

Inhibition of human pancreatic cancer growth in nude mice by boron neutron capture therapy

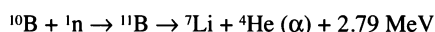
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Summary Immunoliposomes were prepared by conjugating anti-carcinoembryonic antigen (CEA) monoclonal antibody with liposomes containing [¹⁰B]compound. These immunoliposomes were shown to bind selectively to human pancreatic carcinoma cells (AsPC-1) bearing CEA on their surface. The cytotoxic effects of locally injected [¹⁰B]compound, multilamellar liposomes containing [¹⁰B]compound or [¹⁰B]immunoliposomes (anti-CEA) on human pancreatic carcinoma xenografts in nude mice were evaluated with thermal neutron irradiation. After thermal neutron irradiation of mice injected with [¹⁰B]solution, ¹⁰B-containing liposomes or [¹⁰B]immunoliposomes, AsPC-1 tumour growth was suppressed relative to controls. Injection of [¹⁰B]immunoliposomes caused the greatest tumour suppression with thermal neutron irradiation in vivo. Histopathologically, hyalinization and necrosis were found in ¹⁰B-treated tumours, while tumour tissue injected with saline or saline-containing immunoliposomes showed neither destruction nor necrosis. These results suggest that intratumoral injection of boronated immunoliposomes can increase the retention of ¹⁰B atoms by tumour cells, causing tumour growth suppression in vivo upon thermal neutron irradiation. Boron neutron capture therapy (BNCT) with intratumoral injection of immunoliposomes is able to destroy malignant cells in the marginal portion between normal tissues and cancer tissues from the side of ⁴He generation.

Keywords: boron neutron capture therapy (BNCT); immunoliposome; [¹⁰B]compound (Cs₂¹⁰B₁₂H₁₁SH); thermal neutron; local injection

Boron neutron capture therapy (BNCT) is aimed at inhibiting the growth of various cancers (Mishima et al, 1989a; Hatanaka and Nakagawa, 1994). The cytotoxic effect of BNCT is caused by a nuclear reaction between ¹⁰B and thermal neutrons (Locher, 1936; Kruger, 1940). This nuclear reaction is as follows:



The resultant lithium ions and α particles are high linear energy transfer particles with relatively high biological efficiency. Moreover, their short range in tissue (5–9 μm) restricts radiation damage to those cells in which boron is located at the time of neutron irradiation. Therefore, it is theoretically possible to kill tumour cells without affecting adjacent healthy cells, if ¹⁰B atoms can be accumulated selectively in tumour cells. BNCT has been used clinically in patients with malignant brain tumours (Dorn, 1994; Hatanaka and Nakagawa, 1994) and melanoma (Mishima et al, 1989a,b).

Liposomes are useful drug carriers (Bangham et al, 1965; Hashimoto et al, 1983; Konno et al, 1987; Singh et al, 1989; Tomita et al, 1989; Feakes et al, 1993), and it is possible to carry a large amount of [¹⁰B]compound in a liposome, which may be delivered to a tumour cell. We have reported that ¹⁰B atoms delivered by immunoliposomes are cytotoxic to human pancreatic carcinoma cells (AsPC-1) with thermal neutron irradiation in vitro (Yanagi et al, 1991).

Multilamellar vesical immunoliposomes are easily phagocytosed by the reticuloendothelial system (RES), so it is very difficult to accumulate the liposomal contents in the target cancers. Because of this, intravenous injections are no longer used in clinical practice, percutaneous echoic intratumoral injection or transarterial embolization (TAE) are preferred (Hasuie et al, 1992).

In this report, we prepared [¹⁰B]compound solutions in several concentrations and multilamellar liposomes containing [¹⁰B]compound or [¹⁰B]immunoliposomes. Intratumoral injection of ¹⁰B solutions, boronated liposomes and [¹⁰B]immunoliposomes all inhibited tumour cell growth with thermal neutron irradiation in vivo. The [¹⁰B]immunoliposomes showed the strongest suppression of tumour growth in the ¹⁰B treated groups. BNCT with intratumoral injection of immunoliposomes with a high content of [¹⁰B]compound has the ability to destroy malignant cells at the edge of the tumour mass.

MATERIALS AND METHODS

Chemicals

The caesium salt of undecahydro-mercaptopcloso-dodecaborate (Cs₂¹⁰B₁₂H₁₁SH) was kindly supplied by Shionogi Research Laboratories Co. Ltd. (Osaka, Japan). The solubility of the compound in water was 250 mM at 40°C.

Egg yolk phosphatidylcholine (Egg PC) was a gift from Nippon Fine Chemical Co. (Osaka, Japan). Cholesterol was obtained from Sigma Chemical Co. Ltd. (St Louis, MO, USA). Dipalmitoylphosphatidyl-ethanolamine (DPPE) was from Calbiochem-Behring (San Diego, CA, USA). *N*-hydroxy-succinimidyl 3-(2-pyridyl)dithio propionate (SPDP) was purchased from Pharmacia

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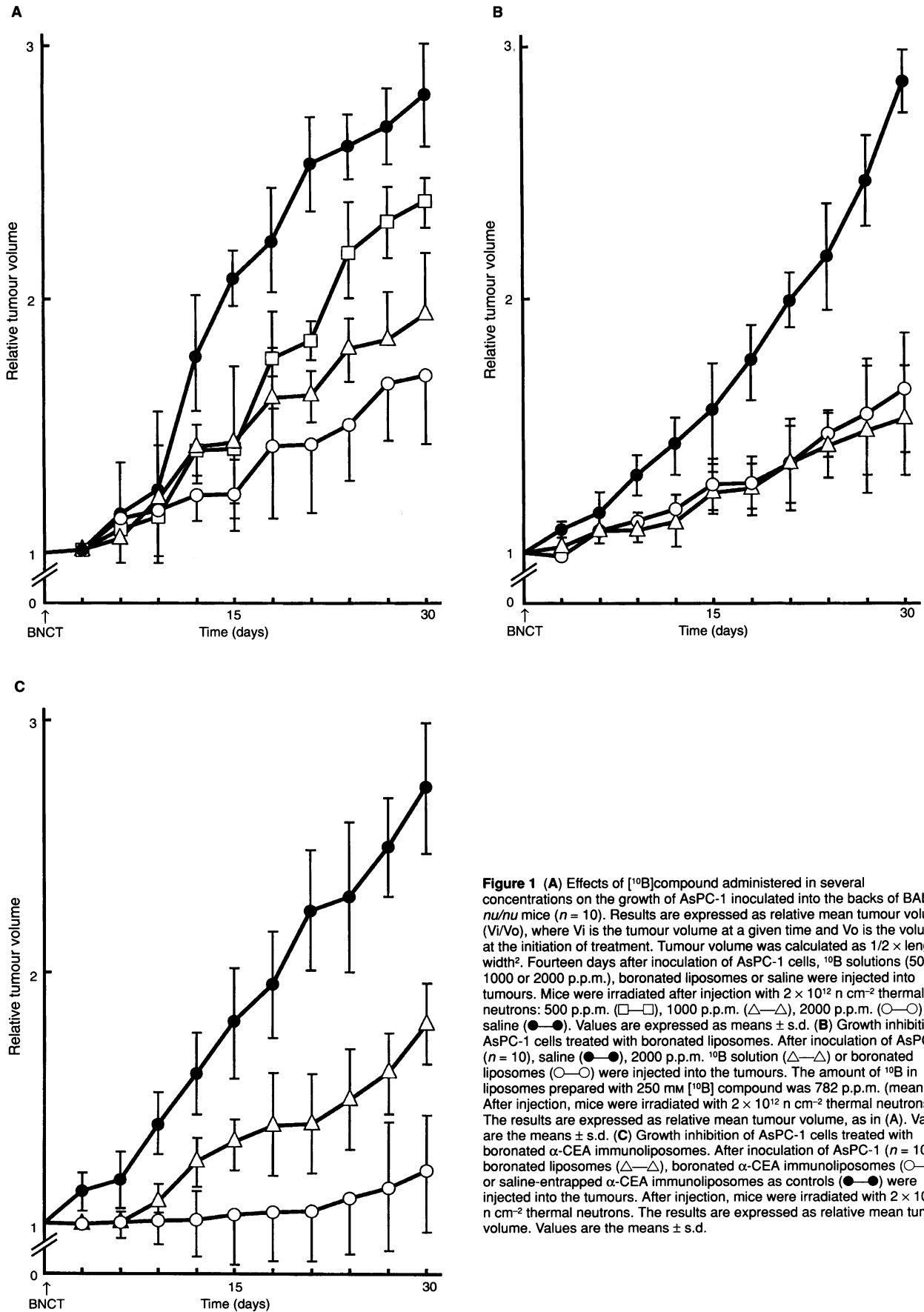


Figure 1 (A) Effects of [¹⁰B]compound administered in several concentrations on the growth of AsPC-1 inoculated into the backs of BALB/c *nu/nu* mice (*n* = 10). Results are expressed as relative mean tumour volumes (V_i/V_0), where V_i is the tumour volume at a given time and V_0 is the volume at the initiation of treatment. Tumour volume was calculated as $1/2 \times \text{length} \times \text{width}^2$. Fourteen days after inoculation of AsPC-1 cells, ¹⁰B solutions (500, 1000 or 2000 p.p.m.), boronated liposomes or saline were injected into tumours. Mice were irradiated after injection with $2 \times 10^{12} \text{ n cm}^{-2}$ thermal neutrons: 500 p.p.m. (□—□), 1000 p.p.m. (△—△), 2000 p.p.m. (○—○) and saline (●—●). Values are expressed as means \pm s.d. (B) Growth inhibition of AsPC-1 cells treated with boronated liposomes. After inoculation of AsPC-1 (*n* = 10), saline (●—●), 2000 p.p.m. ¹⁰B solution (△—△) or boronated liposomes (○—○) were injected into the tumours. The amount of ¹⁰B in liposomes prepared with 250 mM [¹⁰B] compound was 782 p.p.m. (mean). After injection, mice were irradiated with $2 \times 10^{12} \text{ n cm}^{-2}$ thermal neutrons. The results are expressed as relative mean tumour volume, as in (A). Values are the means \pm s.d. (C) Growth inhibition of AsPC-1 cells treated with boronated α -CEA immunoliposomes. After inoculation of AsPC-1 (*n* = 10), boronated liposomes (△—△), boronated α -CEA immunoliposomes (○—○) or saline-entrapped α -CEA immunoliposomes as controls (●—●) were injected into the tumours. After injection, mice were irradiated with $2 \times 10^{12} \text{ n cm}^{-2}$ thermal neutrons. The results are expressed as relative mean tumour volume. Values are the means \pm s.d.

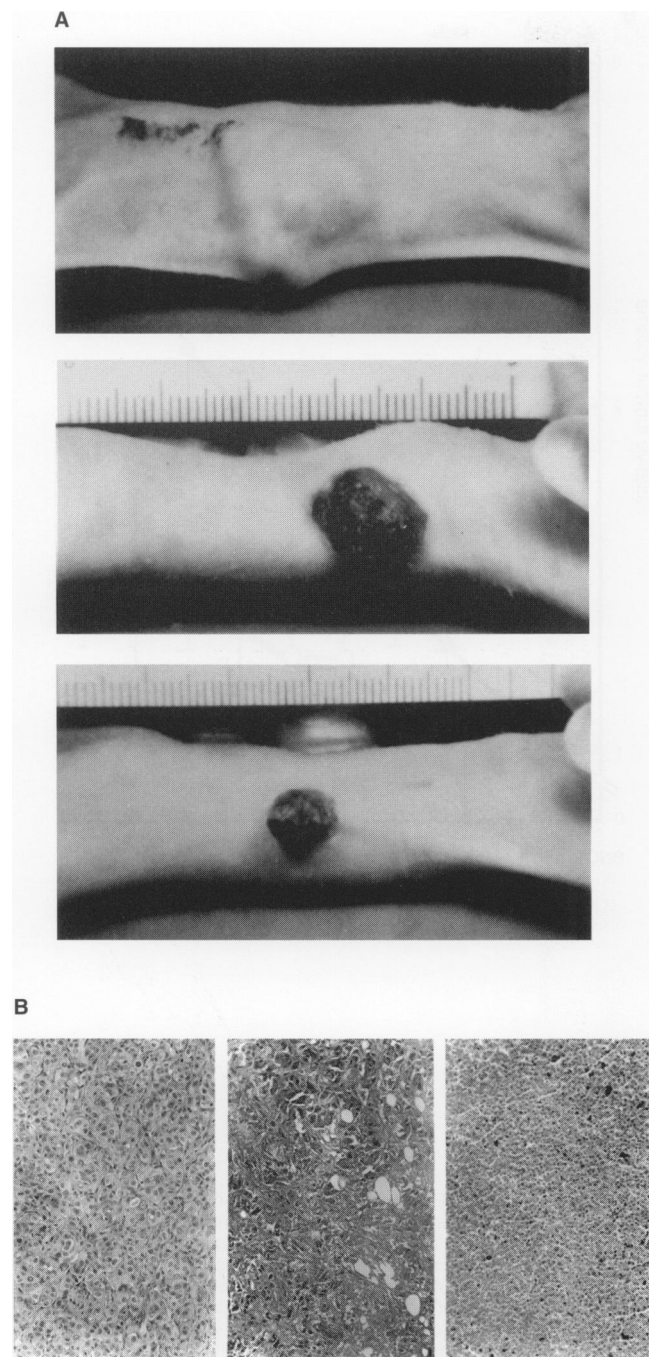


Figure 2 (A) Morphological findings of tumours at 30 days after BNCT. Mice were injected intratumorally with [^{10}B]immunoliposomes and irradiated with 2×10^{12} n cm $^{-2}$ thermal neutrons. Top, saline-entrapped α -CEA immunoliposomes (^{10}B : 0 p.p.m.); middle, boronated liposomes; bottom, boronated α -CEA immunoliposomes. The amount of ^{10}B in liposomes prepared with 250 mM [^{10}B]compound was 782 p.p.m. (mean). (B) Pathological findings of tumours after BNCT. Left, saline-entrapped α -CEA immunoliposomes (^{10}B : 0 p.p.m.); centre boronated liposomes; right, boronated α -CEA immunoliposomes. Original magnification $\times 200$

Fine Chemicals (Uppsala, Sweden); a stock solution (30 mM) in ethanol was stored at -20°C . Dithiothreitol was obtained from Sigma. 3-(2-pyridyldithio) propionyl-dipalmitoyl-phosphatidylethanolamine (DTP-DPPE) was prepared by reacting SPDP with DPPE as described by Barbet et al (1981).

Target tumour cells and mice

The human pancreatic carcinoma cell line AsPC-1 (Chen, 1982), which produces carcinoembryonic antigen (CEA) (Gold and Freedman, 1965), was obtained from Dainihon Seiyaku Co. Ltd. (Osaka, Japan). AsPC-1 was maintained in RPMI-1640 medium (Hazleton Biologics, Kansas, USA), supplemented with 10% fetal calf serum (Cell Culture Laboratories, Ohio, USA) and $100 \mu\text{g ml}^{-1}$ kanamycin. All cultures were incubated in air with a high moisture content and 5% carbon dioxide at 37°C . The medium was routinely changed three times a week.

Male BALB/c *nu/nu* mice were obtained from Nihon SLC (Shizuoka, Japan) and used at 6–7 weeks of age. In each experiment, mice of similar age and weight were selected. Mice were housed in plastic cages and maintained in an air-conditioned room. The procedures for tumour implantation and sacrifice of the animals were in accordance with approved guidelines of the Institution's Animal Ethics Committee.

Preparation of anti-human CEA monoclonal antibody

Detailed procedures for the preparation of the anti-human CEA monoclonal antibody have been described (Yanagië et al, 1991). Briefly, a cell clone, 2C-8, which secretes anti-human CEA monoclonal antibody, was established from somatic cell hybrids between mouse myeloma cells (X63, Ag8 and 653) and spleen cells of a BALB/c mouse that was hyperimmune to AsPC-1. The 2C-8 Ig G antibody was purified on a DEAE-52 cellulose column. The epitope recognized by the antibody was confirmed to be the 200-kDa CEA and 45-kDa non-specific cross-reacting antigen (NCA) by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Preparation of liposomes containing [^{10}B]compound

Egg yolk PC (5 μmol), cholesterol (5 μmol) and DTP-DPPE (0.25 μmol) were dissolved in chloroform-methanol (2:1) and mixed in a conical flask. The organic solvent was removed by evaporation at 40°C . An aliquot of 0.5 ml of 250 mM [^{10}B]compound ($\text{Cs}_2^{10}\text{B}_{12}\text{H}_{11}\text{SH}$) solution was added to the dried lipid film, and multilamellar vesicles were prepared by vortex dispersion (Tomita et al, 1989). Uncapsulated [^{10}B]compound was removed by centrifugation at 20 000 *g*.

Conjugation of the monoclonal antibody to the liposomal surface

Liposomes were centrifuged at 20 000 *g* after treatment for 30 min at room temperature with 20 mM dithiothreitol to ensure functional SH-groups (Yanagië et al, 1991). A sample of 1 ml of monoclonal antibody (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 G 25 column, liposomes were suspended in the antibody solution. Following incubation at 4°C overnight, the boronated immunoliposomes 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 (Yanagië et al, 1991). A prompt-gamma boron analysis technique was used to measure the 478 keV γ -photons produced during the ^{10}B (n, α) ^7Li reaction; this determines the boron content conjugated with anti-CEA monoclonal antibody and entrapped in liposomes and immunoliposomes (Kobayashi and Kanda, 1983).

Gamma-irradiation of cells

Cells irradiated with thermal neutrons were also irradiated with various doses of gamma rays generated concomitantly and dependent on the neutron dose. Detailed procedures for the estimation of the effect of gamma rays generated by thermal neutrons have been described (Yanagië et al, 1991). Briefly, AsPC-1 cells were exposed to gamma rays from a ^{137}Cs source in a Gamma Cell 40 (Atomic Energy of Canada, Ottawa, Canada) equivalent to the fluences of thermal neutron irradiation in an atomic reactor (ICRU, 1964). After irradiation, 0.25 μCi [^3H]TdR was added to each well and incubated for 8 h. The incorporated thymidine was quantified in a liquid scintillation spectrometer. Thermal neutron fluences (2×10^{12} and 5×10^{12} n cm^{-2}) generated 1.03 and 3.36 Gy of gamma ray, respectively, in the thermal column of the TRIGA-II reactor. Doses less than 1.03 Gy of gamma rays had no inhibitory effect on AsPC-1 cell growth. Since the growth of AsPC-1 cells was weakly suppressed by 3.36 Gy gamma ray, thermal neutron fluences less than 2×10^{12} n cm^{-2} were used in the in vivo experiment.

Thermal neutron irradiation

In vitro experiments

Detailed procedures for boron neutron capture have been described previously (Yanagië et al, 1991). Briefly, AsPc-1 cells were incubated in a 96-well microplate at 37°C in 5% carbon dioxide in air for 8 h in the presence of boronated anti-CEA or [^{10}B]immunoliposomes. After washing, the cells were irradiated with thermal neutrons at the TRIGA-II atomic reactor of Rikkyo University. After irradiation, 0.25 μCi [^3H]TdR was added to each well and incubated for 8 h. The incorporated thymidine was then measured as above.

In vivo experiments

AsPC-1 cells (1×10^7) were injected subcutaneously into the back of male BALB/c *nu/nu* mice. At 10–14 days after injection, when the estimated tumour weight reached 100–300 mg, 0.2 ml of ^{10}B solution (0, 500, 1000, or 2000 p.p.m.), boronated liposomes or boronated immunoliposomes were injected directly into the tumours. One hour after injection, mice were irradiated for 37 min with 9×10^8 ns $^{-1}$ cm^2 thermal neutrons (total fluence: 2×10^{12} n cm^{-2}) at the TRIGA-II atomic reactor. After irradiation, the effect of BNCT was evaluated on the basis of tumour volume (calculated as $1/2 \times \text{length} \times \text{width}^2$) and histological findings of the tumours at 3-day intervals (Konno et al, 1987). Boron concentrations of tumours and blood were analysed by prompt-gamma boron analysis technique (Kobayashi and Kanda, 1983).

RESULTS

Growth inhibition of tumour treated with ^{10}B solutions

In order to examine the therapeutic effect of ^{10}B solutions on the growth of AsPC-1 cells, tumour-bearing mice ($n = 10$) were injected intratumorally with ^{10}B solutions. The concentrations of ^{10}B solutions were 0 (control, saline), 500, 1000 and 2000 p.p.m. After injection, mice were irradiated with 2×10^{12} n cm^{-2} thermal neutrons. As shown in Figure 1A, significant inhibition of tumour growth was observed in all three groups treated with ^{10}B solutions compared with the control group. The inhibition was dose dependent.

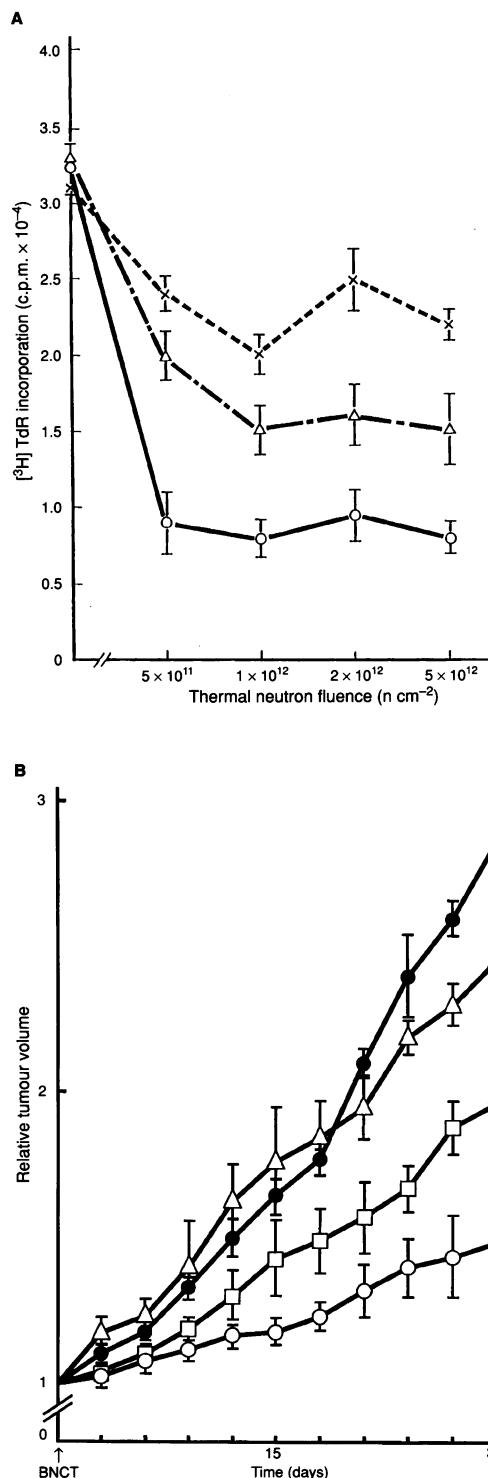


Figure 3 (A) Comparison of boronated antibody and immunoliposomes on growth inhibition of AsPC-1 in vitro. Immunoliposomes were prepared with 250 mM [^{10}B]compound and 4 mg ml^{-1} anti-CEA. Cytotoxicity was estimated by [^3H]TdR incorporation. Each point represents the mean \pm s.d. of triplicate assays. AsPC-1 cells were treated with boronated α -CEA monoclonal antibody (4 mg ml^{-1}) (Δ — Δ) or boronated α -CEA immunoliposomes (\circ — \circ). The growth of irradiated cells without MAb or immunoliposome treatment (X—X) served as a control. (B) Comparison of boronated antibody and immunoliposomes on growth inhibition of AsPC-1 in vivo. After inoculation of AsPC-1 ($n = 10$), boronated α -CEA monoclonal antibody (\square — \square), boronated α -CEA immunoliposomes (\circ — \circ), α -CEA monoclonal antibody (\blacktriangle — \blacktriangle) and saline-entrapped α -CEA immunoliposomes (\bullet — \bullet) were injected. Mice were then irradiated with 2×10^{12} n cm^{-2} thermal neutrons. Values are the means \pm s.d.

Growth inhibition of tumour treated with boronated liposomes

The effect of [^{10}B]compound entrapped in liposomes on AsPC-1 cell growth was examined with thermal neutron irradiation. Liposomes were prepared with 250 mM [^{10}B]compound. The amount of ^{10}B in liposomes was 782.77 ± 34.40 (mean \pm s.d.) $\mu\text{g ml}^{-1}$ liposome by prompt-gamma boron analysis technique. As shown in Figure 1B, intratumoral injection of these liposomes inhibited tumour cell growth to the same extent as 2000 p.p.m. of ^{10}B .

Effect of [^{10}B]immunoliposome on growth inhibition of tumour

In order to examine the therapeutic effect of [^{10}B]immunoliposomes on the growth of AsPC-1 cells, tumour-bearing mice were injected intratumorally with liposomes prepared with 250 mM [^{10}B]compound and 4 mg ml^{-1} anti-CEA, liposomes prepared with [^{10}B]compound only or liposomes prepared with anti-CEA only. After intratumoral injection of [^{10}B]immunoliposomes, we found boron concentrations in tumour tissue and blood of 49.59 ± 6.59 p.p.m. and 0.30 ± 0.08 p.p.m. respectively. After injection, the mice were irradiated with 2×10^{12} n cm^{-2} thermal neutrons. As shown in Figure 1C, tumour growth after BNCT using intratumoral injection of [^{10}B]immunoliposomes had been suppressed, the tumour had not regrown and became necrotic as shown Figure 2A (bottom). The control group treated with saline-entrapped immunoliposomes showed no inhibition of growth.

Morphological and pathological findings of tumour treated with [^{10}B]immunoliposomes

The therapeutic effects of [^{10}B]immunoliposomes were clear from the morphological and pathological findings. As shown in Figure 2, the tumour mass became necrotic and the increment in tumour volume was suppressed in the groups treated with [^{10}B]liposomes and [^{10}B]immunoliposomes, while tumour in the control group (^{10}B : 0 p.p.m.) continued to grow. The histology of tumours after BNCT showed necrosis and hyalinization.

Comparison of boronated antibody and immunoliposomes on growth inhibition of AsPC-1 in vitro

In order to compare the effect of boronated antibody or that of immunoliposomes on the growth of AsPC-1, the cells were treated with 4 mg ml^{-1} anti-CEA monoclonal antibody reacted with 250 mM [^{10}B]compound or liposomes prepared with 250 mM [^{10}B]compound and 4 mg ml^{-1} anti-CEA. The amount of ^{10}B conjugated with anti-CEA was 73.35 ± 13.90 $\mu\text{g ml}^{-1}$, and the amount of ^{10}B entrapped in immunoliposomes was 782.77 ± 34.40 $\mu\text{g ml}^{-1}$ by prompt-gamma boron analysis technique. Cells were then irradiated with thermal neutrons and cultured in vitro. As shown in Figure 3A, AsPC-1 cells treated with boronated anti-CEA and [^{10}B]immunoliposomes showed a reduction in growth by 63% and 35%, respectively, compared with untreated cells at $> 1 \times 10^{12}$ fluence.

Comparison of boronated antibody and immunoliposomes on growth inhibition of tumour in vivo

Tumour-bearing mice ($n = 10$) were injected with boronated anti-CEA or [^{10}B]immunoliposomes intratumorally. After injection,

mice were irradiated with 2×10^{12} n cm^{-2} thermal neutrons. As shown in Figure 3B, tumour growth after boronated anti-CEA and [^{10}B]immunoliposome treatment was suppressed by 51% and 26%, respectively, compared with the control. Tumours treated with anti-CEA alone showed no suppression.

DISCUSSION

We have previously shown that boronated immunoliposomes attach to AsPC-1 tumour cells and suppress their growth in vitro after thermal neutron irradiation (Yanagië et al, 1991). Suppression was dependent upon the concentration of the [^{10}B]compound in the liposomes and on the density of antibody conjugated to the liposomes.

Drugs encapsulated in liposomes are protected from enzymatic attack or immune reaction until they are released (Gabizon and Papahadjopoulos, 1988). Administration of liposomes in vivo is usually non-toxic (Hughes et al, 1989), since they are composed of biodegradable lipids. Immunoliposomes targeted to a specific organ may deliver a high concentration of the encapsulated drug to the organ (Leserman et al, 1981). Drugs released from immunoliposomes can reach target cells by free diffusion over a relatively short distance, thereby producing a localized high dose essential for cytotoxicity. The immunoliposome could serve as a local depot for sustained drug release. An increase in the number of antibody molecules per liposome will prolong the retention of the immunoliposome in the target organ (Hughes et al, 1989).

In the present experiment, intratumoral injection of ^{10}B solutions, boronated liposomes or boronated anti-CEA immunoliposomes suppressed tumour cell growth in vivo with thermal neutron irradiation. Tumours became necrotic and stopped growing for a while. Inhibition of tumour growth after injection of boronated liposomes was dependent upon the concentration of [^{10}B]compound in the solution. Liposomes have the capacity for keeping high concentrations of [^{10}B]compound in the tumour. In our experiments, cytotoxicity of the ^{10}B (n, α) ^7Li neutron capture reaction using the [^{10}B]immunoliposomes was greater than using antibody-free [^{10}B]liposomes. This result suggests that immunoliposomes containing [^{10}B]compounds can be applied to BNCT as effective carriers of those compounds to target cells.

BNCT is dependent on the absolute amount and location of boron in the tumour as well as the tumour-blood and tumour-normal tissue boron concentration ratios. Effective BNCT requires the delivery of a considerable amount (> 15 p.p.m.) of ^{10}B into the targeted malignant tissue (Coderre et al, 1990). Selective tissue cytotoxicity can be achieved in tissues containing ^{10}B that receive irradiation with thermal neutrons. α -Particles and ^7Li recoil nuclei have a very high linear energy transfer, resulting in the rapid transfer of their energy to cells within a diameter of approximately 12 μm . This energy transfer is highly destructive to critical molecular targets, such as nuclear DNA. Therefore, it is desirable to achieve a high gradient between the ^{10}B concentration in tumour compared with adjacent healthy tissues.

Studies in animal models indicate that tumour uptake after administration of the sulphhydryl borane dimer ($\text{Na}_4^{10}\text{B}_{24}\text{H}_{22}\text{S}_2$) is about twice the tumour uptake after administration of equal amounts of boron as a monomer (Joel et al, 1989, 1990). It was observed that the decrease in tumour boron concentration is much slower after infusion of dimer than with monomer. Mercapto-undecahydrododecaborate sodium ($\text{Na}_2^{10}\text{B}_{12}\text{H}_{11}\text{SH}$; BSH) may be oxidized to a dimer and react with disulphide-containing proteins. Joel et al (1989, 1990) measured the ^{10}B concentrations in the

tumours by prompt-gamma spectrometry. The boron-containing amino acid analogue, boronophenylalanine (BPA) has been shown to deliver boron selectively to melanoma tissue (Mishima et al, 1989b). BPA has been successful in animal models and has recently been used in Japan in human trials of BNCT for melanoma (Mishima et al, 1989a). In a study of BPA distribution in a rat glioblastoma, Coderre et al (1990, 1994) demonstrated a tumour–blood ratio of 4:1 compared with 0.7:1 for BSH. Ratios as high as 400:1 in tumour compared with normal brain were obtained 48 h after intravenous injection of 100 mg kg⁻¹ BOPP, while at the same time point, the tumour–blood ratio was only 11:1 (Hill et al, 1992; Malleshe et al, 1993).

Damage to brain endothelium may present particular problems, if reactor exposures take place when blood boron levels are high (Joel et al, 1990). Thus, it is critical to accumulate [¹⁰B]compound in the tumour mass by intratumoral injection and to avoid raising the concentrations of blood boron.

Most treatment failures are caused by local recurrence of the tumour, suggesting that more aggressive local therapy could be beneficial. BNCT using intratumoral injections of [¹⁰B]immunoliposomes will be useful to control local recurrence with minimal damage to adjacent normal tissue. There are several potential clinical applications of [¹⁰B]immunoliposomes for improving further methods for inhibiting the uptake of intravenously administered immunoliposomes by the reticuloendothelial system (RES). To obviate phagocytosis by the RES, we have tried to prepare polyethylene glycol-coated immunoliposomes (stealth immunoliposomes) (Maruyama et al, 1995). Experiments using systemic injection of stealth immunoliposomes are in progress for selective targeting of ¹⁰B atoms in tumours in the application of clinical BNCT.

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