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Biochemical applications of boron cluster chemistry

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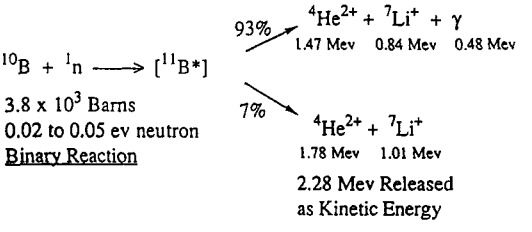
Abstract - Monoclonal antibodies (Mabs) and their immunoreactive fragments continue to be attractive, but as yet unproven, vehicles for the selective delivery of therapeutic quantities of ^{10}B to tumors for the purpose of boron neutron capture therapy (BNCT). Available chemical methodologies and boron-containing reagents for immunoprotein conjugation will be delineated along with the use of designed, precisely synthesized macromolecules which serve as "^{10}B-trailer" reagents for protein conjugation. The latter designed species have been investigated and are either synthetic peptide-like oligomers containing large quantities of boron in their component α-amino acids or they are hydrophilic polyamides comprised of boron-rich repeating units. Both classes of reagent are produced through solid-supported syntheses (Merrifield methodology). Use of these precisely synthesized ^{10}B-trailer species as conjugation reagents equipped with analytically useful fluorescent labels may provide the 10^3 B-atoms per Mab molecule required for effective BNCT. This methodology provides Mab conjugation with a minimal loss of structural information while providing chemical reproducibility. The *in vivo* evaluation of this approach has led to the identification of potential improvements in the use of immunoproteins as vehicles for the selective delivery of boron (or other agents) to tumor cells. Consequently, the synthesis of a hydrophilic and boron-rich peptide which is structurally discrete and immunodirected is in progress.

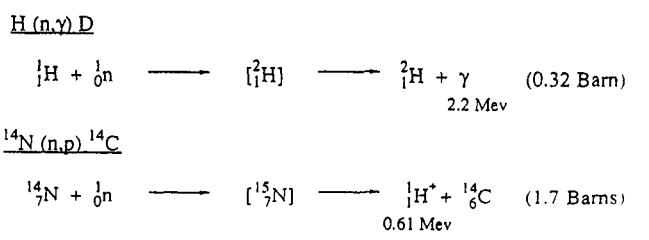
INTRODUCTION

Of the many unusual properties of boron cluster compounds which might make them uniquely suited for specific applications in bioinorganic chemistry, biochemistry or medicine, the proposed use of derivatized polyhedral borane anions or carboranes as carriers of ^{10}B for neutron capture is the most widely known (ref. 1,2). This highly localized cytotoxic binary nuclear reaction was first proposed by Locher in 1936 (ref. 3) as a possible means of cancer therapy and has since been known by the acronym, BNCT (Boron Neutron Capture Therapy). The basis of this proposal is the capture of a reactor-generated slow (thermal) 0.02 to 0.05 eV neutron by a ^{10}B nucleus followed by fission of the unstable $[^{11}B]$ nucleus which results. The required ^{10}B isotope is unique among the light elements since it has the high nuclear cross-section of 3850 Barns and is available in twenty percent natural abundance. This isotope is easily incorporated in enriched compounds at the 95% level.



The 2.28 MeV released as kinetic energy and distributed between the α-particle and the $^{7}Li^{+}$ ion gives these particles an effective distance of travel in tissue of approximately one cell diameter. During their passage through the interior of a cell, these energetic ions cause ionization tracking and cellular damage with associated cytotoxicity. In fact, the α-particle is recognized as the most lethal product of nuclear reactions with a biologic lethality of 2.5 (or greater) that of an identical energy dose provided by γ-radiation. Obviously, the selective concentration of ^{10}B atoms on or within cancer cells followed by exposure of these cells to thermal neutrons provided by an efficient nuclear reactor (10^{11} to 10^{13} $^{1}n/cm^2$ sec) should kill tumor cells in the presence of normal (unlableled) cells.

While thermal neutrons are relatively innocuous to tissue, nuclear reactions capable of delivering significant radiation doses during BNCT are present. These two principal processes are the $H(n,\gamma)D$ and $^{14}N(n,p)^{14}C$ reactions depicted here:



While the nuclear cross-sections of these two processes are exceedingly small when compared to the 3850 Barns cross-section of ^{10}B, their ability to generate cytotoxic products provides a non-selective background radiation dose. In order to administer cytoselective BNCT at a level capable of providing a significant therapeutic advantage, a minimum concentration of ^{10}B must be present in the tumor under attack. Depending upon the neutron current available, the uniformity of ^{10}B distribution within the tumor mass and the position of the targeted ^{10}B atoms with

respect to individual tumor cells, this concentration of ^{10}B may vary from 5 to 30 μg ^{10}B/g tumor. If ^{10}B nuclei are located within tumor cells as opposed to being attached to the tumor cell surface, the concentration of ^{10}B required for therapy is significantly less. Workers in the BNCT field have generally agreed that 30 ppm of ^{10}B in tumor would assure effective therapy with available thermal neutron sources. The analysis of animal tissues obtained from boron localization experiments has been made easier by the development of inductively coupled plasma-atomic emission spectroscopy (ref. 4) for this purpose. Using this method, very small samples can be accurately assayed with boron levels in tissue of 0.1 ppm.

The issue of selective delivery of ^{10}B to tumor as opposed to normal tissue has been addressed in several ways which make use of the physiological differences between tumor and normal cells. These boron-containing delivery systems include, but are not limited to, specific molecules such as porphyrins (ref. 5), amino acids (ref. 6), liposomes (ref 7), as well as tumor specific/associated antibodies and their immunoreactive fragments (ref. 1,2,8,9). Regardless of the boron delivery method employed, the ratio of ^{10}B in tumor to that in blood is critical and tumor to blood concentration ratios less than unity would be undesirable in therapy due to destruction of endothelial cells in that portion of the vascular system subjected to neutron irradiation.

DELIVERY OF BORON TO TUMORS WITH BORON-LABELED IMMUNOPROTEINS

Of the many methods of selective boron delivery to tumor presently under consideration, the use of boron-labeled tumor-targeted monoclonal antibodies (Mabs) and their immunoreactive fragments (Fig. 1) appears to offer the most general, but complex, approach. Immunoproteins of this sort are generally very selective toward complexation with their targeted tumor antigens, form very stable complexes with these cell wall antigens (ref. 1,2,8,10) and at least some of them are capable of gaining entry to the interior of the tumor cell, following complexation, through endocytosis. It is the purpose of this paper to describe recent experimental work in our laboratory which bears upon the use of Mab molecules as boron delivery vehicles, to delineate problems which were encountered and their probable cause. Lastly, we describe work in progress which we hope will correct these deficiencies and, at the same time, evaluate new methodologies for the selective immunoprotein-mediated transfer of effector molecules to tumor cells.

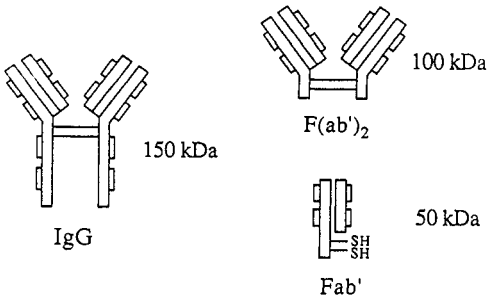
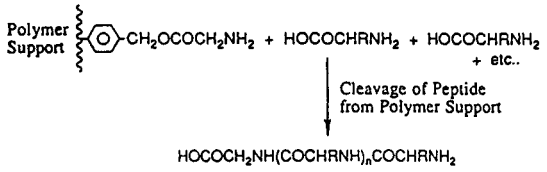


Fig. 1. Schematic representation of monoclonal IgG antibody and its immunoreactive fragments.

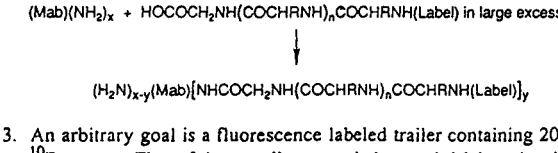
Assuming that tumor cells generally carry 10^6 characteristic antigenic sites of any one type and that there are approximately 10^9 cells per gram of tumor, one calculates that about 600 ^{10}B atoms must be attached to each individual Mab molecule (if all antigenic sites are complexed) for each 10 ppm of ^{10}B supplied to tumor. Rather than randomly attack IgG Mab molecules with a large number of relatively small boron-containing conjugation reagent molecules we have chosen to assemble a series of discrete, precisely synthesized oligomeric reagents ("trailers") each of which contains a fixed number of B-atoms up to approximately 200. These oligomeric reagents

Peptide Trailer Reagents

1. Peptide trailer reagents are synthesized from hydrophilic boron-rich α-amino acids using precision synthesis on solid polymer supports (Merrifield methods) and sequential addition of amino acid residues to a growing peptide chain. R is a boron-containing group.



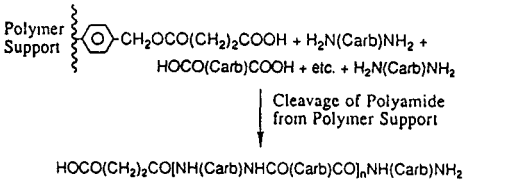
2. The peptide trailer reagent employs its single carboxyl group for attachment to lysine ε-amino groups present on the Mab surface while the terminal amino group is used for the attachment of either a radio or fluorescent label.



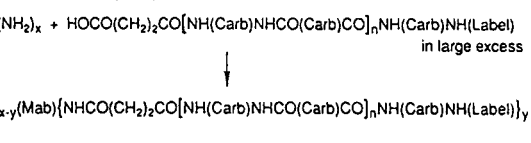
3. An arbitrary goal is a fluorescence labeled trailer containing 200 ^{10}B-atoms. Five of these trailers coupled to each Mab molecule would provide 1000 ^{10}B-atoms as required.

Polyamide Trailer Reagents

1. Polyamide trailer reagents are synthesized from hydrophilic boron-rich dicarboxylic acids and diamines using precision methods based upon Merrifield techniques. Functional group protection and deprotection not required. Carb is a carborane-containing bifunctional group.



2. As in the case of peptide trailer reagents, the polyamide chain is linked to a radio or fluorescence label using the terminal amino group and to the Mab lysine ε-NH_2 groups with the terminal carboxyl group.



3. As in the case of peptide trailer reagents, the goal is y=5 with a 200 ^{10}B-atom polyamide.

Fig. 2. Schemes summarizing the synthesis of boron-containing peptides and polyamides.

would carry a radioactive or fluorescent group for analytical purposes attached to a terminal-NH_2 group of their chain and the remaining -COOH terminus would be free for conjugation with the lysine ε-NH_2 groups of Mab protein. Attachment of an average of five of these oligomeric species to each Mab molecule would provide the desired population of 10^3 B-atoms per Mab and not critically perturb the overall IgG structure due to the small number of oligomers attached per Mab. Two types of oligomeric trailer reagents are envisioned; hydrophilic peptides and polyamides. Both types of reagent are prepared using the solid-supported synthesis methods of Merrifield (ref. 11). Representative peptide trailers are described in Scheme I while Scheme II defines the polyamide reagents (Fig. 2).

Figure 3 illustrates two typical α-amino acids which contain the hydrophobic *closo*-1,2-$C_2B_{10}H_{11}$-cage and the corresponding hydrophilic [*nido*-7,8-$C_2B_9H_{11}$-]$^-$cage fragment. These amino acids are designated *closo*-CB and *nido*-CB, respectively, throughout this paper.

Figure 4 presents the structures of a variety of available bifunctional carboxylic acids and amines which may contain either the hydrophobic *closo*-1,2-$C_2B_{10}H_{11}$-cage or the hydrophilic [*nido*-7,8-$C_2B_9H_{11}$-]$^-$ cage fragment.

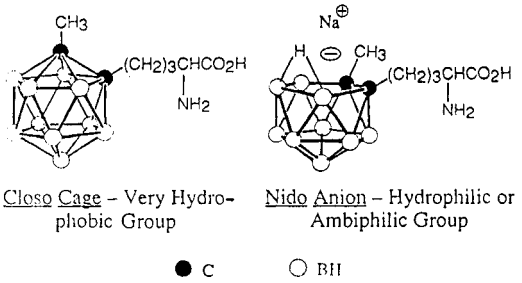


Fig. 3. Two boron-rich α-amino acids employed in peptide synthesis as their *t*-BOC derivatives.

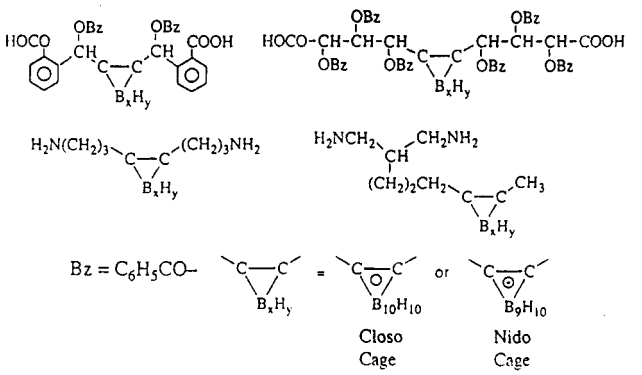


Fig. 4. Carboranyl dicarboxylic acids and diamines for polyamide synthesis.

Figure 5 displays the structures of available tagging reagents useful for labeling oligomer chains by reaction with the -NH_2 terminus of the oligomers. Of these, the fluorescent dansyl group has been most useful for the quantitative determination of trailer species in solution both as a free molecule and when conjugated with protein.

Peptide and polyamide trailers and their derivatives which contained the *closo*-1,2-$C_2B_9H_{11}$-cage group were found to be too hydrophobic to employ in conjugation reactions with antibodies. Consequently, the reaction shown in Fig. 6 was developed which allows the quantitative conversion of *closo*-1,2-$C_2B_{10}H_{11}$-cages to the more hydrophilic [*nido*-7,8-$C_2B_9H_{11}$-]$^-$ anion. As the sodium salts, these anionic groups imparted good water solubility to all species in which they were contained.

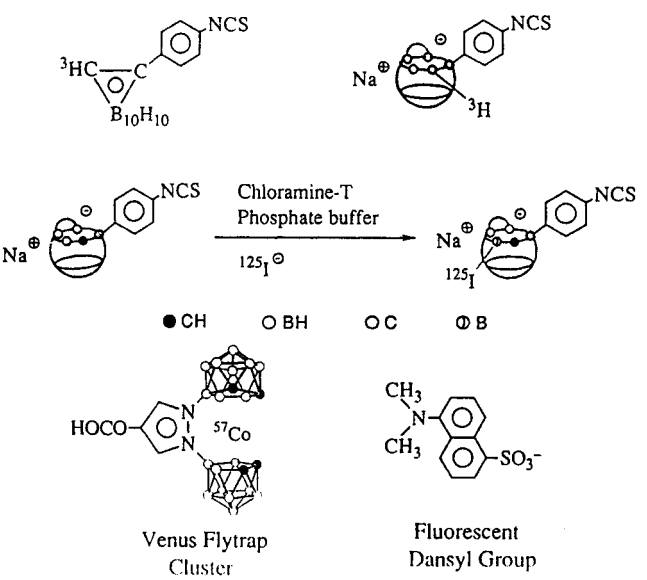


Fig. 5. Representative reagents for radio- and fluorescence labeling of trailers.

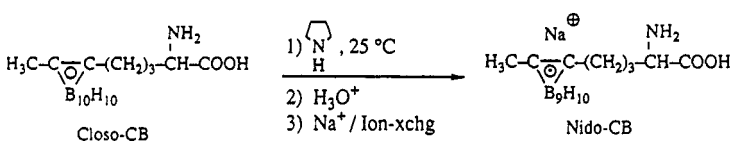


Fig. 6. A general reaction used to convert all closo-cages in peptides to the corresponding nido-anions.

THE SYNTHESIS OF TRAILER PEPTIDES

Having developed the synthesis of the essential α-amino acid, *closo*-CB, and its *t*-BOC derivative, the dansylated dipeptide (nido-CB)$_2$(dansyl) and the dansylated undecapeptide (*nido*-CB)$_{10}$ Lys(dansyl)$_2$ were constructed using standard Merrifield peptide synthesis conditions. These syntheses are outlined in Fig. 7 and 8, respectively. Both peptides were characterized by ^{11}B and ^{1}H FTNMR, mass spectra (as their closo derivatives prior to reaction with pyrrolidine), dansyl fluorescence intensities and HPLC chromatographic properties.

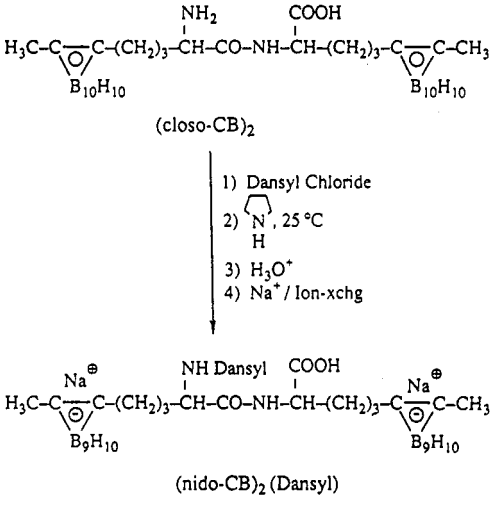
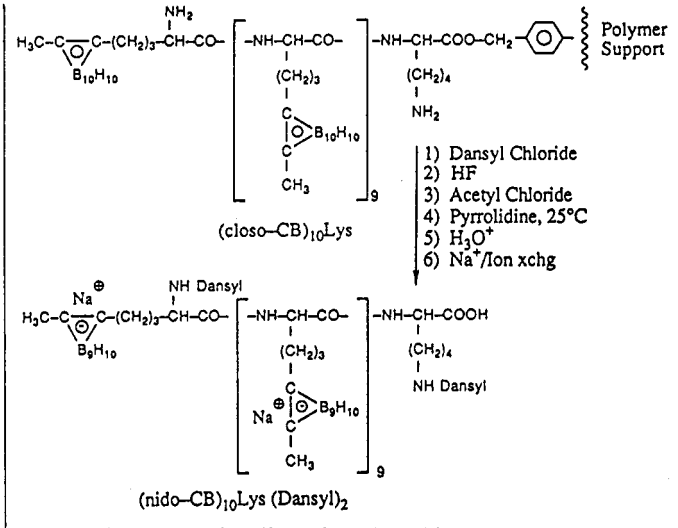


Fig. 7. A dipeptide trailer reagent synthesized using the Merrifield method.



Acetyl may occasionally replace dansyl in these species.

Fig. 8. An undecapeptide trailer reagent synthesized using the Merrifield method.

CONJUGATION OF TRAILER PEPTIDES WITH ANTI-CEA ANTIBODY T84.66

The IgG Mab selected for conjugation with the sodium salts of (*nido*-CB)$_2$(dansyl) and (*nido*-CB)$_{10}$Lys(dansyl)$_2$ was targeted for the carcinoembryonic antigen associated with LS174T human colon tumor xenografts employed in the biodistribution studies described below. For simplicity this Mab may hereafter be referred to by the abbreviated designation T84.

Conjugation of (*nido*-CB)$_2$(dansyl) to T84 Mab. A mixture of the dansylated anionic (*nido*-CB)$_2$ [hereafter (CB)$_2$] and N-hydroxysulfosuccinimide was treated with N,N-diisopropylcarbodiimide in dimethylformamide for one hour. The resulting active ester was incubated with T84 at pH 9.0 for 1h, with the molar ratio of (CB)$_2$ active ester to antibody being 10.5:1. A control reaction of T84 plus unactivated (CB)$_2$ was also prepared. Initial purifications were performed by Centricon-30 diafiltration. Final purifications of the T84-(CB)$_2$ conjugate and the control sample were by Superose 12 gel permeation chromatography. The control sample showed a sharp protein peak corresponding to monomeric antibody with a small amount of high molecular weight (HMW) species preceding the main antibody peak. Very little fluorescence was associated with either protein species indicating that (CB)$_2$ was not bound nonspecifically to the antibody. The T84-(CB)$_2$ conjugate showed a similar protein elution profile; however, a significant fluorescent component was observed indicating covalent attachment of (CB)$_2$ to the antibody. Surprisingly, the HMW species showed a disproportionate amount of the fluorescent label. This suggests that the HMW species is much more reactive towards the (CB)$_2$ active ester; or that a small percentage of the monomeric antibody repeatedly reacts at a disproportionately rapid rate with the (CB)$_2$ active ester, thereby generating a highly conjugated HMW antibody species (accelerated or cascade conjugation).

The monomeric T84-(CB)$_2$ conjugate and the control sample were collected and used for fluorescence, electrophoretic, immunoreactivity, and tumor-targeting analyses. Based on the measured fluorescence intensity and protein concentration of the T84-(CB)$_2$ conjugate, an average of 3.5 (*nido*-CB)$_2$ peptides (63 B-atoms) were incorporated per antibody molecule. SDS polyacrylamide gel electrophoresis (SDS-PAGE) of the monomeric T84-(CB)$_2$ conjugate and the control sample exhibited identical electrophoretic migrations, indicating that there was no gross alteration of protein structure upon conjugation. Enzyme immunoassay of the monomeric T84-(CB)$_2$ conjugate and an untreated sample of T84 Mab showed that they had identical immunoreactivities.

For tumor-targeting analyses, aliquots of monomeric T84-(CB)$_2$ and untreated T84 were radioiodinated using Na^{125}I and chloramine-T. In the case of the T84-(CB)$_2$ conjugate, this procedure is expected to label not only the protein, but also the (*nido*-CB)$_2$ peptide due to the similar reactivity of the *nido*-B$_9^-$ moiety and tyrosine residues towards iodination (Fig. 5). Because the stated goal of this research is the delivery of large amounts of boron-containing compounds to tumor, nude mice bearing CEA-producing LS174T tumor xenografts were administered 96 μg of unlabeled T84-(CB)$_2$ together with 0.4 μg of ^{125}I-T84-(CB)$_2$ as a marker. Similar amounts of native and iodinated native T84 were used as a control. The biodistribution studies are summarized in the accompanying table.

At the three time points studied (24, 48, and 120 hours) tumor was the principal site of uptake. The tumor-to-blood (T/B) ratio increased from 1.9 at 48 hours to 22.6 at 120 hours while the tumor-to-liver (T/L) ratio remained nearly constant at 4.1 to 4.9. The control antibody, which was studied only at 120 hours, showed a T/L ratio of 11.6, although the T/B ratio was 9.3, somewhat less than for the T84-$(CB)_2$ conjugate. The total tumor accumulation was 14.2, 14.5, and 7.0 %ID at the three time points for the T84-$(CB)_2$ conjugate and 10.0 %ID for the control T84 Mab.

Biodistribution of ^{125}I-Labeled T84.66-CB2 in Nude Mice Bearing LS174T Xenografts [a]
% Injected Dose/Gram of Tissue [b]

	48h	72 h	120h	
Antibody	T84.66-CB2	T84.66-CB2	T84.66-CB2	T84.66 (Control)
Tissue:				
Blood (B)	7.35 ± 0.49	6.26 ± 0.75	0.43 ± 0.13	1.28 ± 0.38
Liver (L)	3.48 ± 0.15	2.99 ± 0.32	1.67 ± 0.17	0.87 ± 0.05
Spleen	1.77 ± 0.07	1.56 ± 0.16	0.69 ± 0.11	0.45 ± 0.02
Kidney	1.69 ± 0.05	1.58 ± 0.15	0.33 ± 0.07	0.41 ± 0.08
Lung	2.91 ± 0.22	2.40 ± 0.21	0.41 ± 0.11	0.64 ± 0.10
Tumor (T)	14.21 ± 0.81	14.50 ± 0.91	6.96 ± 0.57	9.87 ± 0.58
T/L	4.10 ± 0.30	4.93 ± 0.27	4.19 ± 0.07	11.57 ± 1.21
T/B	1.94 ± 0.08	2.40 ± 0.19	22.63 ± 5.47	9.26 ± 1.76
Tumor wt (g)	1.52 ± 0.06	1.74 ± 0.13	2.56 ± 0.20	2.15 ± 0.19

[a] 3 μCi (~0.4 μg) of ^{125}I-labeled T84.66-CB2 together with 92 μg unlabeled T84.66-CB2 was injected i.v. in nude mice bearing 14-day-old subcutaneous LS174T tumors. Equivalent amounts of ^{125}I-labeled and unlabeled T84.66 were used as a control. Groups of 4-5 mice were sacrificed at the indicated times after antibody injection, and the tumor and tissues were weighed and counted to determine the percent injected dose per gram tissue.
[b] Values quoted are mean ± standard error.

Conjugation of (*nido*-$CB)_{10}$Lys(dansyl)$_2$ to T84 Mab. Preliminary studies with didansylated anionic (*nido*-$CB)_{10}$Lys [hereafter $(CB)_{10}$] suggested that the hydrophobic nature of the peptide led to its nonspecific binding to antibody, as well as incomplete recovery of the peptide and Mab-peptide conjugates during chromatographic procedures. A number of gel permeation chromatographic media and buffer formulations, including the use of organic solvents and nonionic detergents, were tested in order to alleviate these problems. Optimal results were obtained with Superose 12 in the presence of 0.05% Tween-20. The conjugation of $(CB)_{10}$ to T84 was carried out essentially as described above for $(CB)_2$, but at pH 8.5 with a molar ratio of peptide to antibody of 10:1. Control reactions of T84 alone and T84 plus unactivated $(CB)_{10}$ were also prepared. The conjugate and the two controls were brought to a final concentration of 0.05% Tween-20 and purified by Superose 12 chromatography. A sharp, monomeric antibody peak was obtained for untreated T84 with a minor component of HMW species. Only a trace amount of fluorescence was associated with these peaks. T84 treated with unactivated $(CB)_{10}$ also showed a sharp, monomeric antibody peak. The HMW species was only slightly larger than that seen with T84 Mab alone. A small amount of fluorescence was associated with each of these peaks, with the free peptide eluting as a symmetrical peak after the monomeric antibody peak. For the T84-$(CB)_{10}$ conjugate, a sharp, monomeric antibody peak was obtained with a sizeable peak due to a HMW species. A UV-absorbing peak was later observed for N-hydroxysulfosuccinimide (~37 minutes). A significant amount of fluorescence was associated with the T84-$(CB)_{10}$ conjugate relative to the unactivated $(CB)_{10}$ peptide control. As was seen with the $(CB)_2$ and other $(CB)_{10}$ experiments, the fluorescence was disproportionately associated with the HMW species. The HMW and monomeric antibody species were collected and analyzed separately.

Background fluorescence due to the presence of 0.05% Tween-20 prevented quantitative fluorescence measurements. Recent studies have investigated the use of quantitative amino acid analysis for determining trailer substitution levels in Mab conjugates. Control hydrolyses of *t*-BOC-$(CB)_2$, and -$(CB)_{10}$ revealed a ninhydrin-reactive species eluting before aspartic acid, and therefore consistent with the presence of an anionic amino acid. The same amino acid was present in a hydrolysate of HMW T84-$(CB)_{10}$ conjugate. Quantitation of the amino acid revealed that the HMW conjugate contained an average of 6.3 $(CB)_{10}$ peptides (570 boron atoms) per antibody. Immunoreactivities for the HMW and monomeric conjugates were compared to untreated T84 Mab. The monomeric conjugate retained its native immunoreactivity while there was a slight decrease in the immunoreactivity of the HMW conjugate. For tumor-targeting analyses, aliquots of both the HMW and monomeric T84-$(CB)_{10}$ conjugates and untreated T84 Mab were radioiodinated as described above for the T84-$(CB)_2$ conjugate. Nude mice bearing CEA-producing LS174T tumor xenografts were administered 27 μg of either HMW or monomeric conjugates together with 0.3 μg of the corresponding ^{125}I-labeled conjugate as a marker. A similar amount of boron-free T84 Mab was used as a control. Biodistribution studies were performed at a single time point of 48 hours and are summarized in the accompanying table. Although

Biodistribution of ^{125}I-Labeled T84.66-CB10 in Nude Mice Bearing LS174T Xenografts [a]
% Injected Dose/Gram of Tissue [b]

Antibody	T84.66-CB10 (HMW)	T84.66-CB10 (Monomer)	T84.66 (Control)
Tissue:			
Blood (B)	1.20 ± 0.22	1.90 ± 0.12	7.93 ± 1.42
Liver (L)	43.84 ± 3.78	37.48 ± 1.73	2.89 ± 0.16
Spleen	13.98 ± 1.22	8.07 ± 0.65	1.65 ± 0.07
Kidney	2.30 ± 0.29	3.08 ± 0.18	1.86 ± 0.18
Lung	1.61 ± 0.15	2.36 ± 0.08	3.26 ± 0.26
Tumor (T)	4.35 ± 0.51	5.14 ± 0.33	23.19 ± 5.12
T/L	0.099 ± 0.008	0.139 ± 0.011	7.85 ± 1.23
T/B	3.79 ± 0.28	2.74 ± 0.19	2.91 ± 0.28
Tumor wt (g)	0.64 ± 0.18	0.57 ± 0.15	0.86 ± 0.21

[a] 7 μCi (~0.30 μg) of ^{125}I-labeled T84.66-CB10 samples together with 27 μg of the respective unlabeled T84.66-CB10 samples were injected i.v. in nude mice bearing 10-day-old subcutaneous LS174T tumors. Equivalent amounts of ^{125}I-labeled and unlabeled T84.66 was used as a control. Groups of 5-6 mice were sacrificed 2 days after antibody injection, and the tumor and tissues were weighed and counted to determine the percent injected dose per gram tissue.
[b] Values quoted are mean ± standard error.

tumor showed an appreciable uptake of both the HMW and monomeric T84-$(CB)_{10}$ conjugates, liver and spleen were again the organs of greatest avidity. In comparing the HMW and monomeric conjugates, slightly better tumor retention and decreased liver and spleen uptake were seen with the monomeric T84-$(CB)_{10}$ conjugates. This suggests that the conjugation of $(CB)_{10}$ peptide alone accelerates the deposition of T84 in liver and spleen, and that aggregate formation can further accelerate the deposition. The T84 Mab control behaved as expected and gave a biodistribution similar to that observed in other experiments.

CONCLUSIONS DERIVED FROM TRAILER CONJUGATION AND BIODISTRIBUTION STUDIES

In progressing sequentially through the trailer reagent series $(CB)_2$ to $(CB)_{10}$, the number of boron atoms attached to T84 Mab increased from an average of about 60 to 600.

Progression from $(CB)_2$ to $(CB)_{10}$ introduced the problem of nonspecific binding of Mab with peptide reagent, the formation of increased quantities of HMW species aided by a cascade conjugation reaction sequence and the enhancement of liver uptake of the conjugated Mabs relative to tumor. The nonspecific bonding of both activated and unactivated $(CB)_{10}$ with Mab T84 must arise from the interaction of hydrophobic regions of Mab with ambiphilic $(CB)_{10}$. Conjugation of $(CB)_2$ and $(CB)_{10}$ trailers with Mab led to a disproportionate amount of fluorescent label in the HMW species or aggregate accompanied by the formation of additional aggregate. Mechanistically, the initial lysine conjugation reaction appears to open the Mab structure exposing additional reactive sites which are attacked ever more rapidly as conjugation and the resulting structural perturbation proceeds. The result is a cascade reaction sequence. As the native Mab structure becomes more disarrayed by this process, the number of interchain interactions between Mab molecules will correspondingly increase and the aggregate concentration will increase at the expense of monomeric Mab and its conjugates (Fig. 9).

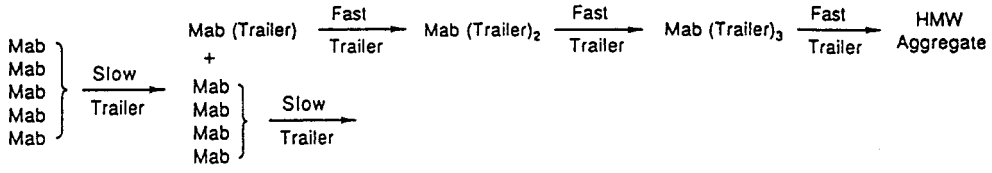


Fig. 9. Possible mechanism for cascade antibody conjugation with a hydrophobic trailer reagent.

Both the HMW and monomeric T84 Mab conjugates derived from $(CB)_{10}$ have good enzyme immunoassay responses and the reduction in tumor uptake is the apparent result of the very effective competitive loss of Mab and HMW conjugates to liver. Tumor/blood ratios were favorably high in all cases.

These collected observations combined with the data of others (ref. 12-14) provide strong support to the conclusion that random substitution of full-sized Mab molecules with large, boron-rich conjugation reagents will become ever more complicated as the boron burden is increased. Therefore, if the splendid versatility of immunochemistry is to be applied to BNCT, radically different approaches will be required. Two such avenues of current research are briefly outlined below.

NEW APPROACHES TO THE IMMUNOCHEMICAL DELIVERY OF BORON

The results presented above suggest that the effective delivery of therapeutic quantities of ^{10}B to tumor might be achieved by (1) use of a greatly simplified chemically monofunctional FabSH antibody fragment related to T84.66 Mab and available by genetic engineering or (2) by synthesizing a bispecific (Fab'_1) (Fab'_2) antibody of the $(Fab')_2$ class (see Fig. 1) which specifically binds tumor cell wall antigen (such as CEA) with one combining site, (Fab'_1), and with the other combining site, (Fab'_2), it binds a discrete, synthesized array of boron-rich oligomers based upon a 20-residue hydrophilic peptide or polyamide (20 mer species). In addition to these modifications of our attack upon the problem, the synthesis of very hydrophilic boron-rich α–amino acids equipped with polyhydroxy side chains (Fig. 10) is underway (ref. 15,16).

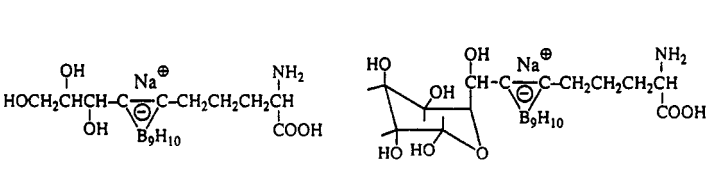


Fig. 10. Carboranyl α-amino acids with enhanced hydrophilicity.

Singly conjugated FabSH. The ability of molecular engineering to provide monoclonal sources for species such as the simple antibody fragment FabSH shown in Fig. 11 suggests the adoption of strongly antigen-binding antibody fragments of this type which are monovalent with respect to cell wall antigen (CEA) complexation and monofunctional in the chemical sense (-SH group). Figure 12 illustrates how a simple FabSH species may be linked to a single large collection of boron-rich $HOCO[Mer]_{20}NH(dansyl)$ peptide chains bonded, in turn, to a small manifold peptide, $HOCOMF(NH_2)_6$, rich in lysine residues. The numerals shown in this figure depict the sequence of coupling reactions required to achieve the 1000 or so ^{10}B-atoms attached to the immunoreactive Fab. The completed molecule to be used in targeting tumor cells is shown in Fig. 13. Advantages which accrue to this

relatively simple BNCT reagent molecule are its low molecular weight giving enhanced mobility and clearance rates coupled with the complete control of the conjugation process since only one thiol function is available per Fab protein for reaction with the maleimide moiety linked to the boron-rich manifold conjugate. Complete structural control appears to be within our grasp. Research based upon this concept is in progress.

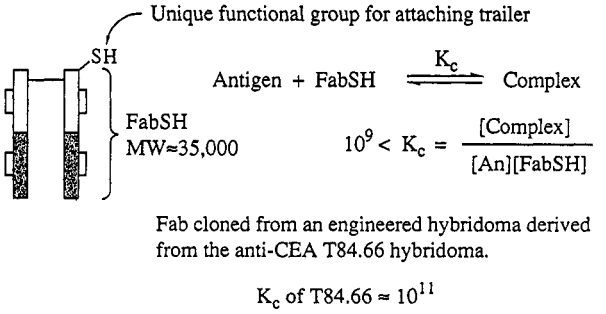


Fig. 11. A simplified immunoprotein, FabSH, engineered and bearing a single functional (-SH) group.

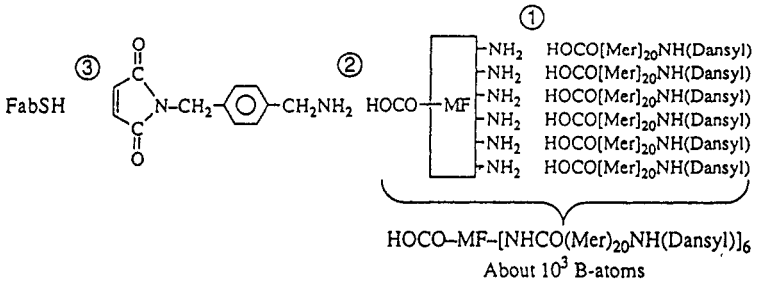


Fig. 12. Sequence of reactions leading to immunodirected boron-rich oligomers. The $HOCO(Mer)_{20}NH_2$ precursor is a peptide or a polyamide with 20 boron-rich repeating units. The $HOCO(MF)(NH_2)_6$ species is a peptide manifold containing five lysine residues.

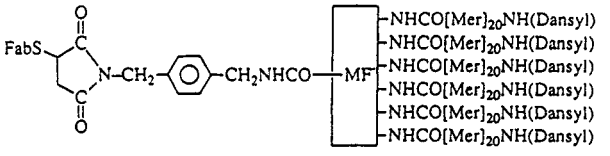


Fig. 13. An immunodirected boron-rich oligomer prepared as shown in Fig. 12.

Bispecific antibodies. The concept of effector molecule delivery by use of a bispecific antibody, such as a $(Fab'_{CEA})(Fab'_{Nido})$ species, which binds simultaneously to two different antigenic sites is relatively new (*17,18*) and has never been employed for the delivery of boron-containing arrays. One route to such systems, which is currently under investigation in our laboratory, may provide a method for binding cell wall antigens, such as CEA, to a large hapten [(*nido*-7,8-$C_2B_9H_{11}$-)$^-$]-bearing molecule such as the MF-$[(Mer)_{20}]_6$ illustrated in Fig. 14. In practice the bispecific antibody would be injected, alone, into the tumor-bearing animal and then binds to tumor antigen with the Fab'_{CEA} portion of its structure. After maximum tumor-loading has been achieved, the animal would then be innoculated with the hapten-bearing boron carrier having approximately 10^3 available ^{10}B-atoms. Binding of this large molecule to the free and uncomplexed (Fab'_{Nido}) arm of the already cell bound (Fab'_{CEA})-(Fab'_{Nido}) should provide each involved cellular antigenic site with 10^3 ^{10}B-atoms without the use of conventional chemical bonds. Fig. 14 displays the desired result viewed at a single cell antigen involved in dual host-guest bonding.

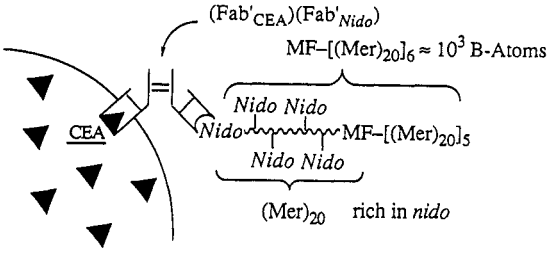


Fig. 14. The bispecific antibody binding hapten-containing boron-rich molecule to cell wall CEA. The synthetic bispecific antibody is initially injected and binds to tumor cell wall CEA. The manifold trailer MF-$[(Mer)_{20}]_6$ is injected later and the *Nido* hapten targets its specific antibody binding site now held to the cell wall by CEA antigen/antibody interaction.

The synthesis of the necessary bispecific antibody, $(Fab'_{CEA})(Fab'_{Nido})$, is outlined in Fig. 15. The critical step in this sequence is the creation of a new hybridoma which produces the precursor IgG Nido-Mab which is targeted for the [*nido*-7,8-$C_2B_9H_{11}$-]$^-$ hapten. The isolation of such a hybridoma is in progress in the laboratory of our colleagues, Drs. Raymond Paxton and Barbara Beatty at the City of Hope National Medical Center, Duarte, California. With Nido-Mab in hand, enzymatic cleavage of this Mab and the chemical synthesis of $(Fab'_{CEA})(Fab'_{Nido})$ using the similarly obtained Fab'_{CEA} fragment should proceed without great difficulty. Use of bispecific antibody systems to carry boron to tumor will avoid the conjugation and product separation problems associated with conventional antibody delivery of effector species.

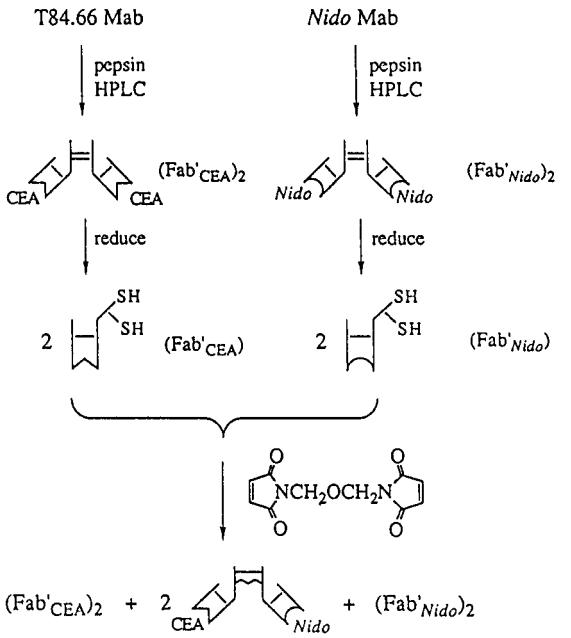


Fig. 15. Synthesis of the bispecific antibody, $(Fab'_{CEA})(Fab'_{Nido})$.

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