Brief Definitive Report

UBIQUITOUS NONIMMUNOGLOBULIN *p*-AZOBENZENEARSONATE-BINDING MOLECULES FROM LYMPHOID CELLS*

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In recent years, a large body of experimental data has suggested that molecules derived from mixed populations of T cells (1–3), T cell clones (4), and T-T hybridomas (5) bind antigen specifically and have molecular weights in the range of 60,000 to 70,000 (reviewed in 6, 7). Some of the molecules appear to bear antigenic determinants encoded within the major histocompatibility complex, and many are thought to share idiotypic determinants with B cells of the same antigenic system.

In an attempt to elucidate the role of idiotype in regulating the immune response of A/J mice to *p*-azobenzenearsonate (ABA), we recently constructed and partially characterized T cell hybrids (5). Several of these hybrids produced a protein that specifically bound hapten (i.e., ABA) and also was directly bound (precipitated) by antisera directed against the arsonate cross-reactive idiotype (CRI). The presence of idiotype on molecules involved in a specific immune response is well established but the precise molecular correlates for idiotypy in most systems are still largely unknown (8). Recent reports (9, 10) have described molecules unrelated to a specific immune response that cross-react with anti-idiotypic reagents. In the course of studies to further characterize the T cell hybridoma ABA-binding proteins recently reported from our laboratory (5), we discovered that these ABA-specific, idiotype-positive molecules are ubiquitous and are found not only in several lymphoid cell lines but also in normal liver and spleen cells.

Materials and Methods

T cell somatic hybrids were generated as described previously (5). Other cell lines used in this study were 1707D, a spontaneous B.10.S T cell lymphoma (D. Johnson, J. Kettman, and J. W. Streilein, unpublished observations); Fs7-6.1B, a T cell somatic hybrid (BW5147 × B10.BR T cells) producing interleukin 2 (11); SP2/O-Ag14, a myeloma cell line used for B cell fusions (12); and 93G7, a B cell somatic hybrid with anti-arsonate specificity (13). A/J spleen cells and homogenized liver were sources of normal cells used in this study. Cells were grown at 37° in a 7% CO_2, humidified atmosphere to a density of ~5×10^5 to 8×10^5 cells/ml. In some cases, cells were labeled with [^{3}H]leucine or [^{3}H]tyrosine in a manner similar to that reported previously (14).

The culture supernatants, cytoplasm, and NP-40 extracts of osmotically lysed cells were studied for the presence of antigen-specific material. Affinity resin was generated by coupling L-lysine to Sepharose 4B (15) and subsequently derivatized with diazobenzenearsonate (16).

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Cell fractions (1×10^8 to 10×10^8 cell equivalents) were applied to the affinity columns and specifically eluted with hapten.

Rabbit antibody affinity columns were prepared by covalently coupling the IgG portion of antisera to Sepharose 4B (15). ^{3}H-labeled ABA binding protein, purified by hapten elution of cell lysates from Sepharose-lysine-ABA columns, was extensively dialyzed and applied to the antibody affinity columns. The bound material was eluted with 0.2 M glycine/HCl, 0.15 M NaCl, pH 2.4.

Affinity purified material was tested for homogeneity by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) (17) and by isoelectric focusing (IEF) under denaturing conditions (18). Photographic negatives of Coomassie Blue-stained IEF gels were scanned on a Transidyne model 2955 densitometer (Transidyne General Corp., Ann Arbor, Mich.). The ABA-binding material was hydrolyzed in 6 N HCl *in vacuo* for 24 h, and its amino acid composition was determined on a Durrum model 500 amino acid analyzer (Dionex Corp., Sunnyvale, Calif.).

Results and Discussion

We previously reported (5) the generation of T cell somatic hybrids that produced ABA-binding molecules that reacted with antisera specific for the CRI of the arsonate system. In that study and in subsequent reports from our laboratory (19, 20), several different anti-CRI antisera were used, including those raised against serum A/J anti-arsonate antibodies and CRI-positive hybridomas. Current efforts have been directed towards chemically characterizing this hapten-binding product from hybridoma line T33F6, which had been selected from the original group (5) because it appeared to produce large amounts of ABA-binding protein. Radiolabeled T33F6 culture supernatants were passed over antigen affinity columns and specifically eluted with hapten. As described previously (5), the eluted material resulted in a single species when analyzed by SDS-PAGE or IEF. The ABA-binding protein had an apparent molecular weight of 62,000 ± 2,000 (determined by SDS-PAGE, Fig. 1 lane b; see also ref. 5) and an isoelectric point of 6.8 (Fig. 2). The product did not appear to be conventionally glycosylated because it failed to bind to several lectin columns (e.g., lentil lectin, concanavalin A, ricin II-A). Amino acid analyses demonstrated the presence of approxiately 15% basic, 14% acidic, and 35% hydrophobic residues (Table I).

Initial characterization of ABA-binding proteins used material derived from radiolabeled culture supernatants from T cell hybrids, but it was subsequently determined that a large amount of material could be isolated from the cytoplasm (0.1–1 $\mu g/10^6$

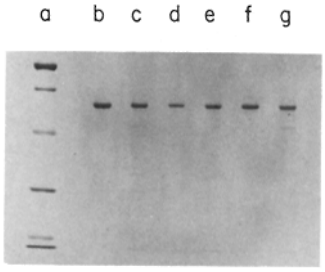

FIG. 1. SDS-PAGE of ABA-binding molecules purified by hapten elution from arsonate-derivatized affinity columns. Molecules were isolated from the cytoplasmic fraction of 5×10^6 cell equivalents. (a) molecular weight standards (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme); (b) T33F6 (T cell hybrid); (c) BW5147 (thymoma); (d) Fs7-6.18 (T cell hybrid); (e) 1707D (T cell lymphoma); (f) SP2/0-Ag14 (myeloma/hybridoma); and g, 93G7 (B cell hybrid). The minor protein at ~50,000 mol wt in lane g probably represents anti-ABA antibody heavy chain.

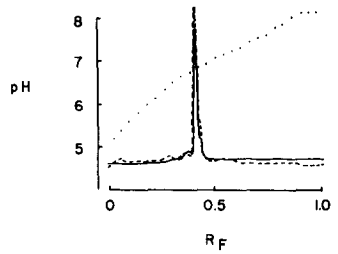

FIG. 2. IEF profiles of affinity purified ABA-binding proteins from BW5147 (solid line) and T33F6 (dashed line); pH gradient (·····).

TABLE I

Amino Acid Composition of ABA-binding Protein *

Amino acid	Percent concentration
ASX	8.9
THR	5.0
SER	9.6
GLX	11.1
GLY	10.9
ALA	11.6
VAL	5.7
MET	2.6
ILE	5.6
LEU	7.7
TYR	2.0
PHE	3.2
HIS	3.7
LYS	8.2
ARG	4.2

* Pro, Cys, and Trp values were not determined. Results of 24-h *in vacuo* hydrolysis in 6 N HCl.

cells). This prompted a search into the cytoplasm of the BW5147 fusion parent. As shown in our original studies, very little ABA-binding material was present in the culture supernatant of BW5147; unexpectedly, however, cell lysates of BW5147 contained large amounts of ABA-binding protein. The ABA-binding proteins isolated from the cytoplasm of the T cell hybrid T33F6 and its fusion parent BW5147 appeared to be identical when analyzed by SDS-PAGE (Fig. 1), IEF (Fig. 2), and amino acid analysis. The presence of this material in BW5147 was initially overlooked because only culture supernatants were studied (5). In culture, the T cell hybrids appear to be less viable than BW5147 and probably release material into their culture supernatants by cell lysis. Analyses of other lymphoid cell lines of both T and B cell origin (Fig. 1), normal lymphoid cells (i.e., A/J spleen cells), and nonlymphoid cells (i.e., hepatocytes) demonstrated the presence of this apparently ubiquitous material. Thus, the T cell hybrid ABA-specific product initially reported from our laboratory does not appear to function in the immune response to *p*-azobenzenearsonate. The initial conclusions concerning this molelcule were the result of the fortuitous presence of a protein that binds to both ABA-derivatized amino acids and anti-idiotypic antibodies.

Carefully controlled fluorescence-activated cell sorter (FACS) analysis of cell surface molecules demonstrated the presence of idiotype on two of the T cell hybrids (T33F6 and T33D5) and absence of idiotype on the fusion parent BW5147 (19, 20). There are

several possible explanations for this observation: (*a*) the T cell hybrids selectively display a membrane form of the ABA-binding protein; (*b*) the lytically released ABA-binding material interacts (binds) with the cell surface; (*c*) the T cell hybrids actually contain idiotype receptors that are independent of the ABA-binding protein and have not yet been isolated; or (*d*) the T cell hybrids are actively secreting the ABA-binding proteins, and the surface staining reflects their transmembrane transport.

Our initial report of the T cell-derived ABA-specific product clearly stated that the identification of idiotype on this product was based on a binding assay (5) rather than the conventional inhibition assay normally used to define the arsonate CRI (21). This material is not inhibitory in the idiotype-anti-idiotype assay normally used in our laboratory (13). The majority of studies in which idiotype has been identified on T cells and T cell products has used direct binding assays (22–24). The binding of anti-CRI described in our previous study was established by immunoprecipitation and has been subsequently verified both by FACS analysis (19, 20) and again in this study by affinity chromatography using a different anti-idiotypic reagent. In the present study, radiolabeled ABA-binding proteins derived from T cell hybrids as well as BW5147 reproducibly bound to anti-CRI affinity columns but did not bind appreciably to an irrelevant affinity resin (i.e., anti-gamma globulin). The products that we isolated, both from T cell hybrids and other lymphoid cell lines and from the anti-arsonate antibodies used to generate the anti-idiotypic reagents, both bind to ABA affinity columns and could have similar binding sites. Some of the molecules in the anti-idiotypic antisera might, therefore, recognize structural similarities shared between these sites.

Two recent reports call attention to the reactivity of a protein that is not part of an idiotypic system with anti-idiotypic reagents. Two phosphocholine-binding proteins, a murine myeloma protein and C-reactive protein, both possess the TEPC 15 idiotype, as defined in a competition assay using a monoclonal anti-idiotypic reagent (9). It also appears that a Thy-1 determinant cross-reacts with a monoclonal antibody that recognizes the V_k region of TEPC 15 (10). The presence of idiotypic determinants on a molecule is a potentially important target for immunoregulation (22, 25, 26). However, the existence of molecules not elicited during specific immune responses that cross-react with anti-idiotype (9, 10) and of idiotype-positive molecules that do not bind antigen (27, 28) makes interpretations based on either antigen binding or idiotype exceedingly difficult until a precise chemical definition of idiotype is available in the system under study. Further, the presence of an antigen-binding, idiotype-positive, 62,000 mol wt protein in virtually all lymphoid tissues studied should encourage caution concerning the nature of T cell receptors and factors.

Summary

A ubiquitous nonimmunoglobulin molecule that binds *p*-azobenzenearsonate (ABA) has been detected in the cytoplasm of several murine cell lines, including T cell hybridomas as well as in normal liver and spleen. Similar to many recently described antigen-specific T cell factors, this ABA-binding protein has a 62,000 mol wt, and, when analyzed by direct binding, the molecule reacts with several different rabbit anti-idiotypic antisera specific to the ABA system. The presence of this antigen-specific, "idiotype positive" moleule in many different cells indicates that it is not an important immunoregulatory molecule.

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