Boron Neutron Capture Therapy: Linkage of a Boronated Macromolecule to Monoclonal Antibodies Directed against Tumor-Associated Antigens

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Two new protein-binding polyhedral boron derivatives, isocyanatoundecahydro-*closo*-dodecaborate(2-) (1) and isocyanato(trimethylamino)octahydro-*closo*-decaborate(1-) (2), were synthesized. These anionic isocyanates have long hydrolysis half-lives at pH 7 and react readily with primary or secondary aliphatic amines resulting in spontaneous urea linkage. Utilizing 1, 1100 boron atoms (7.3% boron by weight) were incorporated per molecule of a polyclonal antibody directed against human thymocytes (anti-thymocyte globulin) without denaturation. However, immunoreactivity of the conjugates was lost. Reaction of 1 and 2 with polylysine yielded boronated macromolecules containing 21-28% boron by weight (up to 2000 boron atoms per molecule). Polylysine boronated with 2 was successfully linked to antibody molecules employing the heterobifunctional linking molecules *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and *m*-maleimidobenzoyl sulfosuccinimide ester (sulfo-MBS). Separation of the conjugated antibody from the free boronated macromolecules and unconjugated antibody molecules has been achieved by gel filtration on a Sephacryl S-300 column. By linking boronated polylysine to antibodies, $>10^3$ boron atoms were incorporated with the attachment of this species to one or more sites on the antibody molecule. The resulting immunoconjugates contained $>10^3$ boron atoms per molecule, retained their immunoreactivity, and potentially might be useful for the selective delivery of large numbers of boron atoms to tumor cells.

The theoretical basis for boron neutron capture therapy (BNCT) is derived from the nuclear reaction that occurs when a stable isotope, boron-10, is irradiated with thermal neutrons to yield an unstable intermediate, boron-11, which undergoes instantaneous fission to yield lithium-7 and α particles ($^{10}B[n,\alpha]^7Li$). The ability to localize boron-10 with a high degree of selectivity for tumors is a key requirement if BNCT is to be successful. In 1952 Bale first proposed that antibodies directed against tumor-associated antigens could be used as carriers of boron.[1] Following this, a number of boron compounds were synthesized which possessed functional groups that could be incorporated into proteins without causing their denaturation.[2-4] Although denaturation did not occur, a major question was whether a large number of boron atoms could be incorporated into an antibody molecule without a significant less of immunoreactivity. In order to achieve concentrations of boron-10 capable of producing a lethal n,α reaction at the cellular level, approximately 10^9 boron atoms must be delivered to each tumor cell.[5] With antigen site densities of 10^6 per cell, 10^3 boron atoms must be incorporated per antibody molecule without adversely affecting its immunoreactivity. Goldenberg et al. have conjugated antibody directed against carcinoembryonic antigen with *p*-[1,2-dicarba-*closo*-(^{3}H)dodecaboran-2-yl]benzenediazonium ion that resulted in 30 boron atoms per IgG molecule.[6] Retention of immunoreactivity and selective localization in tumors were reported, but the small number of boron atoms per molecule of antibody theoretically would be insufficient to sustain a lethal n,α reaction.

Using the heterobifunctional reagent dicesium *N*-succinimidyl 3-(undecahydro-*closo*-dodecaboranyldithio)propionate,[7] we have succeeded in incorporating 10^3 boron atoms per molecule of the monoclonal antibody 17-1A that was produced against colorectal carcinoma.[8] However, there was a 90% reduction in immunoreactivity, as determined by an immunofluorescent binding assay.[7] This marked reduction in immunoreactivity was attributed to the fact that more that 80 sites per antibody molecule were covalently altered in order to incorporate 10^3 boron atoms. While the requisite number of boron atoms could be incorporated into antibodies without denaturation, nevertheless, significantly fewer sites must be modified if immunoreactivity is to be preserved. This has led us to synthesize water-soluble, boron-containing macromolecules that could be linked to either one or very few sites on the antibody molecule by means of heterobifunctional reagents, as described by Tsukada et al.[9] The present report describes the preparation of boronated macromolecules by reacting polylysine with two new protein-binding polyhedral borane derivatives and the linkage of these macromolecules to monoclonal antibodies.

Results and Discussion

Two new polyhedral borane isocyanates were prepared by the reaction of sodium azide on carbonyl-containing polyhedral boranes:

$$B_{12}H_{11}CO^- + NaN_3 \rightarrow \underset{\mathbf{1}}{B_{12}H_{11}NCO^{2-}} + Na^+ + N_2$$

$$Me_3NB_{10}H_8CO + NaN_3 \rightarrow \underset{\mathbf{2}}{Me_3NB_{10}H_8NCO^-} + Na^+ + N_2$$

Anions 1 and 2 were isolated as their tetramethylammonium salts and characterized by IR and ^{11}B NMR spectra and elemental analyses. Both 1 and 2 exhibit the highly characteristic B-H and -NCO stretching bonds (at $\sim$2500 and $\sim$2300 cm^{-1}) in the IR spectra. The ^{11}B NMR spectrum of 1 (Figure 1) is straightforward, as there is no possibility of isomerism in this monosubstituted icosahedral structure. The lone singlet at -8.9 ppm is assigned to the -NCO-bound B_1 atom. The doublet at -17.1 ppm

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(1) Bale, W. F. *Proc. Natl. Cancer Conf.* **1952**, *2*, 967.
(2) Hawthorne, M. F.; Wiersema, R. J.; Takasugi, M. *J. Med. Chem.* **1972**, *15*, 449.
(3) Wong, H. S.; Tolpin, E. I.; Lipscomb, W. N. *J. Med. Chem.* **1974**, *17*, 785.
(4) Sneath, R. L., Jr.; Soloway, A. H.; Dey, A. S. *J. Med. Chem.* **1976**, *19*, 1290.
(5) Tolpin, E. I.; Wellum, G. R.; Dohan, F. C., Jr.; Kornblith, P. L.; Zamenhof, R. G. *Oncology* **1975**, *32*, 223.
(6) Goldenberg, D. M.; Sharkey, R. M.; Primus, F. J.; Mizusawa, E. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 560.
(7) Alam, F.; Soloway, A. H.; McGuire, J. E.; Barth, R. F.; Carey, W. E.; Adams, D. *J. Med. Chem.* **1985**, *28*, 522.
(8) Steplewski, Z.; Koprowski, H. *Hybridomas in Cancer Diagnosis and Treatment*; Mitchell, M. F., Oettgen, H. F., Eds.; Raven Press: New York, 1982; pp 207-211.
(9) Tsukada, Y.; Kato, Y.; Umemoto, N.; Takeda, Y.; Hara, T.; Hirai, H. *J. Natl. Cancer Inst.* **1984**, *73*, 721.
(10) Hertler, W. R.; Knoth, W. H.; Muetterties, E. L. *Inorg. Chem.* **1965**, *4*, 288.

0022-2623/89/1832-2326$01.50/0

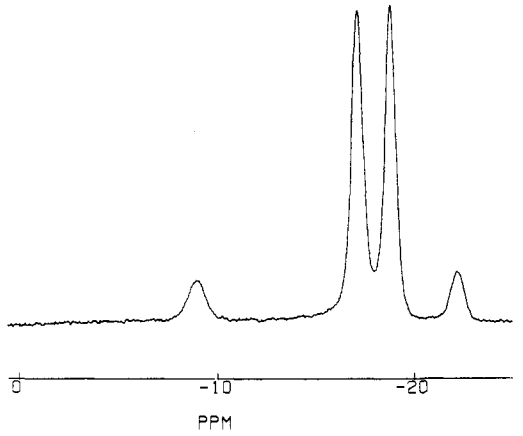


Figure 1. Proton-decoupled ^{11}B NMR of $[Me_4N]_2B_{12}H_{11}NCO$ in $MeOH/D_2O$.

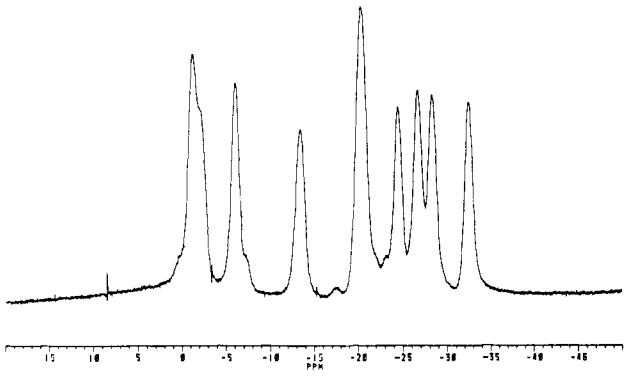
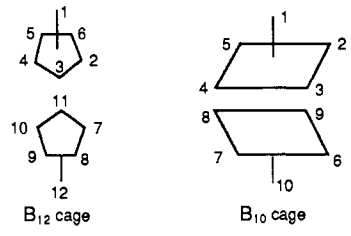


Figure 2. Proton-decoupled ^{11}B NMR of $[Me_4N]$-$Me_3NB_{10}H_8NCO$ is acetone-d_6.

is assigned to the five neighboring boron atoms (B_2–B_6), the doublet at −18.8 ppm is assigned to the next five boron atoms in proximity (B_7–B_{11}), and finally, the doublet at −22.2 ppm is assigned to the B_{12} atom, which is farthest from the −NCO group. The ^{11}B NMR of **2** (Figure 2) is more complex due to the presence of different isomers. This structure has 10 boron atoms forming a cage of bicapped square antiprism geometry. The cage structures of **1** and **2** with their numbering system are shown:



The composition of the precursor compound, $Me_3NB_{10}H_8CO$, has been described at length.[10] It is obtained by the reaction of oxalyl chloride with 2-$Me_3NB_{10}H_9^-$, and the product is a mixture of approximately 34% 2,4-$Me_3NB_{10}H_9CO$ and 66% of the enantiomeric pair 2,7(8)-$Me_3NB_{10}H_{10}H_8CO$; the −CO group is at the 7-position in one enantiomer and at the 8-position in the other.[10] The crude $NaMe_3NB_{10}H_8NCO$ obtained in 100% yield by the reaction of NaN_3 with $Me_3NB_{10}H_8CO$ is expected to have the same isomeric composition. However, purifying **2** by precipitating it as the tetramethylammonium salt followed by recrystallization results in more than 90% of 2,7(8)-enantiomeric pair and less than 10% of the 2,4-isomer. The depletion of the 2,4-isomer in the purified product could be due to greater water solubility of the tetramethylammonium salt of this isomer relative to those of the enantiomeric pair, or simply because a greater fraction of a minor component can be lost during crystallization even when solubilities are similar. The ^{11}B NMR of this salt, $[Me_4N]Me_3NB_{10}H_8NCO$, in acetone-d_6, was recorded at 86.67 MHz. The singlet at −13.5 ppm is assigned to the NCO-bound boron atoms (B_7 or B_8) in the enantiomeric pair, and the small singlet at −17.8 ppm is assigned to the NCO-bound B_4 atom in the 2,4-isomer. The assignment for the remaining peaks that follow apply to the 2,7(8)-enantiomeric pair, since corresponding peaks for the 2,4-isomer (present at less than 10% in the sample) are buried under the major isomer peaks. The other singlet at −2.1 ppm is assigned to the Me_3N-bound B_2 atom. There are two low-field doublets at −6.0 and −1.2 ppm assignable to the unsubstituted apical boron atoms (B_1 and B_{10}). The remaining peaks are due to the unsubstituted equatorial boron atoms.

Both **1** and **2** are relatively stable hydrolytically but react readily with aliphatic amines to form the corresponding ureas. For example, $[Me_4N]B_{12}H_{11}NCO$ reacts completely with a large excess of piperidine in less than 5 min at room temperature, as observed by thin-layer chromatography. This coupling reaction with amino groups makes these derivatives suitable for the boronation of antibodies and other proteins.

$$\underset{\mathbf{1}}{B_{12}H_{11}NCO^{21}} + (H_2N)\text{–antibody} \rightarrow (^{2\text{-}}B_{12}H_{11}NHCONH)\text{–antibody}$$

However, their reaction with amino groups in aqueous buffers is considerably slower, and hydrolysis of a competing reaction. Increasing the pH or temperature does increase the rate of reaction with amines, but it also increased the rate of hydrolysis. In addition, when antibodies or other polypeptides are involved in the reaction, aqueous buffers are necessary, and it may not be practical to use higher temperatures or very high pH. Therefore, most of the boronation reactions of proteins and other polypeptides with these derivatives were carried out at pH 9–10 and at room temperature or below. The reaction of **1** with bovine serum albumin (BSA) resulted in the incorporation of 4.2% boron by weight without any evidence of protein denaturation. Use of an earlier polyhedral borane isocyanate 1-(dimethylsulfono-6-isocyanato-*closo*-decaborate(1−) yielded 1.8% boron but with considerable precipitation of BSA.[3] A similar reaction of **1** with the lectin, concanavalin A, and a polyclonal antibody directed against human thymocytes [antithymocyte globulin (ATG), ATGAM, Upjohn Co., Kalamazoo, MI] yielded boron incorporation levels of 3.0–7.3% (450–1100 boron atoms per protein molecule).

The boron content of boronated BSA was obtained directly by elemental analysis of the freeze-dried material. The boron content of boronated concanavalin A and boronated antibodies was calculated from the ratio of boron concentration (by prompt-γ) and protein concentration of a solution of the conjugate as follows: a boronated ATG solution had a protein concentration of 7.07 μM and a boron-10 concentration (measured by prompt-γ method) of 6.3 μg of ^{10}B/mL, i.e., 0.63 mM. The total boron concentration was (0.63 mM × 5) = 3.15 mM, since ^{10}B is 20% of natural boron. Therefore, the number of boron atoms per ATG molecule was (3.15 mM/7.07 μM) = 446. By converting mole ratios to weight ratios, the weight percent boron in the boronated ATG was ~3.

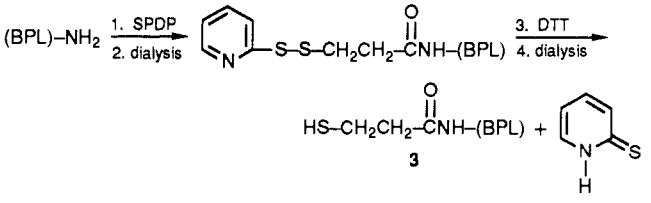
These isocyanate derivatives permit stable covalent incorporation of $>10^3$ boron atoms per antibody molecule. The boronated ATG, however, failed to exhibit any significant binding to lymphocytes, as determined by mem-

brane immunofluorescence.[7] These results confirmed previously reported findings that incorporation of 10^3 boron atoms to antibodies via single polyhedral boron cages, containing 10–12 boron atoms per cage, lead to extensive alteration of the antibody molecule. If immunoreactivity is to be preserved, fewer sites must be modified.

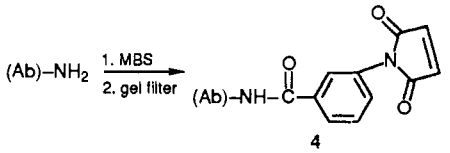
On the basis of these results, the synthesis of boron-containing macromolecules possessing 1000–2000 boron atoms was intitiated. Such structures could be linked to very few sites on the antobody molecule, thereby providing a higher probability for the retention of immunoreactivity. Accordingly, poly(DL-lysine) (degree of polymerization DP = 170) was boronated with the isocyanate 1, resulting in macromolecules containing 28% boron by weight. From the boron content and the degree of polymerization, it is estimated that this boronated polylysine (BPL) contained approximately 1700 boron atoms per molecule, on an average. Simply adding this BPL generated from 1 to the antibodies, ATG or 17-1A, produced immediate precipitation. However, if the less negatively charged BPL that is formed from 2 was used, no precipitation occurred following admixture with either antibody in certain buffers and pH ranges. This latter BPL preparation containing 21–24% boron, therefore, was used to incorporate boron into antibodies.

There are several methods by which macromolecules can be covalently attached to antibodies. One approach involves homobifunctional reagents, such as glutaraldehyde, that can cross-link two different macromolecular species. By use of glutaraldehyde, it has been reported that the retention of immunoreactivity was observed when a serum albumin polymer was incorporated into an antibody.[11] The use of glutaraldehyde to link boronated BSA to ATG resulted in protein denaturation, indicating that this approach was unsuitable. A second approach is the activation of the carboxyl group on BPL via carbodiimide reagents to generate species that could react with amino groups on antibodies. Such reactive carboxyl groups could also form stable linkages with free amino groups on BPL, thereby leading to unwanted homoconjugation. Once again, separation of such a species from the boronated antibody would be essential.

In order to minimize these separation problems, a third approach was used that utilizes two heterobifunctional agents.[9] First, *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was bound via a free amino group to BPL. The disulfide linkage then was cleaved with dithiothreitol (DTT), generating a sulfhydryl-containing BPL, 3:



Second, the antibodies were modified by incorporating *m*-maleimidobenzoyl sulfosuccinimide ester (sulfo-MBS) into them:



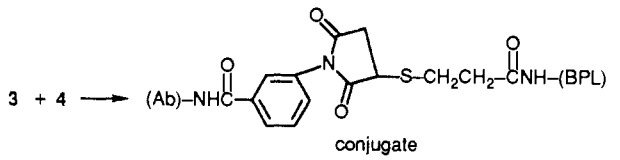
(11) Poznansky, M. J.; Bhardwaj, D. *Biochem. J.* **1981**, *196*, 89.

Table I. Immunofluorescent Assay of Boronated and Nonboronated Monoclonal Antibody IB16-6 against B16 Melanoma Cells

ab concn, μg/mL	immunofluorescent reactivity: boronated IB16-6	immunofluorescent reactivity: native IB16-6	ab concn, μg/mL	immunofluorescent reactivity: boronated IB16-6	immunofluorescent reactivity: native IB16-6
10	3+	3+	0.625	1+/2+	2+
5	3+	3+	0.312	0/1+	1+/2+
2.5	2+/3+	2+/3+	0.156	0	0/1+
1.25	2+/3+	2+/3+			

[a] + fluorescence was scored as follows: 4+, very strong; 3+, strong; 2+ moderate; 1+, weak; 0, no fluorescence. Intermediate reactivity was scored as 2+/3+, 1+/2+, or 0/1+.

The modified antibodies and the sulfhydryl-containing BPL were coincubated, resulting in covalent linkage with the formation of a boronated antibody:



A key question is whether such modified antibodies would retain their immunoreactivity. In order to determine this, it is essential that the immunoconjugates be separated from both unconjugated antibodies and boronated macromolecules. The immunoconjugates synthesized by our method were separated from BPL and from nonboronated antibodies by means of chromatographic techniques using gel filtration. Higher molecular weight species, which may arise from the incorporation of multiple maleimido groups into antibodies or from multiple sulfhydryl groups attached to polylysine, could also be separated on the basis of molecular weight differences. Commercially available polylysine has a broad molecular weight spectrum, and this has necessitated size exclusion purification. It would be preferable to have a macromolecular species with a more defined molecular weight range. A monoclonal antibody IB16-6, produced against the murine B16 melanoma,[12] was boronated by this method. The resulting immunoconjugate contained an average of 2700 boron atoms per molecule. Its immunoreactivity, as determined by a semiquantitative immunofluorescent assay, was similar to that of the native antibody (Table I). Its immunoreactivity was also determined by a quantitative enzyme-linked immunosorbent assay (ELISA) and found to be 58% of the native antibody. Another monoclonal antibody, 17-1A,[8] boronated by this method contained an average of 1550 boron atoms and retained 40% of its immunoreactivity as determined by ELISA. Other boronated antibodies that have been produced by this methodology retained 40–90% of the immunoreactivity of the native antibodies.[13-15] Current efforts are directed toward the preparation of polylysine with a narrower molecular weight range and the synthesis of single boronated macromolecular species that contain the requisite number of boron atoms to sustain a

(12) Johnson, C. W.; Barth, R. F.; Adams, D. M.; Holman, B.; Price, J. E.; Sautins, I. *Cancer Res.* **1987**, *47*, 1111.
(13) Barth, R. F.; Alam, F.; Soloway, A. H.; Adams, D. M.; Steplewski, Z. *Hybridoma* **1986**, *5*, Suppl. 1, S43.
(14) Mafune, N.; Barth, R. F.; Alam, F.; Soloway, A. H.; Blue, T. E.; Adams, D.; Sautins, I.; Curran, J. F.; Steplewski, Z. *Proc. Am. Assoc. Cancer Res.* **1987**, *28*, 391.
(15) Barth, R. F.; Mafune, N.; Alam, F.; Adams, D.; Soloway, A. H.; Makroglou, G.; Oredipe, O. A.; Blue, T. E.; Steplewski, Z. *Strahlenther. Onkol.* **1989**, *165*, 142.
(16) Knoth, W. H.; Sauer, J. C.; Balthis, J. H.; Miller, H. C.; Meutterties, E. L. *J. Am. Chem. Soc.* **1967**, *89*, 4842.

lethal n,α reaction at the cellular level.

Experimental Section

Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. IR spectra were obtained (KBr pellets of samples) on a Beckman IR-4230 spectrometer. Boron-11 NMR spectra of samples were recorded at 96.3 MHz on a Bruker WM-300 spectrometer or at 86.67 MHz on an IBM AF-270 spectrometer; chemical shifts are given in ppm downfield from an external reference, $BF_3(OEt)_2$. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Preparation of $[Me_4N]_2B_{12}H_{11}NCO$. Fifteen grams (0.06 mol) of $[Me_4N]B_{12}H_{11}CO$ was stirred in 125 mL of acetonitrile. Sodium azide (5.0 g, 0.08 mol) was added over a 10-min period, and the mixture was stirred until bubbling ceased (75 min). It was then filtered, and the filtrate was evaporated to dryness. The residue was crystallized from 100 mL of H_2O containing 10 g of tetramethylammonium chloride to obtain 13.0 g (65% yield) of $[Me_4N]_2B_{12}H_{11}NCO$ as a crystalline, colorless solid, mp >300 °C. Anal. ($B_{12}C_9H_{35}N_3O$) C, H, N, B. The sodium salt, $Na_2B_{12}H_{11}NCO$, was obtained by passing a solution of $[Me_4N]B_{12}H_{11}NCO$ in H_2O/acetonitrile through a column containing Amberlite IR-120 (plus) ion-exchange resin (sodium form) and evaporating the effluent to dryness on a rotary evaporator at 40 °C under reduced pressure. This was further dried under vacuum.

The IR spectrum includes a strong isocyanate absorption at 2321 cm^{-1} and B–H absorption at 2510 cm^{-1}. The ^{11}B NMR spectrum in methanolic D_2O consists of a singlet at −8.9 ppm representing the substituted boron atom, two major overlapping doublets of equal intensity at −17.1 and −18.8 ppm (J_{B-H} = 147, 144 Hz), and a doublet at −22.2 ppm (J_{B-H} = 132 Hz).

Preparation of $NaMe_3NB_{10}H_8NCO$. Sodium azide (0.7 g, 10.7 mmol) was added to a solution of $Me_3NB_{10}H_8CO$ [1.7 g, 8.4 mmol; $Me_3NB_{10}H_8CO$ was prepared by a literature procedure,[10] which yields a mixture of the 2,4- and 2,7(8) isomers] in 30 mL of acetonitrile in a nitrogen atomsphere. The mixture was stirred for 1 h and then filtered to remove excess NaN_3. The filtrate was evaporated to leave $NaMe_3NB_{10}H_8NCO$ (2.0 g, 100%).

Boronations of polylysine were carried out with the sodium salt without further purification. For analysis, the sodium salt was precipitated from water with tetramethylammonium chloride, and then the tetramethylammonium salt so obtained was recrystallized from water (mp >300 °C). Anal. ($B_{10}C_8H_{29}N_3O$) C, H, N; B: calcd, 37.09; found, 35.72.

The IR spectrum displayed strong bands at 2475 cm^{-1} (B–H) and 2312 cm^{-1} (–NCO). The strong carbonyl band (2146 cm^{-1}) characteristic of the starting material, $Me_3NB_{10}H_8CO$, was absent. The ^{11}B NMR spectrum of $[Me_4N]Me_3NB_{10}H_8NCO$ in acetone-d_6 recorded at 86.67 MHz exhibits a doublet at −32.6 ppm (1 B, J_{B-H} = 133 Hz); a multiplet from −29.1 to −23.8 ppm that collapses to three peaks at −28.3 ppm (1 B), −26.7 ppm (1 B), and −24.5 ppm (1 B) on proton decoupling; a doublet at −20.4 ppm (2 B, J_{B-H} = 123 Hz); a singlet at −13.5 ppm (1 B); a doublet at −6.0 ppm (1 B, J_{B-H} = 123 Hz); and a singlet at −2.1 ppm (1 B) which overlaps a doublet at −1.2 ppm (1 B, J_{B-H} = 146 Hz). There is also a small singlet at −17.8 ppm and shoulders at 0.5, −5.2, and −23.5 ppm that indicate the presence of the 2,4–isomer (less than 10%).

Determination of Boron Content. The boron content of the boronated antibodies were determined by the prompt-λ method[17] at Brookhaven National Laboratories. In a nuclear reactor, 1.0 mL of the boronated antibody solution in a boron-free glass tube was subjected to a thermal neutron flux of 3×10^7 $n \cdot cm^{-2} \cdot s^{-1}$ for 200 s. The nuclear reaction of ^{10}B with thermal neutrons results in the prompt emission of a 480-keV γ-ray. The boron content was determined from the measurement of the 480-keV peak area in the γ-ray spectrum. For calibration, NBS standard boron-10-enriched boric acid was used with the appropriate buffer as the blank.

Boronation of ATG with $Na_2B_{12}H_{11}NCO$. In a 20-mL vial, 32 mg of $Na_2B_{12}H_{11}NCO$ was dissolved in 0.5 mL of phosphate-buffered saline (PBS), pH 7.5. To this was added 0.5 mL of ATG (4.5 mg) in PBS. The mixture was incubated at room temperature for 30 min and for 3 days at 4 °C. The boronated antibody was purified by gel filtration through two 0.8 cm × 18 cm Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) G-25 columns with PBS, pH 7.5, as eluent. The protein concentration of the purified fraction was 7.07 μM, and the boron-10 concentration, measured by prompt-γ, was 6.3 μg of ^{10}B/mL, indicating that approximately 450 boron atoms were linked per antibody molecule (3.15 mM/7.07 μM). In another experiment, utilizing a longer reaction time (23 days at 4 °C) and a higher pH (9–10), approximately 1100 boron atoms were linked per ATG molecule. Immunoreactivity of these two boronated ATG samples, as measured by an immunofluorecent assay, was lost by chemical modification.

Boronation of BSA with $Na_2B_{12}H_{11}NCO$. In a 20-mL vial, 100 mg of BSA was dissolved in 5.0 mL of PBS, and the pH was adjusted to approximately 9 and made 0.01% in NaN_3. To this was added 250 mg of $Na_2B_{12}H_{11}NCO$ with stirring for a total of 10 h at room temperature and 11 days at 4 °C. The mixture was concentrated to 2 mL on a Minicon (Amicon, Danvers, MA) and passed through a Sephadex PD-10 column, followed by passage through a 0.9 cm × 38 cm Sephadex G-150 column. The protein fraction was freeze-dried. Subsequently, this material was dissolved in 2 mL of H_2O, dialyzed against 2 L of H_2O (four changes), and lyophilized, and a sample was sent for boron analysis. Anal. B: found, 4.2.

Boronation of Concanavalin A with $Na_2B_{12}H_{11}NCO$. In a 20-mL vial, 25 mg of ConA in 3.5 mL of PBS, pH 9, was treated with 50 mg of $Na_2B_{12}H_{11}NCO$. The mixture was stirred at ambient temperature for a total of 6 h and incubated at 4 °C for 3 days. The protein then was concentrated and purified by sequential passage through two 0.8 cm × 18 cm Sephadex G-25 columns, with PBS, pH 7.2, as eluent. Protein analysis and boron analysis by prompt-γ indicate that approximately 1080 boron atoms had been linked per molecule of the lectin.

Boronation of Poly(DL-lysine) with $Na_2B_{12}H_{11}NCO$. In a 20-mL vial, 200 mg of poly(DL-lysine)·HBr (MW = 35 000, DP = 170; Sigma Chemical Co., St. Louis, MO) was dissolved in 15 mL of water. To this was added 250 mg of Na_2CO_3 and 755 mg of $Na_2B_{12}H_{11}NCO$. The mixture was stirred at ambient temperature for 11 days, then filtered to remove suspended solids, and finally purified on a 0.9 cm × 28 cm Sephadex G-25 column with water as eluent. The larger molecular weight fraction was freeze-dried to yield 62 mg of light, fluffy boronated poly(DL-lysine) (BPL). Anal. B: found, 27.52. Other batches of poly(DL-lysine) were boronated with $Na_2B_{12}H_{11}NCO$ as described above, except that the purification was sometimes done by dialysis instead of gel filtration.

Boronation of Poly(DL-lysine) with $NaMe_3NB_{10}H_8NCO$. In a 20-mL vial, 1.0 g of poly(DL-lysine)·HBr (MW = 40 000, DP = 194) was dissolved in a 10 mL of H_2O and adjusted to pH 9 with Na_2CO_3. To this was added 1.5 g of $NaMe_3NB_{10}H_8NCO$, 5 mL of acetone, and 0.5 mL of 0.5% NaN_3 and the mixture stirred at ambient temperature for 13 days. The mixture was filtered to remove suspended solids. The boronated poly(DL-lysine) was then purified by dialysis against 1 L of water/acetone twice, and then against 1 L of water five times, and freeze-dried to yield 890 mg of a fluffy colorless solid. Anal. B: found, 22.69.

Subsequent batches of poly(DL-lysine) (different molecular weights) boronated with $Me_3NB_{10}H_8NCO$ contained 21–24% boron. It sometimes was necessary to add acetone to the reaction mixture to prevent precipitation of polylysine–boron compound complexes at the beginning of the reaction and during the long reaction time.

Conjugation of Boronated Poly(DL-lysine) to Antibody. The BPL described above (DP = 194, boronated with $NaMe_3NB_{10}H_8NCO$; 22.69% boron) was conjugated to monoclonal anti-B16 melanoma IB16-6 by the following three-step procedure:

(a) Introduction of Thiol Groups on BPL. BPL (20 mg) was dissolved in 1 mL of 0.1 M phosphate buffer, pH 7.5. To this was added a solution of 5 mg of SPDP (Pierce Chemical Co., Rockford, IL) in 100 μL of DMF, and the mixture was stirred at room temperature for 3 h, followed by dialysis against water (6 L, three changes). The pH of the dialyzed sample was adjusted to 8.5. This was treated with 4.5 mg of DTT at 45 °C for 1 h. The absorbance at 343 nm (due to pyridine-2-thione released in deprotecting the thiol group; ϵ = 8080) was measured. The results

(17) Fairchild, R. G. Workshop on Radiobiology and Tumor Therapy with Heavy Particles, and IAEA Research Coordination Meeting, Villigen, Switzerland, April 27–29, 1982.

indicated that an average of 2 thiol groups were introduced per BPL molecule. This was dialyzed against water (6 L, three changes) and finally once against 1 L of 0.1 M Tris buffer, pH 8.5.

(b) **Introduction of Maleimido Groups on Antibody.** Antibody IB16-6 (10 mg) was equilibrated in 0.1 M Tris buffer, pH 8.5. To this was added 0.15 mg of sulfo-MBS (Pierce Chemical Co.) and the mixture incubated for 30 min at ambient temperature. It was then passed through a 0.8 cm × 40 cm Sephadex G-25 column with Tris buffer, pH 8.5 (60 mL/h), and the protein fractions containing 4 were pooled.

(c) **Linkage of BPL-SH to 4.** These two species (approximately 20 mg of **3** in 2 mL and 8 mg of 4 in 8 mL of Tris buffer, pH 8.5) were combined, and the mixture was incubated for 24 h at ambient temperature. This was then loaded on a 1.6 cm × 25 cm Sephacryl (Pharmacia Fine Chemicals) S-300 column and eluted with 0.1 M Tris and 0.2 M NaCl, pH 8.5 (45 mL/h). Absorbance at 280 nm showed two peaks. A polyacrylamide gel electrophoresis (10% polyacrylamide to 0.05M Tris/0.2 M glycine, room temperature, 50 mA, 4 h) revealed that the fractions in the first peak contained the conjugated antibody, and the second peak was unconjugated antibody. The fractions in the first peak were pooled and analyzed for protein concentration, boron concentration (by prompt-γ), and antibody activity by an immunofluorescent assay. The protein concentration of the analyzed sample was 1.33 μM, and the boron-10 concentration was 7.2 μg/mL. This indicates 2700 boron atoms per antibody atoms per antibody molecule.

Immunofluorescent Assay of the Boronated Antibody. B16 melanoma cells were disaggregated from monolayer cultures by the addition of 0.1 mM ethylenediaminetetraacetic acid. Cells were sedimented at 350*g* for 10 min and resuspended, and their final concentration was adjusted to 3×10^7 cells/mL. Fifty-microliter volumes containing 1.5×10^6 cells were added to conical microfuge tubes, and then 50 μL of serially 2-fold diluted boronated and nonboronated antibodies were added to the tubes containing cells. They were incubated on ice for 30 min, washed three times, and then resuspended in 100-μL volumes of PBS. An equal volume of fluorescein isothiocyanate conjugated rabbit anti-rat IgG (Cappel Laboratories, Cochraneville, PA) at a dilution of 1:25 was added to the B16 cells, which were incubated on ice for an additional 30 min, washed three times with PBS, and then resuspended in a 1:1 mixture of 50% glycerol and PBS. Fluorescence was scored on a 0–4+ scale by using a Zeiss fluorescence microscope with epiillumination and a halogen light source. The results given in Table I are representative of experiments and show that the immunoreactivity of the boronated IB16-6 was similar to that of the native antibody. Previous studies have shown that the results obtained with this semiquantitative assay correlated well with those obtained with the same antibody using flow cytometry. Antibody IB16-6 showed strong binding to parental B16 cells and the B16-F1, B16F10, and B16-F10FLB sublines, with no nonspecific binding to a broad panel of tumor cell lines, normal thymocytes, and renal adherent cells.[12] Boronated IB16-6 similarly was nonreactive with nonmelanoma cells.

Boronation of 17-1A. Monoclonal antibody 17-1A was boronated with BPL by the identical procedure described above for 1B16-6. The protein concentration of the purified immunoconjugate was 2.17 μM, and the boron-10 concentration was 6.7 μg/mL. This indicates that an average of 1550 boron atoms were linked per antibody molecule.

Determination of the Immunoreactivity of Boronated 17-1A and IB16-6 by ELISA. SW 1116 colorectal cancer cells at a concentration of 1.25×10^6 cells/mL in L15 media containing 10% fetal bovine serum were added in 100-μL aliquots to each well of 96-well microtest plates and incubated at 37 °C for 24 h. Then the medium was poured off, each well was filled with 100 μL of media containing MoAb 17-1A in concentrations of 0.1 to 100 μg, or known quantities of boronated 17-1A, and plates were incubated at 37 °C for 2 h. After removing samples from the wells, they were washed three times with PBS, 80 μL of 15 μg/mL biotinylated horse anti-mouse IgG (Vectastain, Vector Laboratories, Inc., Burlingame, CA) dissolved in 1% normal horse serum in PBS was added, and the plates were incubated at 37 °C for 20 min. Then, the biotinylated antibody was removed and the wells were washed three times with PBS. Forty microliters of avidin–biotin–horseradish peroxidase complex in 0.1% Tween 20 in PBS (ABC reagent, Vectastain) was then added, and the plates were incubated at 37 °C for 20 min. After removal of the supernatant, the plates were washed five times with PBS, 100 μL/well of 3 mg/mL o-phenylenediamine, 0.02% H_2O_2, 0.1 M citrate, and 0.2 M $NaHPO_4$, pH 5.5, was added, and the chromogenic reaction was allowed to develop. This was terminated by adding 40 μL/well 6 N HCl, and color was read at 490-nm absorbance with a ELISA microreader (Model 2550 EIA, Bio-Rad Laboratories).

The absorbance of a 50 μg/mL sample of the boronated 17-1A yielded an absorbance of 0.749. From the standard curve, the concentration of the native 17-1A that gave the same absorbance was 20 μg/mL. The percent immunoreactivity of this boronated 17-1A compared to the native 17-1A therefore was 20/50 = 40%.

Determination of the immunoreactivity of native and boronated IB16-6 also was carried out as described above with the following changes. The target cells were parental B16 melanoma cells, and the biotinylated antibody was rabbit anti-rat IgG (Vectastain). The absorbance of a 12.5 μg/mL of the boronated IB16-6 was 0.79, and from the standard curve the concentration of native IB16-6 that yielded the same absorbance was 7.3 μg/mL. The percent immunoreactivity of this boronated IB16-6 compared to that of the native IB16-6 was therefore 7.3/12.5 = 58%.

Acknowledgment. This work was supported by Grants 1 RO1 CA 41288 and P-30-CA-16058-14 from the National Cancer Institute, DE-ACO2-82ERG0040 from the Department of Energy and the Office of Research and Graduate Studies, The Ohio State University. We thank Dr. Ralph G. Fairchild and Ms. Pessy Micca of the Brookhaven National Laboratory, Upton, Long Island, NY, for boron determinations by prompt-γ spectrometry. We thank the Upjohn Co., Kalamazoo, MI, for generously providing the ATG and Mrs. Ada Morgan and Ms. Kathern Gill for secretarial assistance, and Dr. Yutaka Tsukada for helpful advice.