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Boronation of Antibodies with Mercaptoundecahydro-closo-dodecaborate(2-) Anion for Potential use in Boron Neutron Capture Therapy

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The anionic polyhedral borane derivative, mercaptoundecahydro-closo-dodecaborate(2-), has been evaluated as a boronating agent for antibodies. The objective of these studies was the selective delivery of boron to neoplasms for neutron capture therapy. Incubation of a large excess of this anion with the polyclonal antibody antithymocyte globulin (ATG) resulted in the incorporation of 9–13 mol of the anion per mol of antibody. The extent of boron incorporation into the protein was measured either by tritium-labeled $B_{12}H_{11}SH^{2-}$ or by direct boron determination with neutron activation anlaysis. The nature of the covalent linkage of the anion to the antibody appeared to involve the formation of a new disulfide bond by a thiol–disulfide exchange. The number of boron atoms incorporated into antibodies by this method appeared to be inadequate for neutron capture therapy. However, such boronated antibodies may have potential for the detection of molecules of biologic interest by means of electron energy loss spectroscopy.

Introduction

The theoretical basis for the tumoricidal effects of boron neutron capture therapy (BNCT) depends upon the specific localization of the neutron absorber (boron-10) in the tumor, followed by irradiation with thermal neutrons. A review of earlier work (Soloway, 1964) on BNCT reveals that clinical failures were a direct result of the inability to localize the available boron compounds selectively in neoplastic cells. This limitation prompted the development of protein-binding boron compounds that could be incorporated into antibodies directed against tumor-associated surface antigens. With the advent of hybridoma technology (Köhler *et al.*, 1976; Galfrè and Milstein, 1981), it has been possible to produce monoclonal antibodies directed against a variety of tumor associated antigens. By linking boron-containing compounds to such antibodes, it may be possible to deliver boron-10 selectively to tumor cells for their destruction by BNCT.

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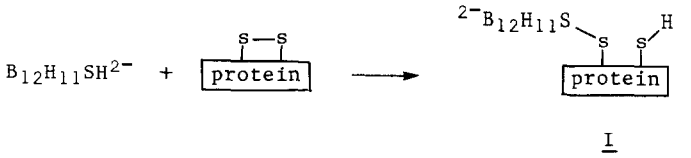
Since 1968, mercaptoundecahydro-closo-dodecaborate, $B_{12}H_{11}SH^{2-}$, has been used clinically (Hatanaka, 1983) in Japan for the chemoradiotherapy of glioblastoma, a highly malignant brain tumor. It has been suggested that this particular anionic polyhedral borane has tumor-localizing properties (Soloway *et al.*, 1967), but the mechanism by which this compound becomes bound to neoplastic cells remains unclear. Since there appear to be distinct biological differences between the unsubstituted icosahedral anion $B_{12}H_{12}^{2-}$ and the sulfhydryl derivatives (Soloway *et al.*, 1967), alteration of the blood–brain barrier in brain tumors may not be the sole basis by which preferential localization is achieved. The involvement of the sulfhydryl moiety is obviously crucial.

One possible mechanism for this ability to localize may involve the interaction of the sulfhydryl moiety with disulfide groups in circulating blood proteins, thereby producing boronated mixed disulfide linkages. Such boronated proteins may be incorporated into tumor cells by pinocytosis, thereby accounting for the differences between these two polyhedral boranes. A possible mechanism was tentatively proposed (Soloway *et al.*, 1967), when it was noted that



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Scheme 1

$B_{12}H_{11}SH^{2-}$ bound covalently to the protein BSA (bovine serum albumin), while $B_{12}H_{12}^{2-}$ did not. A later, more extensive study (Nakagawa and Nagai, 1976a) on the interaction of $B_{12}H_{11}SH^{2-}$ and $B_{12}H_{12}^{2-}$ with BSA supported this finding. A separate report (Nakagawa and Nagai, 1976b), presented theoretical models for the interaction of $B_{12}H_{11}SH^{2-}$ with serum albumin. However, no direct experimental evidence of disulfide linkage of $B_{12}H_{11}SH^{2-}$ to the protein was provided.

The first objective of the present study was to investigate the nature of the interaction of $B_{12}H_{11}SH^{2-}$ with proteins, and to provide experimental verification for disulfide formation of $B_{12}H_{11}SH^{2-}$ to proteins as proposed in earlier studies (Soloway *et al.*, 1967; Nakagawa and Nagai, 1976a). A second objective was to determine whether this anion could be covalently incorporated into antibodies without significant loss of immunoreactivity. The third was to determine whether the extent of boronation would be sufficient for the delivery of enough boron-10 to sustain a lethal n,α reaction at the cellular level.

For the purpose of these studies, it was necessary to have a procedure for measuring both protein and boron concentrations. The former could be determined by a colorimetric protein assay (Bio-Rad Laboratories, Richmond, Calif.). For the latter, in preference to previously described microanalytical boron methods (Soloway and Messer, 1964), we undertook tritium-labeling of these polyhedral boron cages. Direct boron determination (Fairchild, 1982) by neutron activation analysis of antibodies treated with ^{10}B-enriched $B_{12}H_{11}SH^{2-}$ also was carried out to confirm our findings.

Experimental

Tritiation of boron compounds

A 2.0 g sample of $Cs_2B_{12}H_{11}SH$ and a 0.5 g sample of $Na_2B_{12}H_{12}$ each were exposed to 15 Ci tritium gas for two weeks (New England Nuclear, Billerica, Mass.), followed by removal of labile tritium with water. The ^3H-activity of tritiated $Cs_2B_{12}H_{11}SH$ (measured as received from New England Nuclear) was approximately 6.0 mCi/mmol, and that of tritiated $Na_2B_{12}H_{12}$ was 940 mCi/mmol. Tritiated materials were crystallized from H_2O to constant specific activity. Isotopic dilutions of the tritiated samples to obtain material of lower specific activity were carried out by dissolving a mixture of "hot" and "cold" material followed by crystallization.

Thin layer chromatography of boron compounds

TLC of the boron compounds was carried out on Baken-flex DEAE cellulose sheets (J. T. Baker Chemical Co., Phillipsburg, N.J.), developed with 3M NH_4NO_3 and visualized with 1% aqueous $PdCl_2$ spray. The R_f values for $B_{12}H_{12}^{2-}$ and $B_{12}H_{11}SH^{2-}$ under these conditions are respectively 0.44 and 0.34.

Reaction of boron compounds with antibody

For the purpose of these studies, it was decided to use a polyclonal antibody. Antithymocyte globulin has been used clinically to manage acute allograft rejection episodes. Purified, high titer horse anti-human thymocyte globulin (ATGAM®) was kindly provided by The Upjohn Co., Kalamazoo, Mich. In a typical experiment, the solid boron compound was added to a solution of antithymocyte globulin (ATG) in a buffer, stirred for 1 h at ambient temperature and stored overnight at 4°C. The ATG then was separated by sequential passage through Sephadex G-25 columns (Pharmacia Fine Chemicals, Piscataway, N.J.), each time collecting the protein in the void volume.

Determination of boron content

The protein concentration of the purified ATG fraction was measured by means of the Bio-Rad protein assay and the ^3H-activity was determined by counting a 20 μL aliquot in a Beckman LS-6700 liquid scintillation counter. From these two measured quantities, the number of boron cages linked per molecule of ATG was calculated. With non-radiolabeled compounds, the boron concentration of the boronated ATG solution (purified by gel filtration and extensive dialysis) was determined by the prompt-gamma method (Fairchild, 1982), by Dr. Ralph G. Fairchild at the Brookhaven National Laboratories, N.Y. One milliliter of the boronated antibody solution in a boron-free glass tube was irradiated at the high flux beam reactor (HFBR) with a thermal neutron flux of 3×10^7 n·cm^{-2}·s^{-1} for 200 s. 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. Boron-10 enriched $Na_2B_{12}H_{11}SH$ (Shionogi Research Laboratories, Osaka, Japan) was kindly provided by Dr H. Hatanaka, Tokyo, Japan.

Determination of in vitro *binding of boronated antibody by membrane immunofluorescence*

Serial twofold dilutions of the nonboronated and boronated antibodies were prepared by adding 50 μL of antibody to 50 μL aliquots of PBS. Human peripheral blood lymphocytes were separated from whole blood by means of Ficoll-hypaque density gradient centrifugation. Cells were washed in PBS and resuspended and 50 μL volumes containing 1.5×10^6 cells were added to each tube. They were incubated for 30 min at ambient temperature, washed two times, and then resuspended in 100 μL volumes of PBS. An equal volume of fluorescein isothiocyanate (FITC) conjugated rabbit anti-horse IgG (Miles-Yeda Ltd, Israel) at a dilution of 1:30 was added to the lymphocytes. They were allowed to incubate for an additional 30 min at ambient temperature, washed two times with PBS, and resuspended in a 1:1 mixture of 50% glycerol and PBS. Fluorescence was scored 0–4+ with a Zeiss fluorescence microscope with epiillumination and a halogen light source. End point titers were recorded at the dilution of antibody that gave 0 (i.e. background) fluorescence.

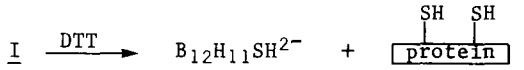
Results and Discussions

The reaction of a large excess of tritium-labeled $Cs_2B_{12}H_{11}SH$ with ATG resulted in the incorporation of 9–13 cages (108–156 boron atoms) per molecule of antibody compared to 4.5 mol of $B_{12}H_{11}SH^{2-}$ per mol of BSA as previously reported (Soloway *et al.*, 1967). ATG has a molecular weight of approximately 150,000 compared to 60,000 for BSA. In another report (Nakagawa and Nagai, 1976a), following extensive dialysis, 4.7 mol of $B_{12}H_{11}SH^{2-}$ were bound per mol of BSA. However, only about 1 mol of $B_{12}H_{11}SH^{2-}$ remained bound after anion exchange chromatography. The stability of the conjugation of $B_{12}H_{11}SH^{2-}$ to ATG is indicated by the observation that even after one month storage at 4°C, there was no significant loss of boron from the protein following either gel filtration or ammonium sulfate precipitation.

The immunoreactivity of the boronated ATG was demonstrated by membrane immunofluorescence. However, there was a reduction in activity as indicated by the fluorescence endpoint titer. The experiments were designed to maximize degree of boronation, even at the expense of some immunoreactivity. The membrane immunofluorescence endpoint titer of boronated ATG with peripheral blood lymphocytes was determined to be 1:1600 after correcting for protein concentration, compared to 1:3200 for the native antibody.

For purposes of comparison, a sample of unsubstituted $B_{12}H_{12}^{2-}$ was tritium labeled and then reacted with ATG in a manner similar to the experiments with $B_{12}H_{11}SH^{2-}$. Surprisingly, using this method (Wilzbach, 1957), a 150-fold higher specific activity was obtained by the tritiation of $B_{12}H_{12}^{2-}$ compared to that for $B_{12}H_{11}SH^{2-}$. However, only 1.6 mol of $B_{12}H_{12}^{2-}$ were bound per mol of ATG after two gel filtration steps. The incorporation of this small number of $B_{12}H_{12}^{2-}$ cages to proteins has been shown to be due to "ionic" binding (Soloway *et al.*, 1967; Nakagawa and Nagai, 1976a). It appears that the sulfhydryl group on $B_{12}H_{11}SH^{2-}$ is indeed primarily responsible for its binding to proteins.

To verify whether $B_{12}H_{11}SH^{2-}$ binds covalently to proteins through the formation of a disulfide bond, ATG conjugated with $B_{12}H_{11}SH^{2-}$ was treated with excess dithiothreitol (DTT), a reagent commonly employed for cleaving disulfide bonds. Approximately 65% of the polyhedral borate content was removed within 24 h, as measured by the decrease in protein-bound tritium activity. This observation supports the hypothesis that $B_{12}H_{11}SH^{2-}$ binds to ATG largely by thiol–disulfide exchange.



Scheme 2

Although the tritium labeled $Cs_2B_{12}H_{11}SH$ was crystallized to constant specific activity, high counting impurities were observed by scanning of a TLC of the compound for tritium activity. The radiochemical purity of freshly crystallized $Cs_2B_{12}H_{11}SH$ was approximately 90%, as measured by the percent radioactivity in DEAE cellulose scraped from a TLC of the material. The radiochemical purity of $Na_2B_{12}H_{12}$ measured in a similar manner was approximately 98%. This raised the possibility that the tritium activity of the boronated ATG might not be an accurate measure of the boron content. Therefore, direct determination of the boron content was carried out by neutron activation anlaysis for boron-10 (Fairchild, 1982). This method utilizes the emission of a 480-keV prompt-gamma from the nuclear reaction of a thermal neutron with a boron-10 nucleus to measure the quantity of boron-10 in a sample. Natural boron contains only 19.8% boron-10. Therefore, to increase the sensitivity of boron determination by this method, ATG was boronated with 95% ^{10}B-enriched $B_{12}H_{11}SH^{2-}$. Another objective of the present study was to determine the feasibility of incorporating sufficient boron-10 nuclides into the antibody for BNCT without destroying antibody activity. Boron determination by the prompt-gamma method also yielded approximately 12 boron cages (144 boron atoms) per ATG molecule treated with $B_{12}H_{11}SH^{2-}$. These results are in agreement with the results obtained with the ^{3}H-labeled compounds, as summarized in Table 1. The slightly higher numbers obtained for boron content when tritium labeled compounds were used may be due to high counting impurities.

Our results demonstrated that sulfhydryl-

Table 1. Molar concentrations of boron compound and protein and their mol ratios in the reaction mixture and in the purified conjugate

	Reaction mixture			Conjugate	
Boron compound	[Boron] mM	[Protein] mM	Mol ratio [Boron]/ [Protein]	[Boron]/ [Protein]	After DTT treatment [Boron]/[Protein]
$Cs_2B_{12}H_{11}SH$ 0.87 mCi/mmol	7.8	0.067	116	9.4	3.2
	49.1	0.333	147	12.0	—
	43.9	0.167	263	13.3	
$Na_2B_{12}H_{12}$ 3.58 mCi/mmol	83.2	0.238	350	1.6	—
$Na_2{}^{10}B_{12}H_{11}SH$ 95% ^{10}B-enriched	235	0.333	705	12.3	4.4
$Na_2B_{12}H_{12}$	276	0.333	800	1	—

containing polyhedral boranes have the capacity of being covalently incorporated into proteins. It appears that the mechanisms for incorporation stems from the cleavage of native disulfide linkages with the generation of a mixed boronated disulfide. Unsubstituted polyhedral boranes lack this capacity. The linkage of $B_{12}H_{11}SH$ to antibodies by disulfide bonds has the disadvantage that they may be cleaved *in vivo* by sulfhydryl containing proteins such as glutathione prior to reaching the targeted tissue. Unlike certain drugs, the boron compounds need not be released from the antibody at the tumor site for effective BNCT.

ATG boronated with 95% ^{10}B-enriched $B_{12}H_{11}SH^{2-}$ contained approximately 140 boron-10 atoms. Calculations show that even with a high antigen site density of 10^6 per target cell, peripheral blood lymphocytes (Technical Report 7830/77/042, The Upjohn Co., Kalamazoo, Mich., 1977), approximately 2 μg ^{10}B/g cell theoretically could be attained with this boronated antibody. The actual figure is likely to be lower, since ATG molecules will be variably boronated (140 ^{10}B-atoms representing an average figure), and the more lightly boronated molecules may preferentially bind to antigen sites. In fact, we were unable to demonstrate a specific cell killing effect due to $^{10}B(n,\alpha)^7Li$ reaction by irradiation with up to 3×10^{12} thermal neutrons/cm^2 of target cells treated with this boronated ATG (unpublished data). For successful BNCT with currently available neutron beams, it has been estimated that selective delivery of approximately 15–30 μg ^{10}B/g tumor would be required (Fairchild and Bond, 1985). With ~140 boron-10 atoms per antibody molcule, such a concentration most likely will not be achievable. Although this would preclude the use of this method for the boronation of antibodies for BNCT, it might, however, be ideal for the incorporation of small numbers of boron atoms as an elemental probe for high-resolution microanalyses of biological specimens by electron energy loss spectroscopy and by electron spectroscopic imaging (Ottensmeyer and Andrew, 1980).

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