Application of boronated anti-CEA immunoliposome to tumour cell growth inhibition in in vitro boron neutron capture therapy model

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Summary An immunoliposome containing a 10B-compound has been examined as a selective drug delivery system in boron neutron-capture therapy. Liposomes, conjugated with monoclonal antibodies specific for carcinoembryonic antigen (CEA) were shown to bind selectively to cells bearing CEA on their surface. The immunoliposomes attached to tumour cells suppressed growth in vitro upon thermal neutron irradiation and suppression was dependent upon the concentration of the 10B-compound in the liposomes and on the density of antibody conjugated to the liposomes. The results suggest that immunoliposomes containing the 10B-compound could act as a selective and efficient carrier of 10B atoms to target tumour cells in boron neutron-capture therapy.

The application of neutron-capture therapy to cancer was first reported by Locher (Locher, 1936). Kruger showed that cancer cells could be killed in vitro by the application of 10B-compounds with thermal neutron irradiation (Kruger, 1940). Cell destruction in boron neutron-capture therapy (BNCT) is due to the nuclear reaction between 10B and thermal neutrons to release alpha-particles (He) and lithium-7 ions (Li). The 10B kills cells in the range of 10 µm from the site of He generation. Therefore, it is theoretically possible to kill tumour cells without affecting adjacent healthy tissues, if 10B-compounds could be selectively delivered.

BNCT has been applied to the treatment of malignant brain tumours (Sweet, 1951; Hatanaka, 1986) and melanoma (Mishima et al., 1983; 1989) by using 10B-compounds selectively taken up by tumour cells. It would be possible to apply BNCT for the treatment of various kinds of tumour, if sufficient amounts of 10B-compound could be delivered to the malignant cells by using monoclonal antibodies reactive to these cells. Takahashi et al. (1987) prepared anti-alpha foeto-protein monoclonal antibody conjugated with 10B-compound, and this produced cytotoxic effects on hepatoma cells in BNCT in vitro. However, in these experiments, antibody was shown to lose activity by the direct conjugation with 10B-compound.

Recently, liposomes have attracted attention as drug delivery systems (Bangham et al., 1965; Hashimoto et al., 1983; Konno et al., 1987; Tanaka, 1989). It is possible to carry a large amount of 10B-compound in a liposome and, therefore, the liposome could deliver a large amount of the 10B-compound to a tumour cell, if it bears specific antibody against the cells on the surface.

In the present experiments, we prepared a liposome which contained 10B-compound and conjugated with a monoclonal antibody specific for the tumour cells on its surface. The immunoliposome was shown to deliver the 10B-compound to target tumour cells and inhibit tumour cell growth on thermal neutron irradiation in vitro.

Materials and methods

Target tumour cells

Human pancreatic carcinoma cell line AsPC-1 (Chen, 1982), producing carcinoembryonic antigen (CEA) (Gold & Freedman, 1965), was obtained from Dainihon Seiyaku Co. Ltd. (Osaka, Japan) and maintained in RPMI 1640 medium (Hazleton Biologics, INC, Kansas, USA) supplemented with 10% foetal calf serum (Cell Culture Laboratories, Ohio, USA) and 100 µg ml⁻¹ kanamycin.

Preparation of anti-human CEA monoclonal antibody

BALB/c mice were immunised intraperitoneally five times with 1 x 10⁵ AsPC-1 cells at intervals of 2 to 3 weeks. Four days after the immunisation, spleen cells from these mice were fused with mouse myeloma cells (X63, Ag8, 653) using polyethylene glyco 4000 (Merk, Parnsteadt, Germany). After 2 weeks growth in selection medium containing hypoxanthine, aminopterine and thymidine, cells that produced anti-CEA were selected by assaying the antibody in the medium by enzyme-immunoassay and they were cloned three times by a limiting dilution technique. A representative hybridome clone, 2C-8, was selected, and grown in the peritoneal cavity of the mouse. The antibody was purified on a DEAE-52 cellulose column (Whatmann, Biosystem Ltd, England) and concentrated to 4 mg ml⁻¹. A monoclonal antibody (MoAb) specific for dinitrophenol (DNP) was used as a control. Specificity of the antibody was confirmed by immunoperoxidase staining of various cell lines. Seven cell lines known as CEA-producers were all stained positive, although the intensity of staining was variable, and eight non-producer cell lines were not stained at all.

The epitope recognised by the antibody was confirmed to be 200 kDa CEA and 45 kDa nonspecific crossreacting antigen (NCA) by SDS PAGE and Western blotting using the soluble antigen of AsPC-1 cells prepared according to Laemmli methods (Laemmli, 1970), purified CEA purchased from Kyowa Hakko Kougou Co. Ltd. (Tokyo, Japan) and purified NCA (Kleist et al., 1972) kindly provided by Dr T. Sugiyama (Sapporo Medical College, Sapporo, Japan).

Immunocytological staining

Reactivity of monoclonal anti-CEA, 2C-8, to various cell lines was examined by immunocytological technique described by Hsu et al. (1981).

Briefly, the cells were fixed with acetone for 2 min at -20°C. After incubation with 5% rabbit serum, the cells were incubated for 60 min at room temperature with 2C-8 mouse ascites (1:200 dilution) or with immunoliposomes. They were then washed in phosphate-buffered saline (PBS) and incubated for 45 min with 1:50 diluted peroxidase-conjugated rabbit antimouse IgGs (DAKO PATTS). The preparations were visualised with diaminobenzine and counterstained with haematoxylin.
**Chemicals**

The cesium salt of undecaacylphosphatidylcholine (Cs 152B10H1s,SH) was kindly supplied by Shionogi Research Laboratories Co. Ltd. (Osaka, Japan). The solubility of the compound in water was 250 mM at 40°C. N-hydroxythiocinnamimid 3-(2-pyridyldithio)propionate (SPDP) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). A stock solution of SPDP (30 mM) was prepared in ethanol and stored at -20°C. Dithiorethiol was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in water to a concentration of 3 mg ml⁻¹.

Hen egg phosphatidylcholine (Egg PC) was a gift from Nippon Fine Chemical Co. Ltd. (Osaka, Japan). Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dipalmitylophosphatidylethanolamine (DPPE) was from Calbiochem-Behring (San Diego, CA, USA). 3-(2-pyridyldithio)propionyl-dipalmitylo-phosphatidylethanolamine (DTP DPPE) was prepared by reacting SPDP with DPPE as described by Barbet et al. (1981).

**Preparation of immunoliposomes containing 10B-compound**

Egg yolk PC (5 μ moles), cholesterol (5 μ moles) and DTP-DPPE (0.25 μ moles) dissolved in chloroform-methanol (2:1) were mixed in a conical flask. The organic solvent was removed by evaporation at 40°C. A half ml of 25, 100, 250 mM 10B-compound (Cs 152B10H1s,SH) solution and 20 μl carboxy fluorescein (CF) (2 μ moles) were added to the dried lipid film, and then multilamellar vesicles were prepared by vortex dispersion. Uncapsulated 10B-compound and CF were removed by washing with centrifugation at 20,000 g. After treatment for 30 min at room temperature with 20 mM dithiothreitol to ensure the functional SH-group, the liposome was centrifuged at 20,000 g. One ml of monoclonal antibody (0, 0.5, 1.0, 4.0 mg ml⁻¹) was incubated with an excess amount of SPDP for 30 min at room temperature. After removal of free SPDP by passage through a Sephadex G25 column, the liposomes were suspended in the antibody solution. After incubation at 4°C overnight, the boronated immunoliposomes (10B-Lip-MoAb) were washed by centrifugation at 20,000 g and suspended in 1 ml of 10 mM veronal buffer, pH 7.4, supplemented with 0.4% gelatin. An average diameter of the liposome was estimated to be 4.7 μ in dynamic light scattering analysis.

The liposome prepared was confirmed to be stable in serum as reported (Yeagle, 1985), and boron was confirmed not to leak out of the liposome.

**The determination of 10B-compound concentration entrapped in liposomes**

The amount of 10B-compound entrapped in liposomes was determined by a colorimetric method in the presence of curcumin (Ikeuchi & Amano, 1978).

After oxidative degradation with potassium permanganate, boron was extracted with chloroform containing 2-ethyl-1, 3-hexanediol. Boron in the extract was converted into the boron-curcumin complex by adding an acetic acid solution of curcumin. Concentrated sulfuric acid was then added to the solution. After dilution with 95% (v/v) ethanol, the optimal absorbance of the solution at 554 nm was measured by spectrophotometry.

**Thermal neutron irradiation**

AsPC-1 cells, 5 x 10⁴ cells/culture, were incubated in a 96 well-microplate at 37°C in 5% CO₂ in air for 8 h in the presence of immunoliposomes. After washing, the cells were irradiated with thermal neutron at the TRIGA-II atomic reactor of Rikkyo University (Yokosuka, Japan). After irradiation, 0.25 μCi ³²PdR was added to each well and incubated for further 8 h. Then, the cells were harvested and the incorporation of thymidine was estimated in a liquid scintillation spectrometer.

**The determination of gamma-ray dose generated at the thermal neutron irradiation in the thermal column**

The gamma-ray generated during thermal neutron irradiation was measured using the ionisation chamber method (ICRU, 1964) at the irradiation points.

**Gamma-ray irradiation of cells**

AsPC-1 cells were irradiated with gamma-rays from the 137Cs source in Gamma Cell 40 (Atomic Energy of Canada, Ottawa, Canada).

**Results**

**Reactivity of immunoliposomes to target tumour cells**

In order to examine the reactivity of immunoliposomes to target cells, AsPC-1 cells were incubated with liposomes for 30 min at room temperature, stained with second antibody and examined under a microscope. The reaction was strongly positive, when the cells were incubated with the original or a 1:10 dilution of the liposome preparation. The cells were stained weakly with the 1:100 dilution. Figure 1 shows positive staining of the AsPC-1 cells with 1:10 dilution of the liposome suspension. AsPC-1 cells incubated with liposomes conjugated with anti-DNP were not stained at all. The reactivity of the immunoliposomes to AsPC-1 cells was also confirmed by the use of liposomes with fluorescent dye, carboxyfluorescein (data not shown).

**The concentration of 10B entrapped in liposomes**

The immunoliposomes were prepared with 100 mM or 250 mM 10B-compound and 4 mg ml⁻¹ SPDP-anti-CEA. These preparations were assayed for entrapped 10B. The amounts of 10B in immunoliposomes prepared with 100 mM and 250 mM

![Figure 1](https://example.com/figure1.png)  
**Figure 1** The reactivity of immunoliposome to AsPC-1 cells. a, anti-CEA MoAb (1:10 dilution); b, anti-DNP MoAb (1:10 dilution). The magnification of all photomicrographs is x 800.


Growth inhibition of AsPC-1 cells treated with immunoliposomes

In order to examine the effect of immunoliposomes on the growth of AsPC-1 cells, the cells were treated with liposomes prepared with 250 mM \(^{10}\)B-compound and 4 mg ml\(^{-1}\) anti-CEA or anti-DNP. After washing to remove free liposomes, the cells were irradiated with various fluences of thermal neutrons and cultured in \textit{vitro}. As shown in Figure 2, AsPC-1 cells treated with the original suspension of immunoliposomes showed a reduction in growth by 50% at \(1 \times 10^{12}\) fluence or more of thermal neutrons. When AsPC-1 cells were treated with immunoliposomes prepared with anti-CEA, they grew as well as untreated cells. As shown in Figure 3, the decrement in cell growth was dependent on the dose of the liposomes used, and liposomes without antibody or \(^{10}\)B-compound exerted little effect on cell growth.

These results indicate that immunoliposomes could carry \(^{10}\)B-compound to the target cells and exert toxic effects on them.

Effect of \(^{10}\)B-compound concentration in immunoliposomes on cytotoxicity

In order to confirm the role of \(^{10}\)B-compound in the immunoliposome, the effect of \(^{10}\)B-compound concentration on the cytotoxicity of the liposome against AsPC-1 cells was examined. Liposomes were prepared by using 25, 100, 250 mM or without \(^{10}\)B-compound and conjugated with 4 mg ml\(^{-1}\) anti-CEA. These immunoliposomes were examined for their cytotoxic effects on AsPC-1. As shown in Figure 4, the liposomes prepared in 25 mM \(^{10}\)B-compound showed little effect on AsPC-1 cell growth. However, liposomes prepared with 100 mM or 250 mM \(^{10}\)B-compound significantly inhibited the growth of the cells and those prepared with \(^{10}\)B-compound under 100 mM and greater than 25 mM may have recognisable dose-dependent effectiveness.

These results indicate that the immunoliposomes must be prepared with a concentration of 100 mM or more of \(^{10}\)B-compound for effective BNCT under our experimental conditions.

Effect of antibody density on the cytotoxicity

Antibody on the liposomes plays a role in their carriage to target cells. In the next experiments, therefore, liposomes prepared with 250 mM \(^{10}\)B-compound were conjugated with various concentrations of anti-CEA, and the effects of these immunoliposomes on AsPC-1 cell growth were examined after thermal neutron irradiation. As shown in Figure 5, AsPC-1 cells showed reduced growth at \(1 \times 10^{12}\) n cm\(^{-2}\) and more of thermal neutron fluences as the increment of the antibody concentration used for the preparation of immunoliposomes. These results indicate that the antibody used for
the preparation of immunoliposomes plays an essential role in targeting the liposomes to tumour cells.

**Effect of gamma-rays generated by thermal neutrons**

There is a possibility that the inhibition of AsPC-1 cells described above was actually due to the gamma-rays generated by thermal neutrons. As shown in Table I, cells irradiated with thermal neutrons were also irradiated with various doses of gamma-rays generated depending on the neutron dose. These doses of gamma-rays did not exert any inhibitory effect on AsPC-1 cell growth. With 3.36 Gy gamma-rays, growth of AsPC-1 cell was suppressed weakly but significantly.

**Failure of inhibition of tumour cell growth with soluble Cs,^10^B,B_2,H_2,S_H**

In order to examine the effects of soluble ^10^B-compound (Cs,^10^B H S H) on the proliferation of AsPC-1 cells, the cells were suspended in various concentrations of ^10^B-compound solution. After they were irradiated with 1 x 10^{12} to 5 x 10^{12} n cm^{-2} of thermal neutron, their growth was examined. As shown in Table II, the soluble ^10^B-compound did not significantly suppress the cell growth even at 2000 ppm (4 mM). If all of the liposomes prepared by using 250 mM of ^10^B-compound were lysed to release ^10^B-compound into culture medium, it would make 312 ppm ^10^B-compound solution.

**Table I** Gamma-ray generated by thermal neutron irradiation and the effect of the irradiation on tumour cell growth

| Thermal neutron fluences (n cm^{-2}) | Dose of gamma-ray* (Gy) | Cell growth (% uptake of ^1-H-TdR) |
|-------------------------------------|-------------------------|----------------------------------|
| 0                                   | 0                       | 100                              |
| 5 x 10^{11}                         | 0.258                   | 95.3 ± 9.0                       |
| 1 x 10^{12}                         | 0.515                   | 93.6 ± 7.0                       |
| 2 x 10^{12}                         | 1.030                   | 96.7 ± 18.0                      |
| 5 x 10^{12}                         | 3.360                   | 70.6 ± 2.0                       |

* Dose of gamma-ray at neutron irradiation site was measured by the ionisation chamber method. 5 x 10^6 AsPC-1 cells/200 ml/culture were irradiated by these doses of gamma-ray from ^13^Cs source in a separate experiment. After the irradiation, cell proliferation in 8 h was measured by the incorporation of ^1-H-TdR. Results on cell growth are presented as the mean ± s.e. in triplicate assays in terms of percentage of ^1-H-TdR uptake of unirradiated AsPC-1 cells.

Because the maximum ^10^B concentration entrapped in immunoliposomes was 623 ppm, and an equal volume of medium suspending target cells was added, the ^10^B-concentration in the medium must be reduced to half. These results may rule out the possibility that soluble ^10^B-compound in the medium emits alpha-particles by thermal neutron irradiation thereby injuring the cells.

**Discussion**

Antibody reactive to tumour cells is one of the most useful vehicles for ensuring selective accumulation of boron in tumours.

The effect of the ^10^B-conjugated antibody on tumour cell growth in BNCT was reported first by Mizusawa et al. (1982), Goldenberg et al. (1984). They conjugated 50 boron atoms directly to an antibody molecule, but the antibody did not work efficiently in BNCT.

It was estimated that 10^{10} ^10^B atoms are required to destroy one tumour cell in BNCT (Alam et al., 1984). When antibody directly conjugated with ^10^B-compound was used in BNCT, the quantity of ^10^B atom delivered to a cell was proportional to the density of cell surface antigen molecules. According to Alam et al., an antibody has to be conjugated with 10^{10} ^10^B atoms to destroy a tumour cell with 10^6 epitopes on its surface. One thousand, three hundred boron atoms were reported to be conjugated to a molecule of monoclonal antibody by using SPDP (Alam et al., 1985).

A monoclonal antibody against alpha-foeto protein was found to exert some cytotoxic effect on AH66 tumour cells in BNCT in vitro (Takahashi et al., 1987). However, a heavy boronation of antibody has been shown to markedly reduce the antibody reactivity (Alam et al., 1985; Takahashi et al., 1987) and the numbers of epitope on the tumour cell surface have been estimated to be at most 10^4/cell (Tsukada et al., 1982; Barth et al., 1990). These results indicate that monoclonal antibody directly conjugated with ^10^B-compound has limited application in BNCT.

A liposome is a vesicle which could entrap various materials, and a method of conjugating protein molecules on the surface by SPDP was developed (Barbet et al., 1981). Therefore, it is possible for liposomes to carry a large amount of substance to the target cell surface, if the substance is entrapped in the liposome conjugated with monoclonal antibody specific against the cells. In fact, liposome-bearing antibody against human β₂-microglobulin was found to bind specifically to human cells but not to murine cells (Leserman et al., 1981). Immunoliposomes containing actinomycin D were reported to exert cytotoxic effect in mammary carcinoma cells in an experimental model (Hashimoto et al., 1983).

In experiments reported here, immunoliposomes suppressed tumour cell growth in vitro after thermal neutron irradiation.
Suppression was dependent upon the concentration of entrapped \(^{10}\)B-compound and also upon the density of anti-CEA conjugated with liposome. These results in \textit{in vitro} experiments suggest that an immunoliposome containing \(^{10}\)B-compound could be applied in BNCT as an effective carrier of \(^{10}\)B-compound to target cells, and the evaluation of the system in animal experiments remains to be carried out. In the present experiment, gamma-rays generated by thermal neutron irradiation did not exert toxic effects on cell growth at less than 3.36 Gy. Therefore, \(1 \times 10^{12} \) or \(2 \times 10^{15} \) cm\(^{-2}\) flux of thermal neutron may be recommended for BNCT, because inhibition of cell growth by concomitant gamma-rays was found to be negligible at these doses. The use of a more powerful atomic reactor may permit generation of more flux of thermal neutrons with less gamma-rays.

In the present experiments, immunoliposomes were found to be potential tools for BNCT, but problems remain to be solved before application to \textit{in vivo} BNCT. First, lipid components of liposomes should be studied more extensively. In the present experiment, multimellar liposomes were used, since small unilamellar ones composed of our lipid component were shown to be unstable in culture, although small unilamellar liposomes could be suitable for BNCT. If heat-sensitive liposomes (Sullivan & Huang, 1985) were used for BNCT, treatment with hyperthermia could possibly be combined with BNCT to improve the efficiency of targeting of \(^{10}\)B to tumour cells. Secondly, the technique of conjugating antibody to liposome has to be improved, since F(ab\(^{\prime}\) ) fragments seem to be better for targeting (Martin et al., 1981), because the depletion of the Fc fragment of the antibody molecule can obviate trapping of the molecule by Fc receptors on phagocytes or other cells.

However, the most important problem to resolve before clinical application of BNCT with immunoliposomes is how to carry the liposome specifically to the target tumour, or to promote penetration of blood vessels to reach tumour cells. It is conceivable that a complement component or some other substance could be conjugated to promote permeability of small blood vessels.

The authors are grateful to Dr Alistair Renwick, Department of Biochemistry, University of Auckland, Auckland, New Zealand, for criticism of the manuscript.

The work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture (No. 01570741).

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