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Coupling of Dextrans Conjugated with Boron to Gamma Globulin: A Model for NCT

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This is a report on the progress of our research grant entitled "Boronated Dextran-Antibody Conjugates for Neutron Capture Therapy", on which we have been working since this past May. The rationale for our project is to meet more effectively the well known primary requirement for treatment with boron-10 neutron capture therapy (NCT): namely, the selective localization of a sufficient amount of boron in or on cancer cells or other target cells. As is evident from earlier presentations, this is needed to ensure that the boron-10-enhanced radiation damage in the target tissue exceeds by a sufficient amount the combined effect on normal tissues of residual boron-10-dependent irradiation plus contaminating background radiation.

Monoclonal antibodies (MCA) to tumor-associated antigens are attractive targeting carriers for boron-10 in terms of the needed selective localization, as several previous papers have emphasized. However, to many investigators in this field, the densities of surface receptors on tumor cells have seemed marginal or deficient to achieve successful NCT. If one seeks the necessary radiotherapeutic ratios by increasing the numbers of boron atoms or carborane cages bound per MCA, then inactivation of the antibody can occur through loss of receptor specificity or avidity and/or by precipitation of the protein, as Hawthorne has pointed out (1).

Fairchild has calculated that for effective tumor therapy by boron neutron capture, the therapeutic gain or "advantage factor" should be at least 1.2 (2). Assuming a uniform distribution of boron-10 within the target and access to an epithermal neutron beam, such as we have at Brookhaven, a range of 14-17 micrograms of boron-10 per gram of target tissue (i.e., ppm) will be required, depending upon the relative uptake of boron by target and normal tissues and upon whether tissue repair is included or not (Table 1). If a scandium-filtered neutron beam is used, only about 1-3 ppm of boron-10 will be needed (Table 1).

On the other hand, most monoclonal antibodies bind to antigenic determinants on the cell surface, and in the absence of information to the contrary, it would be conservative to assume that there is no subsequent internalization by cells. Kobayashi and Kanda (3) and a preliminary evaluation by Gabel have shown that if boron remains on the cell surface, geometric factors reduce its efficacy by approximately three times over that expected from homogeneous distribution. Accounting for this leaves us the goal of reaching about 30-45 ppm of boron-10 for epithermal NCT or only 2-8 ppm if a scandium-filtered beam is available (Table 1).

To achieve the goals calculated by Fairchild while overcoming the limitations of antibody binding capacity, we have elected to use water-soluble dextrans as intermediate carriers. This permits each MCA molecule to target many atoms of boron-10 to the specified antigenic receptors while only 5 to 10 of the amino acid residues of the protein are conjugated by dextrans carrying boron-10. As a result, there should be little of the loss of receptor

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Table 1. Concentrations of Boron-10 Required in Target Tissues for Neutron Capture Therapy Compared with Concentrations Delivered by Boronated Dextran-Monoclonal Antibody Conjugates

CONDITIONS			MIN. PPM NEEDED (ug/g)	COMMENTS
Therapeutic Gain	Quality of neutron beam	Distribution of ^{10}B in target		
1.2	Epithermal	Uniform	14-17	From Bond & Fairchild: 1.Minimum advantage factor=1.2 2.Calculated for 4-cm depth 3.Range due to conc. ratios= 3 to infinite; repair = + or -
1.2	Scandium filtered	Uniform	1-3	From Bond & Fairchild
1.2	Epithermal	Cell surface	30-45	From Koybayashi & Kanda; Gabel: 4.Geometry may reduce efficacy by 2-3 times
1.2	Scandium filtered	Cell surface	2-8	

Dext.: to:MCA ratio	M.W.of Dext.	^{10}B:to :Dext. ratio w/w;no.	Cell size diam. vol. (um);(um^3)	Sites per cell	PPM PREDICTED	
5	$4x10^4$	5%;200	12.4;1000	10^6		1.IgG average M.W. = $1.53x10^5$ 2.Often achieved in this work 3.Used in this work 4.Achieved by Gabel 5.Representative value (V-79) 6.Representative value
5	$4x10^4$	5%;200	12.4;1000	10^6	17	
5	$4x10^4$	5%;200	12.4;1000	$>=10^7$	170	7.Sites/cell are for 3-5 of Ferrone's antimelanoma MCA's
3+	$4x10^4$	5%;200	12.4;1000	$>=10^7$	109	8.Best value of dextran:to:MCA for activated dextran so far
10?	$1x10^5$?					9.Probably achievable 10.Probably not too bulky or unstable in vivo
10?	$1x10^5$?	5%;500	12.4;1000	$>=10^7$	850	

specificity or affinity discussed by Soloway (4). Hurwitz and Arnon and their coworkers from Sela's group at the Weizmann Institute have bound many molecules of cytotoxic antibiotics to each molecule of antibody by way of dextran bridges with excellent retention of antibody specificity (5).

In the preceding presentation from the Ohio State group, Alam described the attachment of many boron atoms to polymers or to albumin (6). That approach is conceptually akin to our project, which utilizes well characterized, hydrophilic dextrans as intermediate carriers.

A secondary gain from the use of boronated dextran-antibody conjugates may be more efficient uptake of MCA's or their $F(ab')_2$ fragments. Several enzymes and other proteins are stabilized against metabolic degradation when conjugated by dextrans (7,8). Hence, slowed clearance of antibody-dextran complexes from the blood may be expected.

As a first step in preparing MCA's or $F(ab')_2$ fragments each carrying a thousand or more boron-10 atoms via dextran bridges, we have developed our chemical methodology by studying the reactions of chemically activated dextrans with human IgG, provided by Sigma as Cohn fraction II of gamma globulin. We shall report on the expermental progress only of the work employing periodate activation (9) to form reactive dialdehydes from some of the

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glucose moieties of the dextran polymers.

On the one hand, these will form Schiff base complexes with the epsilon amino nitrogens of a few of the lysyl residues of antibodies under the right incubation conditions. On the other hand, Schiff base linkages can also form between aldehydes of the activated dextrans and appropriately boronated synthetic amines, which will thereby be bridged to the antibodies. As Detlef Gabel reported (10), his laboratory has already prepared an aliphatic amine and an aromatic amine each of which carries a decachlorocarborane cage, so the stage is set to complete our first synthesis of a boronated dextran-antibody complex. The alternative cyanogen bromide activation of dextrans noted by our abstract is still in preliminary form, although it is already clear that dextrans can also be conjugated to proteins in this way (7). Hence, we cannot tell yet which type of activtion will be preferable in the long run.

METHODS:

We now describe briefly our methods and results utilizing periodate activation of dextrans for conjugation to IgG. Because Schiff base formation is optimized at pH's below 6, we departed from the method employed by the Weizmann Institute group (5,11) and others (12) of coupling periodate-activated dextrans with antibodies at a pH of 8.5 or above. Hydrolysis of the Schiff base linkages and fragmention of the dextrans probably occurred at those alkaline pH's, and this, in turn, may have given rise to some aggregated products (12). Our incubations were carried out at pH 6.0 or at pH 4.5.

First we activated with sodium periodate an aqueous solution of dextran of molecular weight about 40 kilodaltons. The periodate was present at a ratio one half that of the glucose residues of the dextran polymer, whose concentration was determined by an anthrone colorimetric reaction (13). Isolating the partially oxidized dextran by dialysis and lyophilization and then using the Prussian blue method of assaying aldehydes (14), about 16% of the glucose residues were found to contain dialdehyde. A second periodate oxidation afforded an additional 5% conversion of glucose residues to "free" aldehyde for a final yield of 21%.

At slightly acid pH cyanoborohydride has been reported to reduce labile Schiff bases to more stable secondary and tertiary amines without reducing aliphatic aldehydes (15), so on some runs sodium cyanoborohydride was added. Most reactions were terminated by reduction of the remaining free aldehyde groups with sodium borohydride at a pH of 9.0-9.5. Following anion exchange chromatography, the fractions were analyzed for dextran and for protein.

RESULTS:

Table 2 summarizes some typical reactions. The extent of conjugation of IgG by dextran is given on a weight-per-weight basis in the last column. The values shown represent averages of the fractions in the protein-containing peaks, unweighted for their protein contents, along with their standard deviations. We shall return to the significance of these values after examining briefly two representative chromatcgrams.

Figure 1 shows a chromatogram of a reaction carried out at pH 4.5. The elution profile contains two unreacted dextran peaks. The first, probably

Table 2. Coupling of periodate-oxidized dextrans with IgG

Reactions[1]				Product Analyses[2]	
Exp't	pH	Schiff's Bases Reduced by $NaCNBH_3$?	"Free" Aldehydes Reduced?	Protein Recovery (percent)	mg Dextran / mg IgG
1	4.5	No	Yes	80	1.26 ± 0.18
2	4.5	Yes	Yes	73	1.29 ± 0.20 (low I)
					1.03 ± 0.07 (high I)
3	6.0	No	Yes	41	1.01 ± 0.14
4a	6.0	Yes	Yes	81	1.37 ± 0.11
b		Yes	Yes	87	1.21 ± 0.06 (major)
					1.17 ± 0.23 (minor)
5	6.0	Yes	No	22	0.8 - 0.9

[1] Reaction conditions: IgG (4 mg/ml, Cohn Fraction II, human) was reacted with 21% oxidized dextran (20 mg/ml) in 0.1 M sodium acetate buffer, pH 4.5, or 0.1M sodium phosphate buffer, pH 6.0. 1 M sodium cyanoborohydride was added to a final concentration of 0.06 M, when used (Yes in column 3); reaction time: 24-26 hours. Reactions with final reduction of aldehyde groups were terminated by addition of solid sodium carbonate to pH 9-9.5 followed by 5 M sodium borohydride to a final concentration of 0.15 M (Yes in column 4).

[2] Analyses: Reaction mixtures were desalted on G25 Sephadex equilibrated with 0.01 M tris base prior to anion exchange chromatography. Stepwise increases in ionic strength (I) to 1.0 M sodium chloride were used to elute unreacted dextran followed by dextran-IgG complexes. Ion exchangers were DEAE cellulose or DEAE BioGel A. Protein was determined by UV spectra and optical absorbance at 278 nm, using an optical density of 1.3 for one mg/ml at 1 cm pathlength. Total recovery (column 5) was obtained by integrating values from all fractions. Dextran was quantified by the anthrone reaction using as a standard 21% oxidized dextran with its "free" aldehyde groups reduced by sodium borohydride. The extent of conjugation of dextran with IgG is given on a weight-per-weight basis (Column 6). The values shown represent averages of the fractions in the protein-containing peaks, unweighted for their protein contents, along with their standard deviations.

uncharged, elutes at the lowest ionic strength, while the second appears at low ionic strength and presumably contains some charged groups at pH 8-9. However, neither peak has been further characterized.

Although the IgG of Cohn fraction II is not homogeneous, under the conditions used for this run essentially all the protein is eluted in one peak. Quantification of the dextran-to-protein weight ratio was ascertained for each fraction in the peak and is shown at the upper right. The important point to remember from Figure 1 is that there is no evidence for significant variation of the dextran binding ratio in different fractions, and the average value of 1.26 corresponds to 4 or 5 molecules of dextran per globulin.

Under the conditions of the reaction represented by Figure 2 the unconjugated, heterogeneous IgG elutes as three peaks in essentially the same locations as shown here for the gamma globulin conjugated with dextran. Because one of the unreacted dextran peaks overlaps the first protein peak

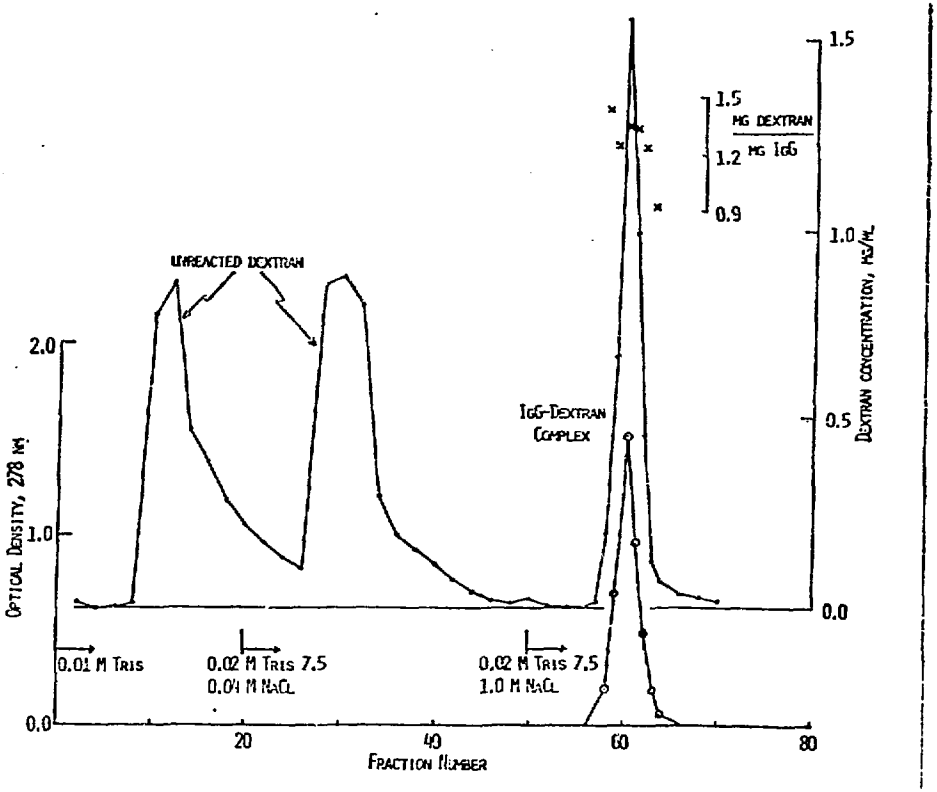

Figure 1. Anion exchange chromatography of a reaction of IgG with 21% oxidized dextran at pH 4.5 in the absence of sodium borohydride. Refer to Table 2, (experiment 1) for reaction conditions. Exchanger: DEAE cellulose; 1.5 x 10.4 cm (diameter x length); 1.8 ml per fraction. Dextran determinations are given by the upper trace with its ordinate at the right in units of mg/ml. Protein elution is represented by the lower trace with its ordinate on the left, in units of optical density at 278 nm.

that is eluted, no meaningful dextran-to-protein coefficients can be calculated for the corresponding fractions. However, the conclusion to be emphasized by this figure is that even for the minor, late-eluting protein fraction, the dextran-to-IgG ratios are not significantly different from those of the main peak, which correspond to about five molecules of dextran per immunoglobulin. In other words, all the IgG molecules appear to be conjugated with dextran to about the same extent. This is consistent with Figure 1 and with other runs not shown.

In experiment 5 (Table 2) IgG was conjugated with dextran and chromatographed with its "free" aldehyde groups still oxidized. Although these are recent data, and it is clear from the poor protein recovery noted in column

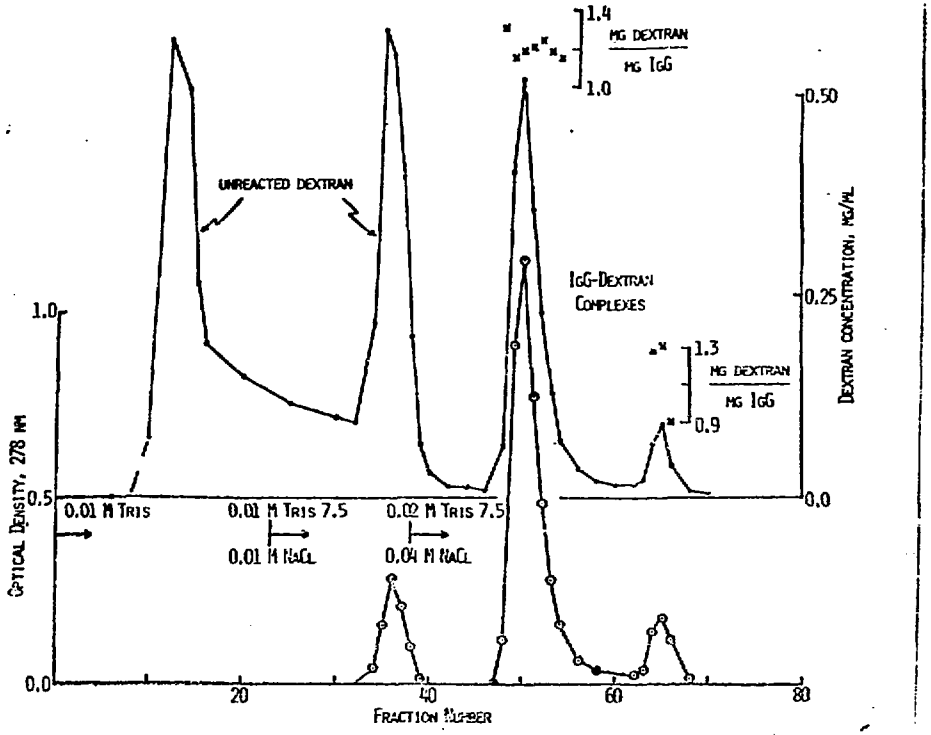

Figure 2. Anion exchange chromatography of a reaction of IgG with 21% oxidized dextran at pH 6.0 in the presence of sodium cyanoborohydride. Refer to Table 2 (experiment 4b) for reaction conditions. Exchanger: DEAE BioGel A; 1.5 x 11.4 cm (diameter x bed height); 1.8 ml per fraction. Dextran determinations are given by the upper trace with its ordinate at the right in units of mg/ml. Protein elution is represented by the lower trace with its ordinate on the left, in units of optical density at 278 nm.

five that we have yet to optimize chromatographic conditions, this preliminary experiment introduces an important extension of our work. It represents the case wherein dextran is conjugated to IgG but still carries many aldehyde moieties which are capable of further reaction to form bridging Schiff base linkages with boronated synthetic amines, such as the aliphatic and aromatic amines complexed to carborane cages that Detlef Gabel and his colleagues have already synthesized (10). Successful achievement of this step may afford boronated dextran-antibody globulin conjugates of the kind needed for NCT.

DISCUSSION:

We return now to the significance of the coefficients in the last column of Table 2. Assuming that the dextrans of 40-kilodalton molecular weight have not been fractionated significantly and that the mean weight of

the somewhat heterogeneous IgG is 153 kilodaltons, a dextran-to-IgG weight coefficient of one corresponds to an average of about 3.8 dextrans complexed to each gamma globulin. Thus a combining ratio of 5 requires a coefficient of 1.31, which is surpassed or nearly equaled by half of the runs terminated by reduction of the "free" aldehydes, as shown by experiments 1-4 in Table 2. Preliminary experiment 5 with IgG conjugated by dextran that is still highly activated for combination with amines carrying carborane cages clearly reflects suboptimal conditions, as noted before. But even so, the fraction of globulin that was recovered in that experiment was attached to more than 3 oxidized dextrans, on the average.

In summary, Table 2 indicates that about five dextrans of 40-kilodalton molecular weight can be bound per antibody. Gabel has already complexed dextrans with decachlorocarboranes to the extent of 5% boron by weight (16), so a projection of at least 1000 boron-10 atoms per lightly conjugated antibody is reasonable. If a characteristic cell volume is taken as 1000 cubic microns, which is typical of a V-79 Chinese hamster cell for example, then the boron-10 content per million antigenic sites per cell will be 17 ppm (Table 1). However, for the antimelanoma MCA's we plan to use, Ferrone has reported 3 million antigenic determinants per cell for each of the first three MCA's whose binding he has quantified (17), and two additional highly specific antibodies have been characterized recently. Hence a "therapeutic cocktail" of boronated dextran-conjugated derivatives of all five of his antimelanoma antibodies might be able to carry as much as 170 ppm of boron-10 to the target if, among the five antibodies, as many as 10 million binding sites are available (Table 1). This compares favorably with the 30 to 45 ppm of boron required for successful antibody-targeted NCT with epithermal neutrons and the 2 to 8 ppm required for scandium-filtered neutrons, and there is a comfortable margin for error.

In short, only the first stage of our project has yet been approached experimentally. Nevertheless, pessimistic projections regarding successful NCT with boronated MCA's appear unwarranted. The use of intermediate carriers to link a thousand or more boron-10 atoms to each antibody with only a handful of amino acid residues on the globulin being conjugated appears very promising for NCT in cases where there are a million or more antigenic sites per cell for the monoclonal antibodies that are available.

In closing, we emphasize that the concept of NCT is not limited to potential cancer treatment, despite the emphasis given to the goal by this symposium and even by this presentation. Extension of the principles developed here should permit NCT to achieve highly specific radiation inactivation of other cellular targets toward which appropriately selective antibodies can be developed, such as clones of cells involved in autoimmune diseases, transplant rejection, etc.

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