly, tumor cells (approximately $1\times10^5$ cells) were cultured in a 24-well flat plate in 1 ml of minimum essential medium. After 24 h, the medium was replaced with RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum, 2 m*M* fresh L-glutamine, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. The spleen cells were added to each well. The wells were washed with the phosphate buffer after 48 h and fixed with 10% formaldehyde solution for 20 min. The wells were then washed with water, and 0.2% crystal violet solution was added for 5 min. After having been washed several times with water until the blank wells became clear, the plates were dried at room temperature overnight and the remaining dye was dissolved in 70% ethanol solution. The absorbance of each well was measured at 570 nm with a spectrophotometer (V-530, JASCO, Tokyo), and cytotoxicity was calculated by use of the following equation:

$$\text{cytotoxicity (\%)}=[1-(\text{absorbance of tumor cells treated with effector cells})/(\text{absorbance of control})]\times100$$

Animal experiments were performed according to the principles laid down in the "Guide for the Care and Use of Laboratory Animals" prepared under the direction of the Office of the Prime Minister of Japan.

## RESULTS AND DISCUSSION

**Heat generation by MCLs in an alternating magnetic field** To detect heat generation by MCLs in an alternating magnetic field, the temperature at the surface of the skin covering the tumor was monitored as described previously.[14] The results are shown in Fig. 1. The temperature of the tumor on the left side was elevated rapidly by mag-

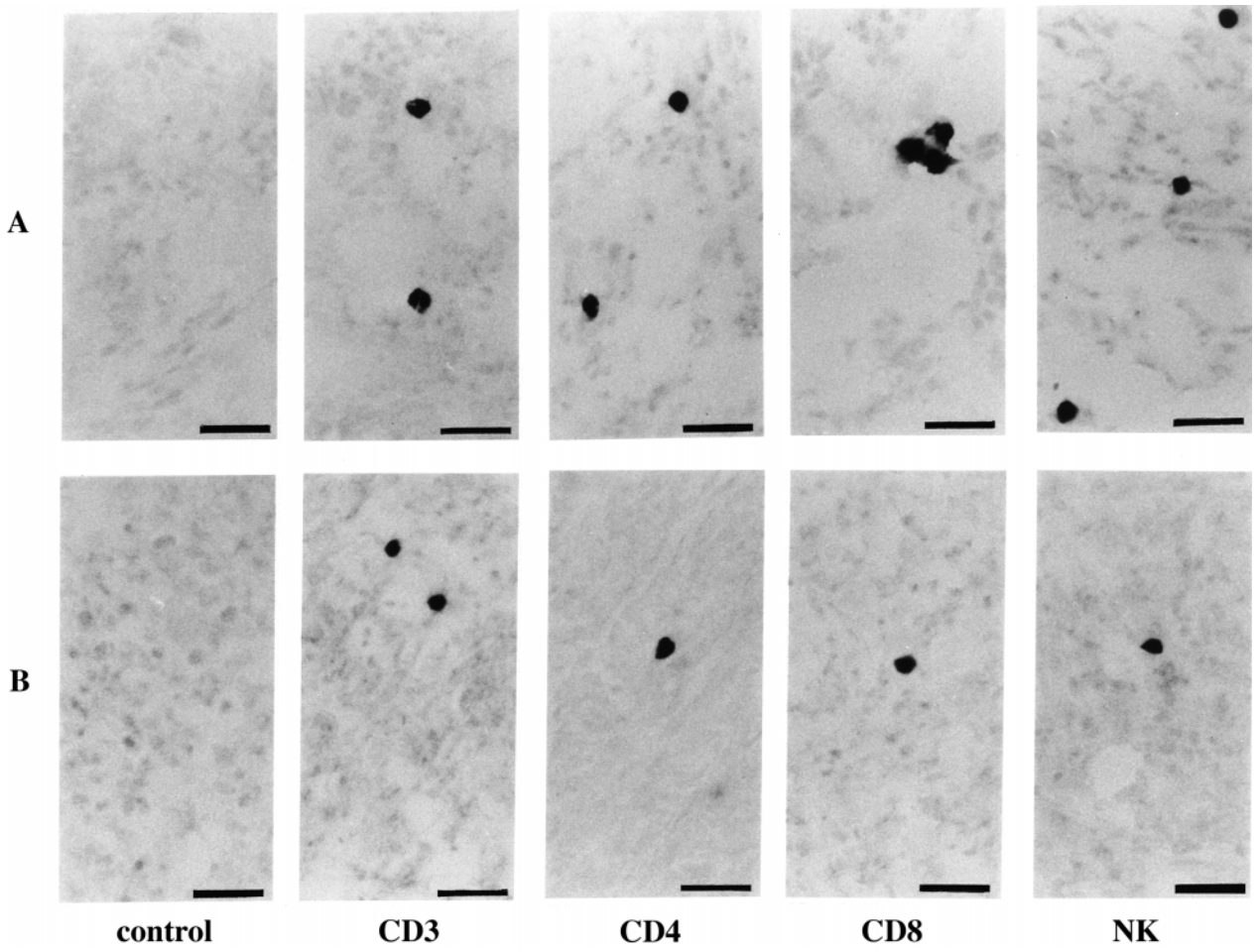


Fig. 4. Immunohistochemical staining for immunocytes. Anti-$CD3^+$, $CD4^+$, $CD8^+$ and NK cells antibodies were used (scale bar, 50 $\mu$m). (A) Tumor tissue in the left femoral region of the rats given the hyperthermic treatment; (B) tumor tissue in the right femoral region of the rats.

netic heating and reached over 43°C after 10 min. In contrast, the temperature of the tumor on the right side or that in the rectum remained at 35–37°C.

**Monitoring tumor growth after hyperthermia** Fig. 2 shows the time courses of tumor growth of 5 rats in each group. In group I rats without irradiation, the tumor volume in each rat steadily increased on both sides, with no evidence of regression. In contrast, in the left femoral region in group II rats, complete tumor regression was observed in all 5 rats. Furthermore, tumor tissues in the right femoral region also completely disappeared, although the MCLs were not injected on this side and the temperature on this side never increased during the hyperthermic treatment, as shown in Fig. 1. Fig. 3 shows photographs of typical rats from groups I and II on the 28th day after the MCL injection. In the cases of complete regression, no regrowth of tumors was observed over a period of 3 months on either side (data not shown).

Table I shows tumor take, ratios of complete regression on the left side, and ratios of remote effect showing complete regression on the right side on the 30th day after the MCL injection. In the case of group I, tumor take was observed in all rats on both sides. In the case of group II, complete regression on the left side was observed in sixteen of eighteen rats, and the remote effect was observed in all of these rats. Complete regression was not observed in two of the eighteen rats in group II, and these rats also showed no remote effect. It is noteworthy that complete regression of the tumor on the left side coincided with the remote effect on the right side. Since the shape of the

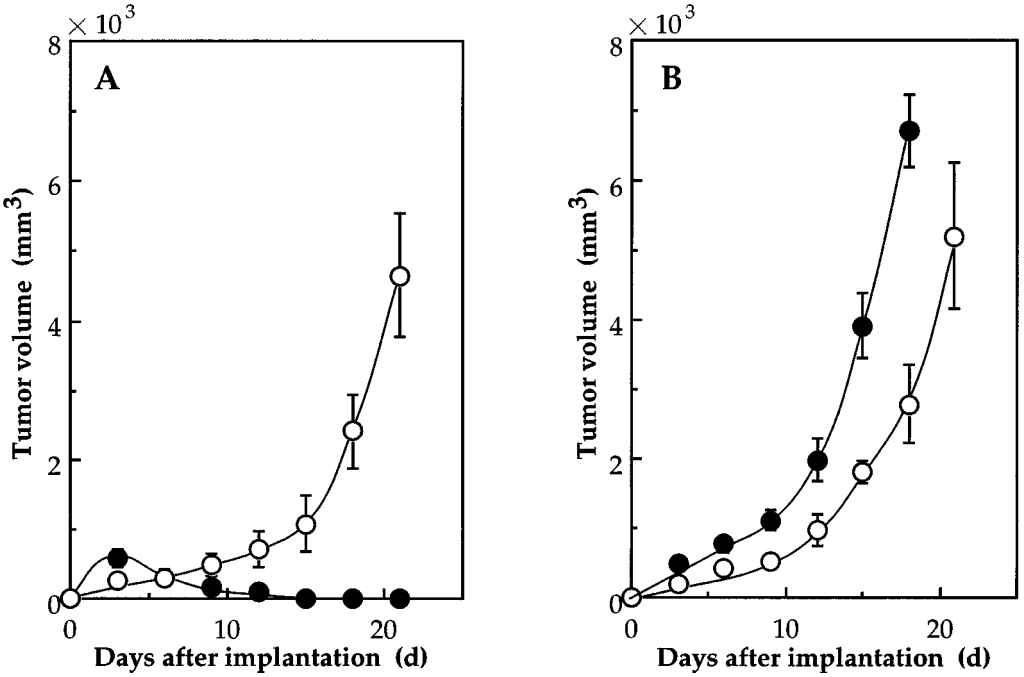


Fig. 5. Time courses of tumor growth after challenge with T-9 or MFH cells 3 months after the treatment. (A) The rats cured by the hyperthermic treatment were challenged with T-9 or MFH cells; (B) naive rats were challenged with the same number of T-9 or MFH cells as a control. Symbols: ○ MFH, ● T-9. Data points and bars are the means and SDs of 5 independent experiments.

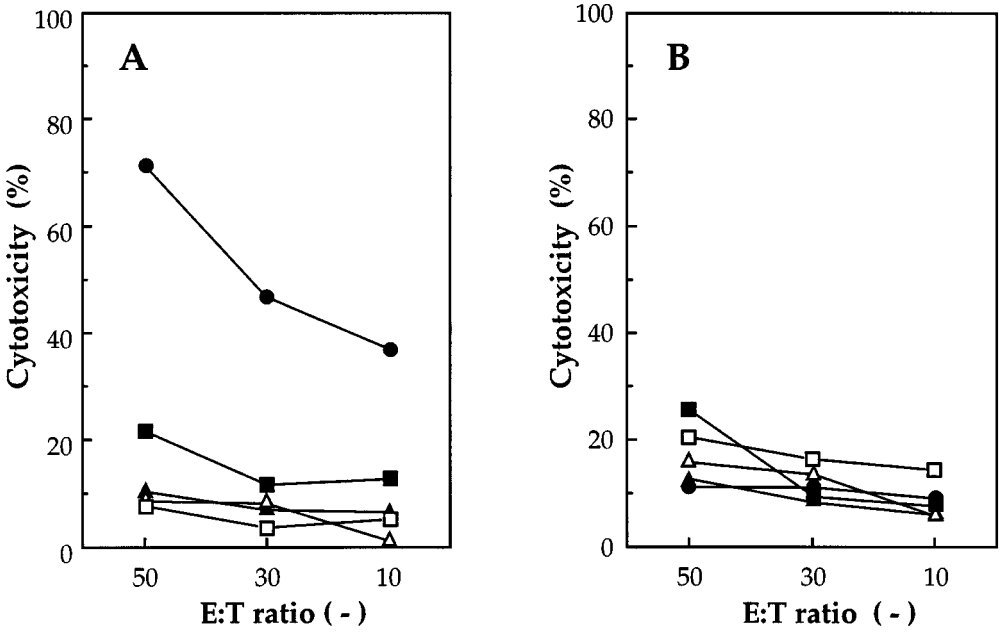


Fig. 6. Increased activity of antitumor CTLs in rats that had been cured by the hyperthermic treatment. (A) The CTL activity from spleen cells of cured rats; (B) the CTL activity from spleen cells of naive rats. Symbols: ▲ GA1, △ C6, ■ COS, □ MFH, ● T-9. Cytotoxic activity against the indicated target cells was examined by means of an established long-term cytotoxicity assay at different effector-target ratios (E:T). Data points are the means of 3 independent experiments.

tumors on the left side in the two rats in group II was not ellipsoidal but irregular, it is likely that the MCLs failed to heat the whole of the tumor homogeneously even though the hyperthermic treatment was applied three times.

**Immunohistological features of tumors after hyperthermic treatment** To examine whether immunocytes exist in tumor tissue, immunohistochemical staining of the tumor tissues was done in rats with and without the hyperthermic treatment. As shown in Fig. 2C, in the cases of complete regression, the tumor volume increased up to the 10th day, then began to decrease and finally the tumor disappeared. Therefore, preparation of specimens for staining was done from rats on the 14th day after the MCL injection, because many immunocytes are expected to be localized in tumor tissue. $CD3^+$, $CD4^+$, $CD8^+$ and NK cells were detected in the left tumor tissues of the rats exposed to hyperthermia, as shown in Fig. 4A. Such immunocytes were also observed in the right tumor tissues of these rats (Fig. 4B), but not in any tumor tissues of the rats given no treatment (data not shown).

$CD4^+$ cells recognize antigen-presenting cells (APC) such as macrophages and when they are activated, they play roles in the activation of B cells to antibody-producing cells and in the activation of $CD8^+$ cells to cytotoxic T lymphocytes (CTL). On the other hand, $CD8^+$ cells recognize antigen-bearing cells such as virus-infected cells and tumor cells, and themselves change into CTL. In the present study, both cell types were observed, so $CD8^+$ cells might have been activated to CTL by $CD4^+$ cells. The appearance of the CTL suggests that the antigenicity of T-9 cells was enhanced by the hyperthermic treatment, i.e., the progression of the memory T cells was promoted by stimulation via APC. The progression of T cells is also caused by expression of heat-shock protein, which is characteristic of heat treatment. For example, Kobayashi *et al*. reported that hsp60-recognizing γδ type T cells existed in peripheral T cells of BALB/c mice.[19] Although the type of T cells observed in tumor tissue was not identified in the present study, it is likely that these cells also contribute to the activation of immunity.

It is considered that NK cells observed in the treatment site resulted from the response to inflammation of the tissue. The appearance of NK cells in the untreated side should reflect the CTL reaction in response to the necrosis of the tumor.

Since cancer is a malignant disease, tumor cells should be completely destroyed to prevent its recurrence. In rats treated with our hyperthermia system, $CD4^+$ and $CD8^+$ cells were detected not only in the treatment side but also in the untreated site, and tumor tissues disappeared at both sides. This means that local hyperthermia at one side enhanced the antitumor immune response in the whole body.

***In vivo* immune response of rats cured by hyperthermic treatment** To examine whether the tumor-specific immunity was persistent, rats that had been cured by the hyperthermic treatment were challenged with T-9 or MFH cells. These cells were transplanted into cured rats 3 months after the treatment. After a period of transient growth, all T-9 tumors regressed (Fig. 5A), as compared with progressive tumor growth in all naive rats (Fig. 5B). This means that the immune response in rats cured by the hyperthermia treatment was retained over 3 months.

As shown in Fig. 5, the tumor volume increased in the cured and naive rats in the case of MFH cells. Therefore, the immune response was found to be specific for the original (T-9) cells. Since this acquired immunity involved the progression of memory T cells and was long-lasting, our hyperthermia system is considered to be a suitable protocol for cancer therapy, including the prevention of recurrence.

***In vitro* immune response using spleen cells from rats cured by the hyperthermic treatment** To examine further the mechanisms underlying the antitumor activity of the hyperthermic treatment, we evaluated its effect on the generation of CTL-killing T-9 cells. As shown in Fig. 6A, the CTL activity for T-9 cells was approximately 7 times higher in spleen cells of the cured rats as compared with that from spleen cells of naive rats (Fig. 6B). The CTL activity was found to be selective for T-9 cells because GA1, C6, COS and MFH cells were not lysed.

In conclusion, our results demonstrated the amplification of an antitumor T cell response by our hyperthermia system, leading to cure. Both $CD8^+$ and $CD4^+$ T cells were detected in the tumor tissue of rats subjected to the hyperthermic treatment, while no immunocytes was observed in that of the untreated rats. Moreover, this acquired immunity was long-lasting and T-9 cell specific. These results suggest that our magnetic particles are potentially effective tools for the treatment of solid tumors, causing both killing of the tumor cells by heat, and the induction of an immune response.

ACKNOWLEDGMENTS

We thank Dr. Makoto Sawada of Fujita Health University for supplying GA1 and C6 cell lines, and Dr. Masahiro Tsutsumi of Nara Medical University for supplying COS and MFH cell lines. This study was partially funded by a Grant-in-Aid for Scientific Research on Priority Areas (No. 10145104) from the Ministry of Education, Science, Sports and Culture of Japan.

*(Received March 19, 1998/Revised May 8, 1998/Accepted May 20, 1998)*

REFERENCES


1) Kobayashi, T. Hyperthermia for brain tumors. *Jpn. J. Hyperthermic Oncol.*, **9**, 245–249 (1993).
2) Baba, H., Siddik, Z. H., Strebel, F. R., Jenkins, G. N. and Bull, J. M. Increased therapeutic gain of combined *cis*-diamminedichloroplatinum(II) and whole body hyperthermia therapy by optimal heat/drug scheduling. *Cancer Res.*, **49**, 7041–7044 (1989).
3) Ikeda, N., Hayashida, O., Kameda, H., Ito, H. and Matsuda, T. Experimental study on thermal damage to dog normal brain. *Int. J. Hyperthermia*, **10**, 553–561 (1994).
4) Lin, J. C. and Wang, Y. J. Interstitial microwave antennas for thermal therapy. *Int. J. Hyperthermia*, **3**, 37–47 (1987).
5) Stauffer, P. R., Cetas, T. C., Fletcher, A. M., DeYoung, D. W., Dewhirst, M. W., Oleson, J. R. and Roemer, R. B. Observation on the use of ferromagnetic implants for inducing hyperthermia. *IEEE Trans. Biomed. Eng.*, **BME-31**, 76–90 (1984).
6) Tazawa, K., Takemori, S., Sawadaishi, M., Nagase, T., Kasagi, T., Suzuki, Y., Saito, M., Kato, H., Maeda, M., Honda, T. and Fujimaki, M. Intracellular hyperthermia for the treatment of cancer (II): raising the high temperature with exciting submicron particles. *In* "Hyperthermia in Cancer Therapy," ed. S. Egawa, pp. 248–249 (1986). Shinohara Publishers Inc., Tokyo.
7) Jordan, A., Wust, P., Fähling, H., John, W., Hinz, A. and Felix, R. Inductive heating of ferromagnetic particles and magnetic fluids: physical evaluation of their potential for hyperthermia. *Int. J. Hyperthermia*, **9**, 51–68 (1993).
8) Mitsumori, M., Hiraoka, M., Shibata, T., Okuno, Y., Masunaga, S., Koishi, M., Okajima, K., Nagata, Y., Nishimura, Y., Abe, M., Ohura, K., Hasegawa, M., Nagae, H. and Ebisawa, Y. Development of intra-arterial hyperthermia using a dextran-magnetite complex. *Int. J. Hyperthermia*, **10**, 785–793 (1994).
9) Shinkai, M., Matsui, M. and Kobayashi, T. Heat properties of magnetoliposomes for local hyperthermia. *Jpn. J. Hyperthermic Oncol.*, **10**, 168–177 (1994).
10) Shinkai, M., Yanase, M., Honda, H., Wakabayashi, T., Yoshida, J. and Kobayashi, T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: *in vitro* study. *Jpn. J. Cancer Res.*, **87**, 1179–1183 (1996).
11) Yanase, M., Shinkai, M., Honda, H., Wakabayashi, T., Yoshida, J. and Kobayashi, T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: *ex vivo* study. *Jpn. J. Cancer Res.*, **88**, 630–632 (1997).
12) Koshizaka, T., Hayashi, Y. and Yagi, K. Novel liposomes for efficient transfection of β-galactosidase gene into COS-1 cells. *J. Clin. Biochem. Nutr.*, **7**, 73–77 (1989).
13) Yagi, K., Hayashi, Y., Ishida, N., Ohbayashi, M., Ohishi, N., Mizuno, M. and Yoshida, J. Interferon-β endogenously produced by intratumoral injection of cationic liposome-encapsulated gene: cytocidal effect on glioma transplanted into nude mouse brain. *Biochem. Mol. Biol. Int.*, **32**, 167–171 (1994).
14) Yanase, M., Shinkai, M., Honda, H., Wakabayashi, T., Yoshida, J. and Kobayashi, T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: an *in vivo* study. *Jpn. J. Cancer Res.*, **89**, 463–469 (1998).
15) Yoshida, J., Mizuno, M. and Yagi, K. Secretion of human β-interferon into the cystic fluid of glioma transfected with the interferon gene. *J. Clin. Biochem. Nutr.*, **11**, 123–128 (1991).
16) Chikazumi, S. Empty coil. *In* "Magnetism," ed. S. Chikazumi, pp. 37–45 (1968). Kyoritsu Shuppan, Tokyo.
17) Sawada, M., Suzumura, A., Yoshida, M. and Marunouchi, T. Human T-cell leukemia virus type *trans* activator induces class I major histocompatibility complex antigen expression in glial cells. *J. Virol.*, **64**, 4002–4005 (1990).
18) Aruga, A., Aruga, E. and Chang, A. E. Reduced efficacy of allogeneic versus syngeneic fibroblasts modified to secrete cytokines as a tumor vaccine adjuvant. *Cancer Res.*, **57**, 3230–3237 (1997).
19) Kobayashi, N., Matsuzaki, G., Yoshikai, Y., Seki, R., Ivanyi, J. and Nomoto, K. γδ5$^+$ T cells of BALB/c mice recognize the murine heat shock protein 60 target cell specificity. *Immunology*, **81**, 240–246 (1994)