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Preparation of Tumor-Specific Boron Compounds. 1. *In Vitro* Studies Using Boron-Labeled Antibodies and Elemental Boron as Neutron Targets†

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The diazonium salt from 1-(4-aminophenyl)-1,2-dicarba-*closo*-dodecaborane(12) has been incorporated into anti-bovine serum albumin and antibody proteins specific to human and mouse histocompatibility antigens. The incorporation of the B label (natural isotopic abudance) into the antibody is accomplished under sufficiently mild conditions so as to have minimal effect on the activity and specificity of the resulting B-labeled antibody. The amount of B incorporated into the antibody is sufficient to cause *specific* cellular destruction when subjected to neutron radiation *in vitro*. It has also been demonstrated that particulate B (natural isotopic abundance) is incorporated into Walker 256 cells which can be specifically destroyed with neutron radiation. The significance of these results using B-labeled antibodies and elemental B strongly suggests that the concept of incorporating boron into proteins which exhibit marked tumor specificity is of great potential for use in neutron-capture therapy.

The potential use of boron-containing compounds in cancer therapy is based on the unique nuclear property of the ^{10}B nucleus to absorb thermal neutrons. This approach to cancer therapy is based on the liberation of high-energy fission fragments following neutron capture. The incident thermal neutrons have a relatively low energy (0.025 eV) which gives rise to a very high energy α-particle (2.4 MeV) following capture by ^{10}B and fission.

The essential factors for the selective destruction of tumors have been reviewed.[1,2] The major requirements for the successful application of neutron-capture therapy are: first, there must be a large concentration of ^{10}B in all areas of the neoplasm (15 mg of ^{10}B/kg of tumor[2]). This requires that the B compound be injected iv and not directly into the suspected tumor area since the precise dimensions and configuration of the invasive neoplastic process is not clearly delineated. Second, a source must be available to irradiate the neoplastic area with a sufficient number of thermal neutrons to cause cellular destruction (10^{12} neutrons/cm^2).[3] Third, a sufficiently large concentration differential of ^{10}B between the neoplasm and adjacent normal tissue must exist to permit the complete eradication of the tumor without adversely affecting tissue surrounding the neoplasm.

The primary emphasis for all of the previous studies of neutron-capture therapy is the syntheses of B compounds which exhibit suitable structure-activity relationships for specific binding to neoplasms. This approach has met with only minimal success in fulfilling the requirement for neoplasm destruction.[4] The possibility of using ^{10}B-containing antibodies for use in neutron-capture therapy has been suggested.[1] The basis for this approach is that antibodies are formed in response to the administration of an antigen and react *specifically* with that antigen. Consequently, if ^{10}B could be incorporated into a tumor-specific antibody protein, the antibody would subsequently concentrate the boron in the region of the tumor cell antigen and the cell might then be destroyed by subsequent thermal neutron radiation. This paper describes our initial exploratory research based upon this concept and provides encouragement for future work.

Results and Discussion

BSA–Anti-BSA. The chemical modifications required for ^{10}B incorporation are based on the extensive research which has been performed in the area of fluorescent protein tracing.[5,6] Proteins, including serum antibodies, can be labeled with fluorescent dyes without material effect on their biological properties. The fluorescent proteins are then detectable in tracing experiments by fluorescence microscopy. Tracing may be carried out directly by injection of labeled proteins and subsequent histological examination, or by the application of immunological principles in which labeled antibody is used as a specific histochemical stain for antigenic materials in tissues.

Our first investigation of the applicability of this method used the standard precipitin reaction.[7] The purpose of this experiment was to determine if a B-containing compound could be attached to an antibody protein under sufficiently mild conditions so as to have minimal effect on the resulting labeled antibody specificity. The modification of the antibody was based on the results of antihapten antibody

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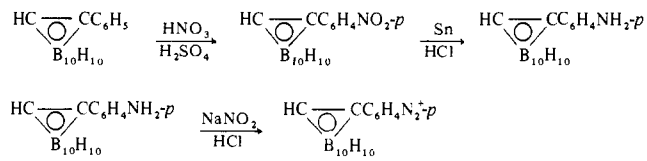

Figure 1. Preparation of boron-labeling reagent.

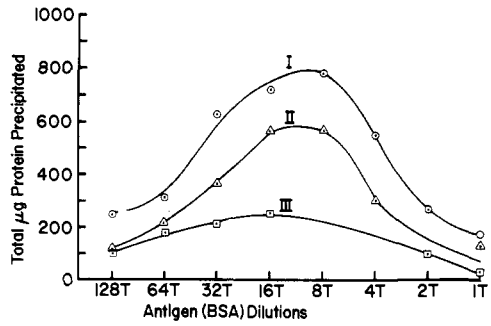

Figure 2. Precipitin reaction between bovine serum albumin (BSA) and rabbit anti-bovine serum albumin (anti-BSA). I, BSA + 7S anti-BSA; II, BSA + 7S anti-BSA (labeled) complex contg 0.35% B; III, BSA + 75 anti-BSA (labeled) complex contg 0.38% B.

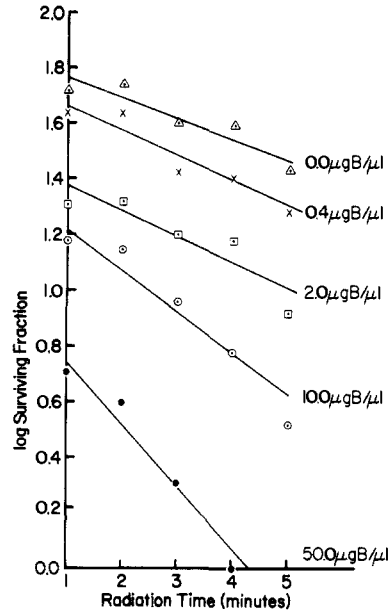

Figure 3. Survival curves for peripheral blood lymphocytes with $K_2B_{10}H_{10}$ solns.

labeling.[8] The B compound which served as the precursor in subsequent diazo coupling reactions was 1-(4-aminophenyl)-1,2-dicarba-*closo*-dodecaborane(12) which contained a natural abundance of boron isotopes (20% ^{10}B) and was converted to the corresponding diazonium ion for use as a labeling reagent containing a high total boron content. The preparation of this compound has been reported previously,[9] and the synthesis is outlined in Figure 1. The diazonium salt could couple to antibody proteins through tyrosine, histidine, and lysine groups.[8]

The 7S fraction of rabbit anti-bovine serum albumin (anti-BSA) was coupled with the diazonium salt in a phosphate-buffered (pH 8.0) aqueous solution at 4°. The resulting yellow (λ_{max} 350 nm), B-labeled antibody solution was purified and the quantitative precipitin reaction was performed on the various preparations. Two labeled antibody solutions were prepared by varying the molar ratio of the diazonium salt to the antibody. The first was prepared at a molar ratio of 20:1; the second with a ratio of 100:1. The quantitative precipitation curves are shown in Figure 2. These results indicated that about 0.4% B (natural isotopic abundance) had been incorporated into the protein system with a concomitant loss of about 25% of the precipitating ability of the antibody.

These preliminary results with the BSA-anti-BSA system proved that suitable B-containing compounds can be incorporated into antibody proteins under sufficiently mild conditions such as to maintain the specificity of the antibody.

Human Lymphocyte System. Since nearly all neoplastic cells thus far studied in detail appear to possess tumor-associated antigens which induce an immune response in competent hosts,[10] cancer research should benefit by the development and exploitation of this technique. Neutron-capture therapy using ^{10}B-conjugated antibodies allows a new approach to cancer therapy; one that combines the great tumor specificity of antibodies with the known ^{10}B thermal-neutron-capture reaction. This method would thus limit and localize the destruction of tissue to tumor cells which carry ^{10}B-labeled tumor-specific antibodies at the time of neutron irradiation.

Human histocompatibility antigens are also cellular antigens and invoke antibodies when introduced into an allogeneic host. Since more is known about the antigens and antibodies formed in multiparous females, the transplantation system was adopted as a model for the development of the test. The test for lymphocyte killing was adapted from Terasaki's lymphocytotoxicity test and possesses the advantage of microassays. It is presently used in most histocompatibility testing.[11]

To ascertain the feasibility of *in vitro* lymphocyte killing by the α particle emitted from neutron-irradiated ^{10}B, purified peripheral blood lymphocytes were tested in B-containing solutions of varying concentrations. In this test the boron was added as $K_2B_{10}H_{10}$, a compound which exhibits a low toxicity[12] (LD_{50} = 1025 mg/kg) and nonspecific absorption to cellular material. Similar studies using sodium pentaborate *in vivo* have been reported.[3] The thermal neutron source was the UCLA R-1 100 KW nuclear reactor with a core flux of 1.5×10^{12} neutrons/cm^2 sec.

The results of the neutron radiation experiment using lymphocytes and $K_2B_{10}H_{10}$ are shown in Figure 3

For the initial studies with B-conjugated antibodies, a strong multispecific antiserum was selected. Among the specificities possessed by this antiserum were HL-A 1, 2, 3, 9, and Te55. Since nearly 50% of the population carry the HLA-2 antigen, lymphocytes needed for the tests were relatively easy to obtain.

The 7S fraction of the serum was isolated by passage of the serum through Sephadex G-200. The coupling reaction between the antibody containing fraction and the B-containing diazonium compound was allowed to proceed at 4° in an aqueous, phosphate-buffered, saline (pH 7.4) media. The antibody was purified and analyzed for total B (0.2%).

The antibody preparation was tested against cells which carried the HL-A specificity. The results of the neutron radiation experiments which varied both the antibody concentration and radiation time are shown in Figure 4. The initial antibody concentration was 15 mg/ml.

These results indicate that the binding of the B-conjugated specific antibody brought the boron atoms close enough to the cells so that the α-particle disintegration product formed from neutron capture by ^{10}B destroyed the lymphocytes. That the conjugation of the B-containing label to antibody

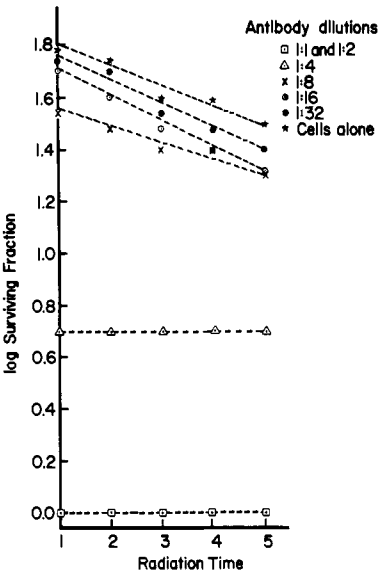

Figure 4. Effect of titration of B-labeled antibody on survival of peripheral blood lymphocytes. Initial antibody concn = 15 mg/ml; 0.20% B.

molecules did not affect the antibody specificity was shown by the cytotoxic effect in the test with complement employing this antibody preparation. The complement cytotoxic test using normal and B-labeled antibodies gave similar results for the antibody activity.

The results obtained by varying the total B concentration and the radiation time shown in Figures 3 and 4, were compared in order to demonstrate the ability of the antibody to concentrate the B-containing label on the cell. From Figure 3, 2 μg of B/μl gave 30% survival after 2 min of neutron radiation. From Figure 4, an antibody titer of 1:8 gave 30% survival after 2 min of radiation. The antibody contained 0.2% B. The concentration of the antibody and B at this dilution (1:8) was 1.87 μg of Ab/μl and 3.8×10^{-3} μg of B/μl. Comparison of the total B concentration required to give a comparable level of cellular destruction in the two experiments indicates that if the B is first incorporated into a specific antibody the B is approximately 500 times more effective in causing cellular destruction than a nonspecifically bound B compound.

Mouse Histocompatibility System. Antibodies directed against the C57 B110 mouse strain were prepared in the C3H mouse strain. The C3H anti-C57 B110 antibody (7S fraction) was conjugated with the B-containing labeling reagent in the usual manner. The B-labeled antibody solution containing 20% of available B as ^{10}B was then incubated with C3H lymphocytes and C57 B110 lymphocytes. The cells were washed with tissue culture media and subjected to neutron radiation. The results obtained are shown in Figure 5. The C3H lymphocytes survived the radiation since the B-containing antibody was completely removed by washing with tissue culture media. The C57 B110 lymphocytes exhibited a marked decrease in survival owing to the specific binding of B-containing antibody to the histocompatibility antigens against which the antibody was directed. This procedure of removal of excess antibody prior to radiation experiments indicates that specific antibodies, when labeled with ^{10}B, will destroy only those cells which exhibit the antigen necessary for binding the B-labeled antibody. Thus, in principle, the excess B-containing antibodies can be removed prior to radiation of cancer patients in order to limit cellular destruction in nonneoplastic regions.

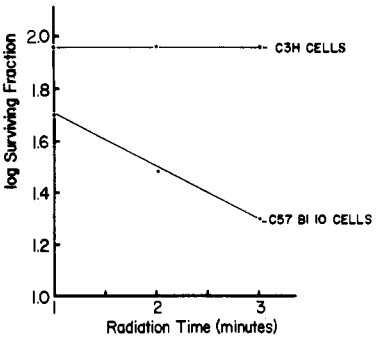

Figure 5. Survival of C3H and C57B110 peripheral blood lymphocytes after incubation with B-labeled C3H anti-C57B110 antibody.

Cellular Incorporation of Elemental Boron. An additional mechanism which is operative in the immune response is the promotion of phagocytic activity.[13] Phagocytosis is performed by polymorphonuclear leukocytes and macrophage cells; the latter include monocytes in the bloodstream, the lining of cells of the sinusoids of the liver, bone marrow, lymph nodes, and spleen. It was anticipated that owing to increased phagocytic activity it would be possible to incorporate elemental boron *within* the cell owing to its particulate nature.

Elemental B (natural isotopic abundance) (1-10 μ particle size) was added to Walker 256 cloned cells in tissue culture media. After 2.5 hr 78% of the cells exhibited visible B incorporation within the cell. The cells containing the B were removed with trypsin and replated, washing away the excess B particles which had not been absorbed. One-half the cells were subjected to a 30-min neutron irradiation, the other half were used as a control. Immediately following the neutron radiation both sets of cells appeared normal and no differences were noted under an inverted microscope with 40X objective. However, after 21 hr the cells subjected to neutron radiation had fragmented and were dead, whereas the B-containing cells which had not been irradiated were normal. In a separate control experiment it was determined the W256 cells suffered no ill effects when subjected to 1 hr of neutron radiation.

The difference in the time limit for neutron radiation between peripheral blood lymphocytes and W256 cells reflects only a major difference in the radiosensitivity of cells.[14] In addition, the α particle produced from ^{11}B fission causes observable damage to lymphocytes immediately, while the α particle damage in W256 cells is more delayed.

Conclusions

These results obtained using B-labeled antibodies and elemental B *in natural abundance* strongly suggest that the concept of incorporating B into proteins or polymers which exhibit marked tumor specificity is of great potential for use in neutron-capture cancer therapy. One additional and important aspect of the use of B-labeled antibodies is that the antibodies when bound to the cell define the region of cellular destruction so that the exact dimensions of the neoplasms do not need to be determined as in other forms of radiation treatment.

The B-containing labeling compound used in this study and the method of conjugation to the protein had little effect on the specificity of the antibody and exhibited no cellular toxicity at the concentrations used in these studies. In addition, use of highly ^{10}B enriched labeling compound

could increase the effectiveness of cellular destruction by as much as fivefold.

Experimental Section

1-(4-Aminophenyl)-1,2-carborane was prepared by the literature procedure.[9] N and B analyses were performed using standard analytical procedures.[15,16] Protein concns were detd spectrophotometrically.[17] The neutron radiation experiments were performed in the thermal column of the UCLA R-1 reactor. The thermal neutron flux with the reactor operating at 100 KW is 1.5×10^{10} neutrons/cm^2 sec, which was detd by standard Au foil radiation techniques at the irradiation site.

Diazotization of 1-(4-Aminophenyl)-1,2-carborane. A general procedure was used for diazotization and suitable aliquots of aqueous solns of the diazonium salt were used in subsequent labeling experiments.

1-(4-Aminophenyl)-1,2-carborane (24 mg, 0.1 m*M*) was added to 0.3 ml of 1 *M* aqueous HCl in an ice bath. A $NaNO_2$ soln (7 ml, 1 mg/ml) was added and the mixt allowed to stir for 0.5 hr. The soln was filtered and dild to 25.0 ml with 0.01 *M* aqueous HCl. Owing to the reactive nature of the diazonium salt, the solns must be kept cold and slightly acidic to retard decompn. The diazonium soln was stable for at least 3 hr if kept in an ice bath.

The concn of the diazonium salt was detd spectrophotometrically. A suitable aliquot (~10 μl) of the above soln was added to a 0.01 *M* (10 ml) soln of β-naphthol in 95% EtOH. After 30 min the absorbance was detd at 485 mμ (ϵ_{485} 1.98×10^4).

BSA-Anti-BSA Reaction. Bovine serum albumin (BSA) and the 7S fraction of rabbit anti-bovine serum albumin (lyophilized) were obtd commercially and used as received.‡ All solns were prepd in an aqueous phosphate buffer (pH 8.0). The precipitin reaction was performed by the literature method.[7] The initial concns of BSA and anti-BSA were 10 mg/ml and 15 mg/ml, respectively.

Two separate B-labeled anti-BSA solutions were prepared by varying the molar ratio of the diazonium salt to anti-BSA during the coupling reaction. The labeled anti-BSA solutions obtd from the coupling reaction with a molar ratio (diazonium/anti-BSA) of 20/1 (II) and 100/1 (III) were used in the precipitin reaction. A third precipitin reaction was performed using BSA and unmodified anti-BSA (I).

Suitable aliquots of the diazonium soln were added to the anti-BSA soln at 4° at pH 8.0. The pH was maintd at 8.0 by dropwise addn of 0.1 *M* NaOH. The resulting yellow soln (λ_{max} 350 mμ) was allowed to stir for 24 hr at 4°. The mixt was centrifuged to remove the decomposed diazonium salt which had not coupled to the anti-BSA. The clear yellow supernatant was dialyzed for 48 hr against phosphate-buffered saline. The soln was evapd to the original vol and the precipitin reaction performed.

Human and Mouse Histocompatibility Boron-Labeled Antibodies. The 7S fraction of serum was obtd by gel filtration through Sephadex G-200 with phosphate-buffered saline. The 7S fraction was concd by negative pressure dialysis.

The labeling experiments were performed as outlined previously using a molar ratio of 10 diazonium/1 antibody. The coupling reaction was allowed to proceed for 3 hr at 4° in a phosphate-buffered saline soln (pH 7.4). The yellow soln was passed through Sephadex G-25 with phosphate-buffered saline to remove the unreacted diazonium salt. The effluent was again concd by neg pressure dialysis.

B-labeled antibody solns (1 μl) of different concns were added to the wells of a microtest plate§ under 5 μl of mineral oil. The oil is added to the well first to prevent the minute amts of reagents from drying. One thousand purified, peripheral blood lymphocytes suspended in 1 μl of McCoy's medium were added to each well. Following neutron radiation, Eosin Red was added to each well and the test terminated by fixation of the cells with formalin. The tray was prepd for reading in an inverted phase microscope. Live cells in this test appear bright, clear, and unstained while dead cells are dark and diffuse.

Lymphocytotoxic assays were performed on all B-labeled antibody solns. After 0.5 hr incubation of the cells with the antibody, 5 μl of rabbit complement was added to each well followed by an additional 1 hr of incubation. Eosin Red was then added to each well to stain dead cells and the test was fixed with formalin. Each of the B-labeled antibody prepns gave an antibody activity titer identical to the unmodified antibody.

‡ Nutritional Biochemicals Corporation, Cleveland, Ohio, 44128.
§ Falcon Plastics, Los Angeles, Calif.

For neutron radiation experiments with the excess B-labeled antibody removed (mouse histocompatibility system), B-labeled antibody solns at full strength were allowed to react with cells for 1 hr. The cells were then centrifuged and washed twice in tissue culture media. Antibody-coated lymphocytes were then adjusted to give 1000 cells in 1 μl and dispensed into the wells of a microtest plate under oil. The plates were treated in the same manner as described previously.

Intercellular Incorporation of Particulate Boron. Six control and 6 experimental small flasks were each plated with 0.28×10^6 Walker 256 cloned cells. The cells in the experimental flasks were allowed to take up B particles from a saline soln added to their medium. The cells were left in contact with the particulate boron for 2.5 hr and then removed with trypsin and replated on small flasks. Of the cells 78% had visible uptake B. About 40 min after the small flasks were incubated at 37° in growth medium contg 20% fetal calf serum, the cells were subjected to neutron radiation for 30 min. The controls which had no B incorporation showed no change after the radiation. Three flasks contg cells with B were not irradiated to determine any toxic effects of B, the 3 other flasks with B were irradiated for 30 min. All flasks were examined following the radiation experiment and no differences were noted under inverted scope with 40× objective. An addl 2 ml of regular growth medium was added to each flask before replacing them in the incubator. After 21 hr both sets of control cells (no B and irradiated, and B with no irradiation) all appeared normal, while the cells with B and irradiation were all now fragmented, floating, and dead.

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References

(1) A. H. Soloway, "Progress in Boron Chemistry," A. L. McCloskey and H. Steinberg, Ed., Pergamon Press, New York, N. Y., 1964, pp 203-234.
(2) G. L. Brownell, A. H. Soloway, and W. H. Sweet, "Modern Trends in Radiotherapy," T. J. Deeley and C. A. P. Wood, Ed., Butterworths, Inc., London, 1967, pp 132-145.
(3) L. E. Farr and T. Konikowski, *Int. J. Appl. Radiat. Isotop.*, 19, 459 (1968).
(4) A. H. Soloway, H. Hatanaka, and M. A. Davis, *J. Med. Chem.*, 10, 714 (1967).
(5) "Fluorescent Antibody Techniques and their Applications," Akiyoshi Kawamura, Jr., Ed., University of Tokyo Press, Tokyo, 1969.
(6) R. L. Nairn, "Fluorescent Protein Tracing," 3rd ed, The Williams and Wilkins Company, Baltimore, Md. 1969.
(7) D. H. Campbell, J. S. McGarvey, N. E. Cremer, and D. H. Sussdorf, "Methods in Immunology," W. A. Benjamin, Inc., New York, N. Y., 1964.
(8) D. Pressman and A. L. Grossberg, "The Structural Basis of Antibody Specificity," W. A. Benjamin, Inc., New York, N. Y., 1968.
(9) M. F. Hawthorne, T. E. Berry, and P. A. Wegner, *J. Amer. Chem. Soc.*, 87, 4746 (1965).
(10) G. Klein, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 28, 1739 (1969).
(11) P. I. Terasaki and J. D. McClelland, *Nature (London)*, 206, 998 (1964).
(12) E. L. Muetterties, J. H. Balthis, Y. T. Chia, W. H. Knoth, and H. C. Miller, *Inorg. Chem.*, 3, 444 (1964).
(13) W. S. Hammond, "Biology of the Immune Response," P. Abramoff and M. LaVia, Ed., McGraw-Hill, New York, N. Y., 1970, Chapter 13.
(14) A. P. Casarett, "Radiation Biology," Prentice-Hall, Englewood Cliffs, N. J., 1968.
(15) G. Ingram, "Organic Elemental Microanalysis," Reinhold, New York, N. Y., 1962, Chapter 3.
(16) A. Kaczmarczyk, J. R. Messer, and C. E. Pierce, *Anal. Chem.*, 43, 271 (1971).
(17) T. H. The and T. E. W. Feltkamp, *Immunology*, 18, 865 (1970).