T CELL HYBRIDS WITH ARSONATE SPECIFICITY

I. Initial Characterization of Antigen-specific T Cell Products that Bear a Cross-Reactive Idiotype and Determinants Encoded by the Murine Major Histocompatibility Complex*

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The immune response of A/J mice to the *p*-azophenylarsonate (Ar)[1] hapten has become one of the most extensively characterized idiotypic systems. As originally defined by Nisonoff et al. (1–3), the system takes advantage of the fact that 20–70% of the serum anti-Ar antibodies bear a cross-reactive idiotype (CRI) when an appropriately absorbed rabbit antiserum is used. This finding has prompted a number of studies on the induction, regulation, and structure of such antibodies (4–9). Recently, our attention has been directed toward a serologic and chemical study of the products of somatic cell fusion (10–12). Through a thorough understanding of the relationship of these monoclonal B cell products to each other and to the induced serum antibody, we hope to approach, in molecular terms, an understanding of the precise chemical nature of the Ar idiotype.

Several laboratories have recently studied the role of T cells and their products in the arsonate system. Suppressor T cells have been shown to bear idiotypic determinants similar to those found on B cells and/or their antibody products. Furthermore, arsonate-specific suppressor factors have been characterized as proteins of between 33,000 and 68,000 mol wt, bearing determinants encoded within the major histocompatibility complex (13–16). However, a serious limitation of these studies has been the lack of single clonal products—especially ones that could be propagated indefinitely.

The production of T cell hybrids has led several investigators to use this technique to study T cell functions, T cell surface markers, and T cell products (17–21). The present study was undertaken with the aim of identifying such antigenic-specific nonimmunoglobulin T cell products in the A/J arsonate system. Our long-range goal is to understand the molecular relationships between such T cell products and the products of B cell hybridomas with similar idiotypic characteristics. Such studies are

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[1] *Abbreviations used in this paper:* ABA and Ar, used interchangeably for *p*-azophenylarsonate; BSA, bovine serum albumin; CRI, cross-reactive idiotype; DMEM, Dulbecco's modified Eagle's minimal essential medium; DTH, delayed type hypersensitivity; HAT, hypoxanthine aminopterin thymidine; HGG, human gamma globulin; HPLC, high-pressure liquid chromatography; MHC, major histocompatibility complex; PBS, phosphate buffered saline; PEG, polyethylene glycol; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

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fundamental to a complete dissection of the interrelationships of T-B interactions in this genetically and chemically characterized idiotypic system.

Materials and Methods

Mice. A/J mice (H-2^a) were obtained from The Jackson Laboratory, Bar Harbor, Maine. All animals were 8–10 wk old at the time of initial immunizations.

Preparation of Antigen and Antigen-coupled Cells. The diazonium salt of Ar (Eastman Kodak Co., Rochester, N. Y.) was prepared as described by Bach et al. (13). Activated Ar was conjugated to single cell suspensions of erythrocyte-free A/J splenocytes. Briefly, a 40-mM solution of Ar diazonium salt was prepared from Ar. A/J spleen cells were washed and resuspended in Dulbecco's modified Eagle's minimal essential medium (DMEM) and the erythrocytes lysed with ammonium chloride. Cells were then suspended in borate-buffered saline, pH 8.4, at a concentration of 1×10^7/ml and mixed with the solution of Ar diazonium salt. The final concentration of Ar was 10 mM. The reaction was stopped by the addition of cold phosphate-buffered saline, and the cells were washed and counted before use. The same preparation of Ar-coupled cells was used to prime for suppression, or alternatively to induce delayed type hypersensitivity (DTH) as described by Bach et al. (13).

Immunization and Challenge. A group of mice was primed for suppression by intravenous injection of 5×10^7 Ar-coupled syngeneic cells (13). 5 d later, the mice were challenged with 25 μl of 10 mM Ar diazonium salt in one footpad, and with 25 μl of saline in the other footpad. A second group of mice were primed for DTH by subcutaneous injection of 3×10^7 Ar-coupled cells and similarly challenged 5 d later. Footpad thickness was measured 24 h after challenge. The first group of mice showed minimal swelling, whereas the control group displayed a normal DTH. Spleens were removed from the suppressed mice for hybridization.

Antisera. Rabbit anti-mouse immunoglobulin, rabbit anti-CRI, and goat anti-rabbit Fc were prepared as previously described (2, 8–12). Monoclonal antibody (HP93G7) anti-Ar was previously produced in our laboratory and kindly supplied by Pila Estess. A.TH anti-A.TL was provided by three separate laboratories (Dr. E. S. Vitetta, Southwestern Medical School, Dallas, Tex., J. A. Frelinger, University of Southern California, Los Angeles, Calif., and Dr. H. O. McDevitt, Stanford University School of Medicine, Stanford, Calif.), as well as the Research Resources Branch of the National Institutes of Health, Bethesda, Md. A.TL anti-A.TH was provided by Dr. D. C. Shreffler, Washington Univ. School of Medicine, St. Louis, Mo. Anti-Thy 1.1 and 1.2 antisera were kindly provided by Dr. D. H. Sachs, National Institutes of Health, and Dr. J. Forman, Southwestern Medical School, Dallas, Tex., B10.BR anti-B10.D2 was provided by Dr. Richard Cook, Southwestern Medical School, and was further absorbed with the parent BW cell line before use.

Immunoadsorbents. Ar was coupled to human gamma globulin (HGG) and it, as well as HGG, were coupled to activated Sepharose 4B according to the method described by Cuatrecasas (22).

Cell Fusion. Spleen cells from suppressed mice were fused with the azaguanine-resistant thymoma line of AKR origin, BW 5147 (phenotype: H-2^k, Thy 1.1^+, Ig^-, Ly 1^-, Ly 2^-) (obtained from the Salk Institute, San Diego, Calif.) by a modification of the method of Pontecorvo (23). Briefly, lymphocytes and BW 5147 cells were mixed at a ratio of 10:1, centrifuged, and resuspended in 2 ml of a 40% solution of polyethylene glycol 1,000 in DMEM (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.). After a 2-min incubation at 37°C, the suspension was slowly diluted by adding 6 ml of DMEM. The cells were again pelleted, resuspended in hypoxanthine aminopterin thymidine (HAT) medium, and distributed into 96-well tissue culture plates (Costar, Data Packaging, Cambridge, Mass.) at a concentration of 3×10^5 cells/well. HAT medium was changed every fourth day. Microscopic cell growth was apparent within 3 wk. All the hybrids were assayed for binding to bovine serum albumin (BSA)-Ar (see below), and positive wells were cloned by growth at limiting-dilutions.

Radioimmunoassays. Culture supernates were tested for nonimmunoglobulin anti-Ar activity on BSA-Ar-coated polyvinyl microtiter plates by a modification of the method of Klinman et al. (24). An anti-Ar titration curve was constructed of affinity-purified anti-Ar antibody (HP93G7) and radiolabeled rabbit anti-mouse Ig as a detecting reagent (Fig. 1). On the basis

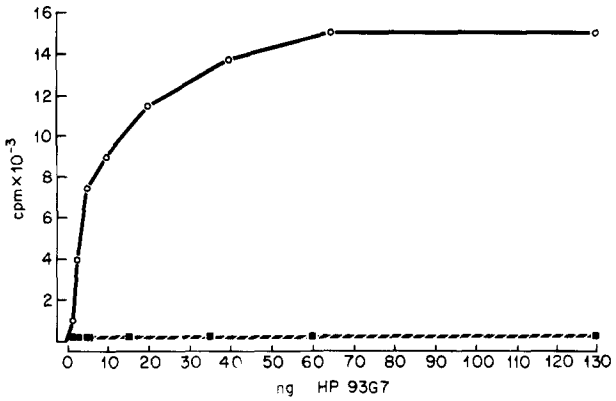

FIG. 1. Binding of HP93G7 anti-Ar antibodies to BSA-Ar (open symbols) or BSA (closed symbols) to coated polyvinyl microtiter plates. Dilutions of specifically purified 93G7 hybridoma product with anti-arsonate activity were added to each well. The ordinate indicates the number of counts per minute of ^{125}I-labeled rabbit anti-mouse immunoglobulin bound to each well. From this experiment, 40 ng of HP93G7 was chosen for the inhibition assay.

of this curve, a fixed point of 40 ng of HP93G7 was chosen, and culture supernates were tested for their ability to inhibit the binding of this murine anti-Ar to the test plate. 50 μl of culture supernate plus 50 μl of the anti-Ar monoclonal antibody were added to each well and incubated 3 h at room temperature. After further washing, 100 μl of ^{125}I-rabbit anti-mouse Ig was added. The plates were left overnight at 4°C, then washed extensively, and each well counted in a gamma counter. In the assays used in this report, the 100% figure (no inhibition) varies from 10,000 to 20,000 cpm because of variation in specific activity of the ^{125}I-rabbit anti-mouse Ig reagent.

Biosynthetic Labeling. Cells were centrifuged, washed twice with spinner salt solution, and then resuspended at a concentration of 3×10^6/ml in labeling medium (25) supplemented with a mixture of ^{3}H- (0.1 mCi/ml) or ^{14}C- (0.025 mCi/ml) labeled amino acids (New England Nuclear, Boston, Mass.). For most experiments, this mixture included arginine, lysine, and leucine. After 14–16 h incubation at 37°C, the supernates were harvested by centrifugation and stored at −70°C until used.

Gel Electrophoresis. Samples were subjected to electrophoresis on 10% acrylamide slab gels, as described by Laemmli (26). The following molecular weight standards were used: BSA (68,000), immunoglobulin heavy and light chain (56,000 and 25,000), ovalbumin (44,000), and carbonic anhydrase (30,000). The sodium dodecyl sulfate (SDS)-polyacrylamide gels were fluorographed as described by Bonner and Laskey (27).

Immunoprecipitation. Appropriately titered antisera were used to immunoprecipitate either biosynthetically radiolabeled Ar eluates or whole supernates. Second-step precipitations were accomplished with goat anti-mouse Ig, goat anti-rabbit Ig, or rabbit anti-mouse Ig as indicted in the individual experiments. All precipitates were washed by procedures described by Shackelford and Strominger (28).

High-Pressure Liquid Chromatography (HPLC). The ^{3}H- and ^{14}C-labeled T cell products eluted from a HGG-Ar column were mixed with a ^{3}H:^{14}C cpm ratio of 3:1 or 4:1 with 1.0 mg HGG as carrier and 0.6 ml of 0.1 M ammonium bicarbonate, pH 8.5. The samples were digested at 37°C for either 4 or 24 h with Tos-Phe-CH_2Cl-trypsin (Worthington Biochemical Corp., Freehold, N. J.) at a ratio of 1:10 the amount of HGG and lyophilized. Peptides were then dissolved in 2% acetic acid and resolved on a high-pressure liquid chromatograph equipped with either a micro-bondapak C_{18} column (Waters Associates, Inc., Milford, Mass.; column dimensions 0.4 × 30.0 cm) or a radial compression module system (Waters Associates, Inc.) using a reverse-phase cartridge. Three different HPLC systems were used for these analyses: a standard C_{18} column in which the aqueous phase was 2% acetic acid and the organic phase acetonitrile, and a second standard C_{18} column system where the aqueous phase was ammonium bicarbonate and the organic phase acetonitrile. Finally, the acetic acid-acetonitrile system was

used with the radial compression module. In each system, over a period of 45 min, a linear gradient of 0–25% acetonitrile was generated using a curve 6 of the model 660 solvent programmer (Waters Associates, Inc.). Isocratic elution at 25% acetonitrile was continued after completion of the gradient for a period of 15 min. The flow rate varied between 0.5 and 1 ml/min, depending upon the particular experiment. Fractions were collected into scintillation vials at 0.5-min intervals. Water (0.5 ml) and scintillation fluid were added to the vials and samples were counted under double label (^{3}H-^{14}C) conditions (29).

Results

Construction of T Cell Hybrids. Initially, mice were immunized intravenously with Ar-coupled spleen cells and tested by footpad swelling induced by Ar-coupled spleen cells. Those mice that clearly demonstrated absence of the DTH reaction were killed by cervical dislocation, and their spleens were removed for hybridization with the AKR thymoma, BW5147.

Three separate fusions were performed and cell growth was observed in 324 of 1,080 wells, and the hybrids in the 324 positive-growth wells were transferred to larger wells. A radioinhibition assay (Materials and Methods) was performed on the 238 hybrids that survived passage. A sample of the initial screening of 48 of these 238 wells tested for inhibition of the binding of the hybridoma product to BSA-Ar-coated polyvinyl plates is shown in Fig. 2. Wells 5, 36, and 41 showed considerable inhibition of binding, and the cells in these wells were selected for further study. Several other hybrids inhibited in intermediate range (for example, those in wells 1, 4, 16, 18, 38, 39, 42, and 47). These latter clones were frozen in liquid nitrogen for future analysis.

The hybrids in wells 3, 5, and 41 were cloned by limiting-dilution; for this study, the hybrid clone originally derived from well 5 is referred to as T34G6, and the clone derived from well 36, T33D5. This latter clone was subsequently subcloned. After subcloning, the T33D5 hybrid continued to produce nonimmunoglobulin antigen-binding material. As shown in Fig. 3, this product could be detected in culture supernates even at dilutions of 1:16.

Characterization of the T cell Hybrids. The hybrid nature of each clone was confirmed by growth in HAT medium. A subclone of T33D5 was tested for Thy-1 antigens and

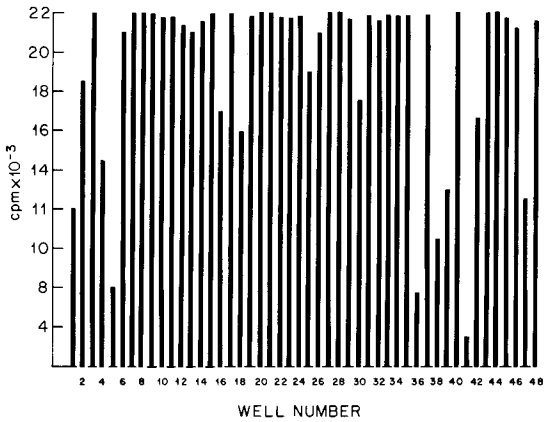

FIG. 2. An example of the screening assay for nonimmunoglobulin Ar-specific T cell products. This illustrates tests on 48 of the 238 growth positive wells. No inhibition was observed in over two-thirds of the wells. Of the approximately one-third that did inhibit the reaction, some (for example, wells 5, 36, and 41) inhibited markedly and were chosen for further study.

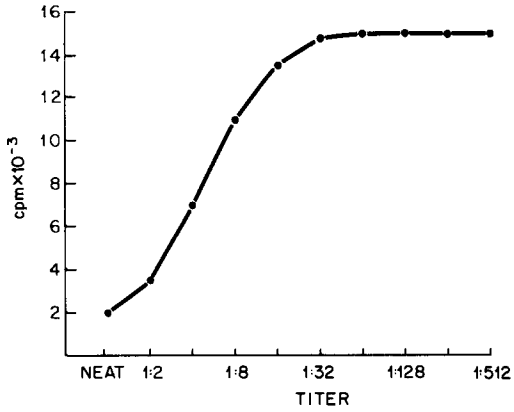

FIG. 3. Titration of the culture Ar eluate of a subclone at T33D5 in the radioinhibition assay. Activity could be detected at a 1:8 to 1:16 dilution. The Ar eluate was derived from 2×10^7 cells.

>95% of the cells contained both Thy-1.1 and Thy-1.2 on their surface by indirect immunofluorescence. To test for the presence of the H-2 complex of the A/J parent in the hybrids, the T33D5 subclone was tested by indirect immunofluorescence with B10.BR anti-B10.D2 previously absorbed with the BW (AKR) parent line. A weak but clearly positive reaction was evident. Controls done during this set of experiments indicated that the T33D5 clone did not express immunoglobulin or Fc receptors on its surface.

Sixteen clones, nine of which secreted the Ar specific-product and seven that did not, were tested for direct cytotoxic effector cell activity following the method of Forman and Möller (30) in which individual clones are mixed with P815 target cells in the presence of phytohemagglutinin. This procedure detects cytotoxic activity of any specificity. Because none of the clones tested caused lysis of the P815 target cells, it is unlikely that any express cytotoxic T cell activity. (These studies were kindly done by Dr. James Forman.)

On the basis of biosynthetically incorporated leucine, arginine, and lysine, we estimate that from 0.1 to 0.2% of the material in culture supernates is the arsonate-specific product. Whether this is actively secreted, shed from living cells, or released after cell death and lysis has not been investigated. The amount was reasonably constant over the 4 mo these clones have been extensively studied and, in addition, the Ar-specific product appears stable as no proteolytic breakdown has been observed in stored culture fluids.

Isolation of Ar-specific T Cell Hybrid Products. Antigen-specific T cell products were isolated from three of the cloned T cell hybrids by biosynthetic labeling with either [^{3}H]leucine or a mixture of [^{3}H]leucine, [^{3}H]arginine, and [^{3}H]lysine. Other biosynthetic studies involved [^{14}C]amino acids as outlined below. In addition, unlabeled T cell hybrid products were isolated from one clone (T33D5).

Table I illustrates one experiment with the T33D5 clone biosynthetically labeled with [^{3}H]leucine. 1 ml of culture supernate was collected and a portion tested in the radioimmunoinhibition assay; in this particular experiment ~31% inhibition was found. After dialysis, 1 ml of this radioactive supernate was placed on an HGG-Ar column that was then exhaustively washed with phosphate-buffered saline (PBS). The

TABLE I

Inhibition of Binding by T Cell Hybridoma Products

Inhibitor		Inhibition
	cpm	%
PBS	15,000	0
T33D5 supernate	10,300	31.4
T33D5 Ar Eluate	1,400	90.7
T33D5 effluent	12,600	16.0
BW51 supernate	14,600	2.7
T33B3 supernate	14,600	2.7

Ar-BSA-coated plates plus HP93G7 anti-Ar + ^{125}I-rabbit anti-mouse Ig.

T cell product was then isolated by hapten elution with 0.1 M Ar. Both the effluent and the eluate were collected, dialyzed, lyophilized, and resuspended in 250 μl of PBS. As shown in Table I, the T35D5 arsonate eluate inhibited the reaction >90%, whereas the column effluent was inhibitory at the level of only 16%. Both the BW5147 supernate and T33B3, a T cell hybrid that formerly had been producing the Ar-specific product but that had ceased to produce the specific product, gave background inhibition levels.

When the Ar-specific eluate was applied to an HGG column with no hapten coupled, all radioactivity appeared in the effluent. In addition, after elution of an active T cell supernate with the Ar hapten, further stripping of the column with 5 M guanidine resulted in the recovery of relatively few counts.

Serologic Characterization Arsonate-specific T Cell Hybrid Products. Because one is able to isolate relatively large amounts of biosynthetically labeled T cell hybridoma products with antigen specificity with the techniques employed, experiments could be done on the purified material rather than on whole culture supernates. In typical experiments, 3,000 cpm of leucine-, arginine-, and lysine-labeled Ar eluates were placed in serial tubes and antisera added. Controls always included the use of hyperimmunized mouse or rabbit antisera. After overnight incubation (Materials and Methods), the pellets were counted and invariably all (3,000 ± 200 cpm) the counts were in the pellet or none (400 ± 50 cpm) of the counts were in the pellet. This is a substantially different system than employed by other investigators studying T cell products and is one of the significant advantages of isolating hapten-specific material.

As can be seen, of all the antisera tested, only rabbit anti-CRI and A.TH anti-A.TL resulted in specific precipitation of the T33D5 T cell hybridoma product. Similar studies have been done on the products of three clones. Similar experiments were also performed on unfractionated biosynthetically labeled supernates. These studies demonstrate that all three T cell hybrids produce a product that has determinants that are recognized by a rabbit anti-CRI, as well as contain determinants that are recognized by antisera directed against the whole *I* region of the murine major histocompatibility complex.

Chemical Characterization of Ar-specific T Cell Hybrid Products. The Ar eluates from all three products were run both on polyacrylamide slab as well as tube gels with appropriate molecular weight markers, and the apparent molecular weight of each is 62,000. Fig. 4 illustrates an SDS slab gel of whole biosynthetically labeled T cell supernate (lane 1) as well as the Ar eluate from an HGG-Ar column (lane 2). As can

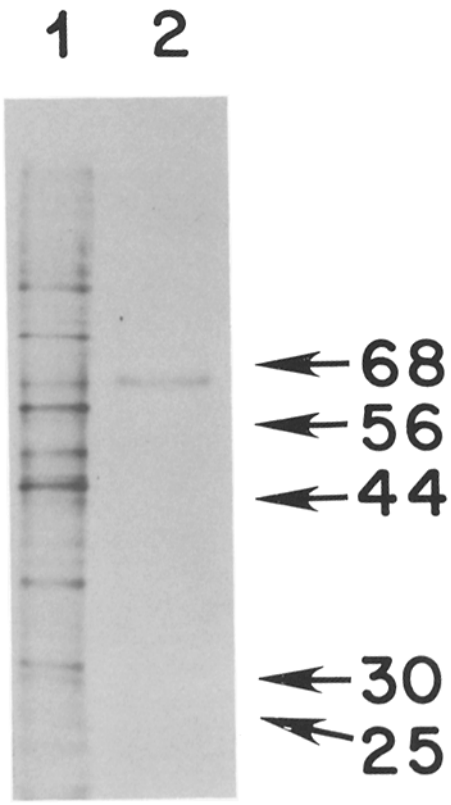

FIG. 4. Fluorographic analysis of biosynthetically labeled T35D5 supernate (lane 1) and the Ar eluate (lane 2). Molecular weight markers are indicated on the right.

be seen, the Ar eluate is a single band of 62,000 daltons. This band is clearly evident in the whole cell supernate.

In some experiments with biosynthetically labeled material, a stained band appeared to coincide with the radiolabeled band. In addition, when cold Ar eluate was run on SDS-polyacrylamide gel electrophoresis (PAGE), a single band was evident in the 62,000 dalton range. When the BW line was biosynthetically labeled, its culture supernate never showed a band at 62,000 daltons, and when its culture supernate was passed over an HGG-Ar column and eluted with Ar hapten, no such material was noted.

Complete reduction and alkylation of the Ar eluate did not appreciably change its apparent molecular weight. Thus, the T cell product appears to be either a single 62,000-dalton molecule, or multiple 62,000-dalton subunits noncovalently associated.

Tryptic Peptide Analysis of Two Ar-specific T Cell Hybrid Products. Fig. 5 shows the results of an HPLC analysis of the tryptic peptides of two of the T cell products. Each of these clones had been run several times under single label conditions, and four separate analyses were performed with double labels. In each instance, 14–20 peptides were clearly resolved by HPLC and, in all cases, the vast majority of the peptides were shared by both T cell products. In each instance, however, from two to four peptides were clearly distinct. HPLC resolves peptides primarily on the basis of their

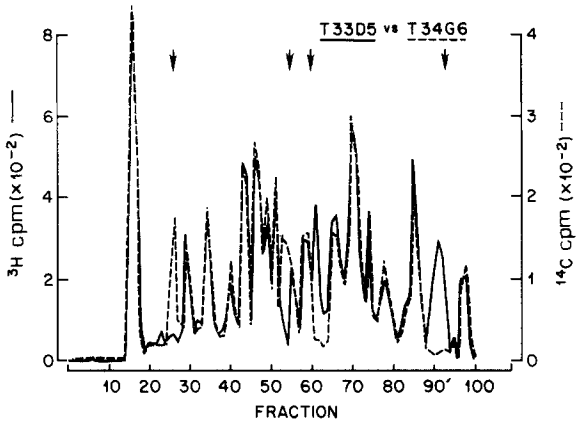

FIG. 5. HPLC analysis of tryptic digests of the T35D5 and T34G6 specifically purified T cell products. ^{3}H-labeled (—) T33D5 is compared with ^{14}C-labeled (---) T34G6. The four peptides that are clearly distinct between the two T cell products are indicated by arrows. All other peptides appear identical by this analysis. This particular analysis was performed on a radial compression module with an acetic acid-acetonitrile gradient (Materials and Methods).

hydrophobicity, and has proven in our hands to be a rapid and simple method of separating tryptic peptides. Its resolution capacity is equal to or exceeds that of ion exchange chromatography. These results indicate that, whereas these two T cell products are remarkably similar, they have unique features as well.

Discussion

This report concerns the initial characterization of the products of three T cell hybrids. Each T cell product is Ar-specific and bears determinants related to both the Ar CRI and determinants encoded by the murine major histocompatibility complex. The ease of production of these hybrids, their clonal nature, the substantial amounts of product produced, and the stability of the cell lines provide an opportunity for extensive chemical and serologic investigation of what are likely to be important immunoregulatory molecules in the arsonate idiotypic system.

Evidence that These Cell Lines are T-T Hybrids. Growth in HAT media supports the view that these cell lines are somatic cell hybrids. Additional characterization of one T cell hybrid (T33D5) indicated the presence of at least a portion of chromosome 17 of the A/J parent by the presence of cell surface antigens that reacted with antisera to H-2D^d glycoproteins. The evidence that these are T cell hybrids is as follows: (*a*) Immunochemical characterization of the product indicates the presence of no known immunoglobulin constant region markers; (*b*) Neither immunoglobulin nor Fc receptors could be demonstrated on the cell surface on any of the hybrid lines; (*c*) all hybrid lines tested expressed both the Thy-1.1 and Thy-1.2 cell surface markers, indicating that, unless the A/J T cell genes in a B cell have been derepressed, the fusion partner for the AKR thymoma line was an A/J T cell. This possibility must be considered in view of the recent report by Taussig et al. (31) who demonstrated that this same AKR thymoma could be successfully hybridized to the IgM-bearing (BALB/c × NZB) F_1 B cell lymphoma WH1231. Surprisingly, they were able to demonstrate the presence of both the Thy-1.1 and Thy-1.2 allelic products in at least some of their hybrid lines.

Thus, the evidence is conclusive that these are, indeed, somatic cell hybrids and, very likely, that they are T-T hybrids.

A Single Chain Bears Both the Serologic Characteristics of Immunoglobulin-Variable-Region and MHC Determinants. A significant advantage of the system employed in this study is that a highly purified biosynthetically labeled T cell product isolated by its antigen-binding characteristics can be tested essentially in an all or none fashion in a serologic assay. As shown in Table II, the only two antisera that immunoprecipitated the Ar eluate were directed against the A/J Ar CRI or the entire *I* region of the major histocompatibility complex (MHC). The immunoprecipitates in both instances were run on SDS polyacrylamide gels and demonstrated a single band of 62,000 dalton, whereas the supernates, in both instances, revealed no bands whatsoever. In all instances, where no counts were recovered in the immunoprecipitates, SDS slab gel analysis revealed no bands. These and other data unequivocally document that the same molecules that bear the CRI bear determinants encoded by the *I* region of murine MHC.

Several different A.TH anti-A.TL antisera were used in these studies with identical results. Although there is a remote possibility that the specificity we are detecting is at the *Qa-1* locus, the strain combinations used argues that the more likely specificities are directed against the *I* region. Further evidence for the specificity of the *I* region determinants being of A/J origin comes from the demonstration that the *D* region of the A/J strain is expressed on the cell surface of at least one of the hybrid clones, T33D5. This indicates that at least a portion of the A/J 17th chromosome is present in one of the T cell hybrids.

The binding of the Ar-specific T cell product to rabbit anti-CRI does not indicate idiotypic identity between the T cell and B cell product to which the anti-CRI was raised. This is an important issue and one generally confused in idiotypic studies in general, and in idiotypic analyses of T cells and their products in particular. For example, in standard inhibition assays employed in our laboratory for both the serum Ar antibodies as well as anti-Ar hybridoma products, the T cell products generated in these experiments gave little, if any, inhibition. Thus, although in a binding assay

TABLE II

Serological Characterization of T33D5 Arsonate-specific T Cell Product

Antiserum	Precipitate
	cpm
Rabbit anti-CRI	2,857 ± 275
A.TH anti-A.TL	1,981 ± 127
A.TL anti-A.TH	405 ± 37
Anti-H-$2K^k$	330 ± 44
Anti-H-$2D^d$	487 ± 56
Anti-Slp	550 ± 65
Rabbit anti-human C4	604 ± 93
Rabbit anti-mouse Ig	783 ± 83
Mouse anti-human IgGK	468 ± 50
Goat anti-mouse Ig	403 ± 47
Goat anti-rabbit Ig	409 ± 56

Each reaction system included 3,000 cpm of ^3H leucine, arginine, and lysine biosynthetically labeled Ar eluate and 50 μl of appropriate antisera.

rabbit anti-CRI can recognize these T cell products, they are not identical to the B cell idiotype. It should be emphasized that in the well-defined Ar idiotypic system, isolated heavy chains are not completely inhibitory and rarely even inhibit to 50%. In addition, certain hybridomas are known to bind to the rabbit anti-CRI reagent, but not inhibit the standard assay (32). Thus, it is not at all surprising that the single chain T cell product does not bear all of the determinants that comprise the CRI. Nonetheless, it is likely that simple binding would be sufficient either to stimulate, to suppress, or to kill (with complement or with an auxiliary cell) cells and, thus, could explain the effect of anti-B cell idiotypic antisera on T cell function.

Preliminary experiments designed to narrow the specificity within the *I* region have been limited primarily by the nature of the antisera available. 3R anti-5R as well as antisera directed against the *E/C* subregion (anti-Ia.7) provided by several investigators as well as the Division of Research Resources, National Institutes of Health, specifically immunoprecipitated these T cell products. However, most Ia.7 antisera potentially contain *I-J* reactivity, and the anti-I-J sera employed contain other specificities, particularly to viral proteins. Experiments are in progess with recently available hybridoma antibodies to the murine *I-A* and and *I-E/C* subregion. Thus, the precise *I* region determinants located on these T cell products are unclear at the present time.

Chemical Characterization of the T Cell Products. By SDS-PAGE, the molecular weight of the Ar-specific material derived from the three T cell lines appeared identical. They reacted similarly in serological analyses as well. Preliminary analysis of one of the T cell supernates by molecular exclusion chromatography in neutral buffers indicates that the bulk of the arsonate-specific product elutes in the molecular weight range of 50–70,000. Some activity was detected in the 120–150,000 range as well (A. Pacifico and J. D. Capra. Unpublished observations). Thus, by both SDS-PAGE analysis as well as by column chromatography, the active material appears to be primarily a single chain, and probably does not exist, at least in appreciable amounts in the culture supernates, as a two-chained dimer. It may, however, exist in that form on the cell surface.

Although these products could not be distinguished either serologically or by molecular weight analysis, peptide map comparisons clearly demonstrated that, whereas the vast majority of the tryptic peptides were common to the products of all three of the T cell lines, each contained unique peptides. Thus, for example, as shown in Fig. 5, an HPLC comparison of T33D5 and T33G6 indicates that 15 tryptic peptides are common to the two molecules, whereas each molecule has two peptides not found in the other molecule. These results could be explained by a difference of as few as two amino acids between the two hybridoma products. Analysis of a third clone indicated further differences. These experiments suggest that, whereas these separately derived Ar-specific T cell products are remarkably similar both serologically and chemically, there are individual differences in their primary structures, just as there are in the idiotype positive B cell hybridoma products in the Ar system (10–12).

Barring unexpected serologic cross-reactivity, the data presented strongly suggest that a single polypeptide chain bears antigenic determinants thought to be encoded on different chromosomes. Thus, the antigen-binding function and the CRI are encoded on chromosome 6 and 12 (light and heavy chain loci), whereas *I* region structures are encoded on chromosome 17. Four possibilities should be considered for

this unprecedented finding: (*a*) the variable region loci have been duplicated and they, or structures related to them, are encoded on chromosome 17; (*b*) the *I* region loci have been duplicated and exist on chromosomes 6 and/or 12 as well as chromosome 17; (*c*) protein or RNA ligases exist that splice either nucleic acids or proteins from different chromosomes or (*d*) the loci (possibly *I-J* because previous results seem to rule out *I-A* or *I-E/C* [33]) do not encode the structural genes for the polypeptide chains in question, but rather encode modifying enzymes that actually dictate the serologic specificities. This latter possibility has been suggested previously by Parish et al. (34) and may be the most conservative explanation of our data.

The relationship of these T cell products to molecules described by other investigators is speculative at present because we have not determined any functional activity for these Ar-specific products. Our approach has been to search for a specific molecule and now, with this homogeneous product available, design experiments to test potential function. Others have searched for a function, and then attempted to characterize the molecules. Nonetheless, the relationships of these antigen-specific idiotype-positive *I* region-containing structures to suppressor, helper, or allogeneic effector factors, or to the T cell receptor problem itself, is approachable now that cloned cell lines are available that secrete significant amounts of homogeneous molecules that appear to link two important loci of the immune system.

Summary

T cell hybrids have been constructed between the BW5147 thymoma cell line and A/J splenocytes from mice suppressed with the *p*-azophenylarsonate hapten. Three independently derived cloned lines have been characterized. Each secretes or sheds a 62,000-dalton antigen-specific product bound by rabbit antisera directed against the arsonate cross-reactive idiotype. In addition, each of the antigen-specific molecules contains determinants encoded within the *I* region of the murine major histocompatibility complex. Peptide mapping analysis indicates that, whereas these molecules are remarkably similar, each is individually distinct in primary structure.

The availability of cloned T cell lines that produce antigen-specific idiotype-positive *I* region-containing products should facilitate a more thorough dissection of the interrelationships of T-B interactions in the arsonate idiotypic system.

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