ACTIVATION SPECIFICITY OF ARSONATE-REACTIVE
T CELL CLONES

Structural Requirements for Hapten Recognition and Comparison with Monoclonal Antibodies

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T cells can discriminate among antigens that differ only slightly in structure (1, 2). Direct binding of antigen to T cells has been difficult to demonstrate (3). Antigen recognition by T cells has therefore been indirectly studied by measuring activation (i.e., DNA synthesis or cytolytic/inducer function), which occurs only after corecognition of antigen and histocompatibility proteins (4–12). To understand why corecognition is required for T cell activation, it will be necessary to identify physiological binding sites for antigen and histocompatibility proteins, and to study their structure and association on the T cell surface.

The binding sites of antibodies have been most successfully mapped by using hapten-specific antibodies and haptens of systematically varied size and structure (13–17). A similar approach may provide structural information about a potential T cell hapten-binding site, if the following requirements are met: (a) clones of hapten-reactive T cells (7–10) are used instead of heterogeneous populations (1, 2, 4–6); (b) their recognition of hapten is not materially altered by varying the carrier protein to which the hapten is conjugated (8); (c) all structural analogues of the hapten are recognized in conjunction with the same histocompatibility protein on antigen-presenting cells (APC);[1] (d) hapten conjugates that weakly activate or do not activate a clone are immunogenic for other T cells of the same strain or haplotype, i.e., lack of activation is not due to inefficient presentation by APC (10). Since all activating haptens must interact with a physiological binding site on the T cell surface, analysis of their structure may suggest complementary structural features of the site (1, 2).

In this study, we describe inducer T cell clones specific for the *p*-azobenzenearsonate hapten that possess the properties described above. We have used analogues of the arsonate hapten to identify structural features important for

[1] *Abbreviations used in this paper:* APC, antigen-presenting cells; BGG, bovine gamma globulin; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; HIS, histidine; IL-2 and -3, interleukin 2 and 3; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; OVA, chicken ovalbumin; PBS, phosphate-buffered saline; RGG, rabbit gamma globulin; TYR, tyrosine. Structures and abbreviations for arsonate and related haptens are summarized in Table IV.

activation. In subsequent papers,[2,3] we use this information to identify and characterize a cell-surface binding site for arsonate on one of the clones, which may function as the physiological receptor for antigen.

Materials and Methods

Mice. D2.GD and B10.GD mice were generously provided by M. Dorf, Harvard Medical School. B10.A(4R) mice were bred in the animal facility at the Dana-Farber Cancer Center. All other mice were obtained from The Jackson Laboratories, Bar Harbor, ME and used between 6 wk and 4 mo of age.

Antigens. p-Arsanilic, sulphanilic, and p-aminobenzoic acids were obtained from Eastman Laboratory and Specialty Chemicals, Rochester, NY, and o-arsanilic acid, sulphanilamide, p-aminobenzamide and p-nitrobenzenediazonium hexafluorophosphate from Aldrich Chemical Co., Milwaukee, WI. Haptens were conjugated to bovine gamma globulin (BGG), ovalbumin (OVA), bovine serum albumin (BSA), and keyhole limpet hemocyanin (KLH) using the diazonium salts (18, 19). The isothiocyanate of p-arsanilic acid was synthesized using thiophosgene (20) and conjugated to KLH and BSA at 20 mM for 1 h at 23°C. Noncovalently bound haptens were removed by extensive dialysis against phosphate-buffered saline (PBS), pH 7.4. Conjugation ratios for the azo-linked haptens (moles hapten)/mole protein) were estimated using an extinction coefficient of 8.15 mM^{-1} in 0.1 N NaOH at the isosbestic point of 350 nm (18, 19, 21). The presence of the benzenearsonate hapten on the thiourea-linked conjugated [AR(NCS)] was determined by binding to the nonselective class of monoclonal antiarsonate antibodies (see Table VI). Structures and abbreviations for all haptens are listed in Table IV.

Derivation and Culture of T Cell Clones. Arsonate-reactive helper T cell clones were derived from (BALB/c × A/J) F_1 (CAF$_1$) mice suppressed for production of the cross-reactive idiotype (22) (clones Ar-3, Ar-5, Ar-7) or from unsuppressed CAF$_1$ mice (clone Ar-4). Conditions for derivation and culture have been described (21, 23). Clones Ar-3 and Ar-7 were derived by cloning from a single mass culture; clones Ar-4 and Ar-5 were derived independently from two other cultures.

T Cell Activation. Activation of the clones was measured as incorporation of [³H]-thymidine by 3–5 × 10^4 cloned T cells between 20 and 40 h after stimulation with 5 × 10^5 irradiated (2,000 rad) spleen cells and antigen (21, 23). Alternatively, 10^6 irradiated spleen cells were pulsed with 30–50 µg/ml antigen at 37°C for 1–2 h; excess antigen and nonadherent cells were removed by washing and 3–5 × 10^4 cloned T cells were added. Direct haptenation of splenic APC was performed by treating adherent cells derived from 10^6 irradiated spleen cells with 13 mM diazotized arsanilic acid, pH 8.5, for 20 min at room temperature (24).

Lymph Node Proliferation Assay. CAF$_1$ and D2.GD mice were primed at the base of the tail and in the footpads with a total of 100 µg of S-BSA, SNH$_2$-BSA, or CONH$_2$-BSA in complete Freund's adjuvant (CFA) (7). Inguinal and popliteal lymph nodes were removed 7 d after immunization. Lymph node cells (2–4 × 10^5) were assayed for DNA synthesis on day 4 in response to AR-OVA, S-OVA, SNH$_2$-OVA, or CONH$_2$-OVA.

Long-term T Cell Lines. Draining lymph node cells from CAF$_1$ and D2.GD mice immunized with S-BSA, SNH$_2$-BSA, or CONH$_2$-BSA as described above were cultured at 10^6 cells/ml with 50 µg/ml S-OVA, SNH$_2$-OVA, and CONH$_2$-OVA in Dulbecco's modified Eagle's medium containing 0.5% normal mouse serum, 2 mM glutamine, and 5 × 10^{-5} M 2-mercaptoethanol. Surviving cells (enriched for hapten-reactive T cells) were tested at day 12 of culture for DNA synthesis in response to AR-OVA, S-OVA, SNH$_2$-OVA, or CONH$_2$-OVA presented on syngeneic splenic APC (21, 23).

Monoclonal Antiarsonate Antibodies. B cell hybridomas were derived by fusion of immune A/J spleen cells (from mice primed and boosted with AR-KLH in CFA) with X63-Ag8.653

[2] Rao, A., W. Ko, S. Faas, and H. Cantor. Antigen binding by arsonate-reactive T cell clones in the absence of histocompatibility proteins. Submitted for publication.

[3] Rao, A., S. Faas, and H. Cantor. Analogues which compete for antigen binding to an arsonate-reactive T cell clone inhibit the functional response to arsonate. Submitted for publication.

myeloma cells (25, 26). Culture supernatants of hybridomas and subclones were screened for antiarsonate antibodies using a solid-phase radioimmunoassay (27). Monoclonal antibodies were purified from ascites fluids (28) by affinity chromatography over individual columns of Sepharose coupled to AR-rabbit gamma globulin (AR-RGG). Binding constants of the antibodies for the haptens AR-tyrosine (AR-TYR) and AR-histidine (AR-HIS) were determined by quenching of tryptophan fluorescence (29, 30). The content of the cross-reactive idiotype for arsonate (31) was determined in a solution radioimmunoassay, by measuring the ability of monoclonal antibodies to inhibit the binding of iodinated idiotype-positive serum antiarsonate to rabbit anti-idiotype antibodies (31, 32). Selectivity of the antibodies for haptens was monitored in a solid-phase radioimmunoassay (27). Binding to AR-BSA was compared with binding to S-BSA and C-BSA. Similarly, binding to AR-KLH and AR(NCS)-KLH were compared. Antibodies were considered selective if 30-fold or more antibody was required to give equivalent binding to the analogues vs. the arsanylated proteins (see Fig. 6A), and nonselective if the ratio was <30-fold (see Fig. 6B).

Results

Generation of T Cell Clones. We derived hapten-reactive helper T cell clones by immunizing CAF_1 mice with AR-KLH, and subsequently stimulating the immune spleen cells with AR-BGG in the absence of exogenous serum proteins or growth factors. This protocol selects for survival of arsonate-reactive helper T cells. T cells were cloned from the enriched cultures by limiting dilution. Clones were screened for DNA synthesis in response to arsonate-coupled proteins.

Characteristics. All the arsonate-responsive clones possessed properties of helper T cells: their cell surface phenotype was $Thy-1^+Ly-1^+Ly-2^-$ and they produced interleukin 2 (IL-2) and IL-3 in response to stimulation (21 and unpublished results). All clones recognized I region major histocompatibility complex (MHC) products; clones Ar-5 and Ar-7 are specific for $I-A^d$, while clones Ar-3 and Ar-4 recognize $I-A^k$. Clones specific for I-E proteins or "hybrid" Ia antigens on F_1 cells (33) were not obtained. Specificity for MHC products was established both by using APC from inbred strains of mice (Table I) and by inhibiting the response with monoclonal antibodies specific for I-A and I-E proteins (21).

Recognition of the Arsonate Hapten. All the arsonate-reactive clones we obtained responded to arsonate coupled to several different carrier proteins but did not respond to the carrier proteins alone (Table II). However, there was some dependence on the protein carrier: Clone Ar-5 responded to lower concentrations of AR-OVA than of AR-BGG, while the reverse was true for the other clones tested (see Figs. 3 and 4). All the clones also recognized the arsonate hapten directly conjugated to splenic and tumor APC. The response to directly conjugated hapten was MHC-restricted, since it required APC of the appropriate haplotype (Table III), and it was inhibited with monoclonal antibodies to appropriate I region proteins (not shown).

Thus arsonate-reactive clones appear to be hapten specific rather than conjugate specific, according to earlier criteria (8). Small molecules containing the hapten were not generally immunogenic; none of the clones were activated by AR-HIS (1–300 μM), and only clone Ar-4 responded to AR-TYR (half-maximal activation at 3–5 μM AR-TYR). Like the response to arsanylated proteins, the response of clone Ar-4 to AR-TYR required the presence of APC bearing $I-A^k$.

TABLE I

DNA Synthesis by T Cell Clones in Response to Antigen Presented on
APC from Different Strains of Mice

Strain	I region proteins ex-pressed	[³H]thymidine incorpora-tion	
		Clone Ar-4	Clone Ar-5
		cpm	
	—	570	707
CAF₁	I-Aᵈ, I-Eᵈ; I-Aᵏ, I-Eᵏ	122,827	131,201
A/J	I-Aᵏ, I-Eᵏ	124,802	1,590
BALB/c	I-Aᵈ, I-Eᵈ	352	143,148
B10.D2	I-Aᵈ, I-Eᵈ	1,450	78,169
D2.GD	I-Aᵈ	3,508	108,482
B10.A	I-Aᵏ, I-Eᵏ	100,273	783
B10.A(4R)	I-Aᵏ	105,470	ND

Clones Ar-4 and Ar-5 were incubated with 50 µg/ml Ar-BGG and irradiated spleen cells from the indicated strains of mice. [³H]thymidine incorporation (mean of duplicates) was measured between 20 and 40 h after stimulation. The results are representative of four similar experiments. ND, not done.

TABLE II

DNA Synthesis by T Cell Clones in Response to Arsanylated Proteins
Presented on Syngeneic APC

Antigen	[³H]thymidine incorporation	
	Clone Ar-5	Clone Ar-4
	cpm	
—	1,570	827
BGG	2,436	552
AR-BGG	186,096	149,159
OVA	2,219	696
AR-OVA	172,672	146,647
KLH	2,516	1,962
AR-KLH	184,188	191,793

Clones Ar-4 and Ar-5 were incubated with irradiated CAF₁ spleen cells and antigen (10–30 µg/ml). [³H]thymidine incorporation (mean of duplicates) was measured between 20 and 40 h after stimulation. The results are representative of three similar experiments.
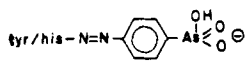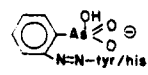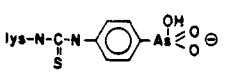
Activation by Structurally Related Haptens. Several structural analogues of the arsonate hapten, shown in Table IV, were conjugated to carrier proteins and tested for their ability to activate arsonate-specific clones. The analogues can be grouped into two categories: (a) those, like the benzenesulphonate, benzoate, benzenesulphonamide, benzamide, and nitrobenzene analogues (S, C, SNH₂, CONH₂, and NO₂, respectively), which differ from the arsonate hapten only in the most distal portion of the hapten (Table IV *A*), and (b) those derived from diazotized o-arsanilic acid (OAA) or from the reactive isothiocyanate of p-arsanilic

TABLE III

DNA Synthesis by Clone Ar-5 in Response to Directly Haptenated Splenic APC

Treatment	[^{3}H]thymidine incorporation		
	Source of splenic adherent cells		
	CAF$_1$	A/J	BALB/c
		cpm	
None	836	770	949
AR-BGG	99,161	874	61,899
AR-N$_2$$^+$	122,924	887	73,095

Adherent spleen cells were treated with diazotized arsanilic acid or pulsed with AR-BGG (see Materials and Methods). [^{3}H]thymidine incorporation (mean of triplicates) by 3×10^4 clone Ar-5 cells was measured between 20 and 40 h after stimulation. Results are representative of three similar experiments.

TABLE IV

Structural Analogues of the p-Azobenzenearsonate Hapten Used in this Work

STRUCTURE	ABBREVIATION	NAME	LINKAGE
A. tyr/his—N=N—⬡—As⟨^O_O⟩OH ⊖	AR–	Benzenearsonate	p-azo
—S⟨^O_O⟩—O ⊖	S–	Benzenesulfonate	p-azo
—C⟨^O_O⟩ ⊖	C–	Benzoate	p-azo
—S⟨^O_O⟩—NH$_2$	SNH$_2$–	Benzenesulfonamide	p-azo
—C⟨$^O_{NH_2}$⟩	CONH$_2$–	Benzamide	p-azo
—N⟨^O_O⟩	NO$_2$–	Nitrobenzene	p-azo
B. ⬡—As⟨^O_O⟩OH ⊖ N=N—tyr/his	OAA–	Benzenearsonate	o-azo
lys—N—C—N—⬡—As⟨^O_O⟩OH ⊖ S	AR(NCS)–	Benzenearsonate	thiourea

acid (AR(NCS)), which retain the distal benzenearsonate moiety but differ in their mode of attachment to carrier proteins (Table IV*B*).

Activation of one of the arsonate-reactive clones, Ar-5, in response to these analogues is shown in Fig. 1. The response to AR-BGG was detectable at 0.2 μg/ml and was maximal at 5–10 μg/ml (Fig. 1). C-BGG, S-BGG, and SNH$_2$-BGG also activated clone Ar-5; however, C-BGG was 10-fold less effective while S-BGG and SNH$_2$-BGG were 100–300-fold less effective than AR-BGG, as judged by the concentrations required to stimulate an equivalent response. CONH$_2$-BGG, NO$_2$-BGG, and OAA-BGG (Table IV) did not stimulate significant re-
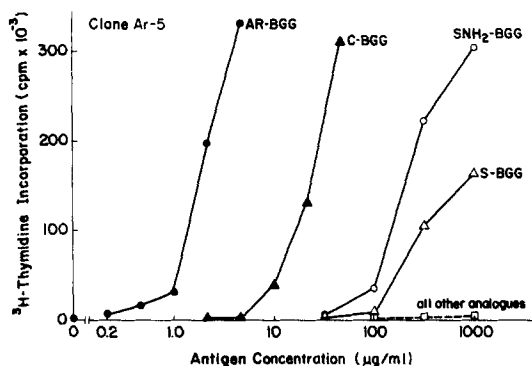
FIGURE 1. DNA synthesis by clone Ar-5 in response to structural analogues of arsonate conjugated to BGG. [^{3}H]thymidine incorporation by 3×10^4 clone Ar-5 cells was measured in response to irradiated CAF$_1$ spleen cells and antigens as indicated. (□) CONH$_2$-BGG, OAA-BGG, NO$_2$-BGG, and AR(NCS)-KLH.
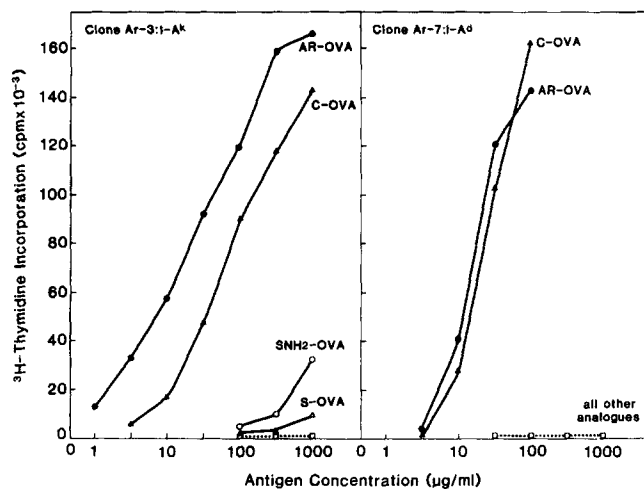

FIGURE 2. DNA synthesis by clones Ar-3 and Ar-7 in response to structural analogues of arsonate conjugated to OVA. [^{3}H]thymidine incorporation by 3×10^4 cloned T cells was measured in response to irradiated CAF$_1$ spleen cells and antigens as indicated. To emphasize the cross-reaction with C-OVA, responses to AR$_4$OVA and C$_{21}$OVA are compared. Haptenation ratios for other conjugates were between 7 and 9 mol of hapten per mol OVA. (□) CONH$_2$-OVA, NO$_2$-OVA, OAA-OVA and AR(NCS)-KLH for both clones, and in addition, S-OVA and SNH$_2$-OVA for clone Ar-7.

sponses at concentrations up to 1 mg/ml. Similarly the thiourea-linked conjugate AR(NCS)-KLH did not activate clone Ar-5 even at high concentrations (Fig. 1), although similarly haptenated AR-KLH, conjugated via the p-azo group, caused detectable DNA synthesis at 1 μg/ml (see Table II). The same results were obtained using OVA conjugates, showing that carrier proteins did not contribute significantly to the specificity of activation.

Two other arsonate-reactive clones (Ar-3 and Ar-7) were cross-reactively activated by p-azobenzoate conjugates (Fig. 2). To emphasize the cross-reaction, Fig. 2 compares responses to highly haptenated C$_{21}$OVA and lightly haptenated

AR$_4$OVA (the subscripts refer to moles of hapten conjugated per mole of protein). Comparison of equivalently haptenated conjugates (AR$_7$OVA with C$_9$OVA) showed that ~30–100-fold greater concentration of C-OVA than AR-OVA was required to stimulate an equivalent response by the clones (not shown). Clone Ar-3, like clone Ar-5, responded also to high concentrations of S-OVA and SNH$_2$-OVA, while clone Ar-7 did not (Fig. 2).

A fourth T cell clone, Ar-4, had more stringent requirements for activation. This clone responded to the arsonate hapten on BGG, OVA, KLH, and BSA, but not to any of the analogues of arsonate conjugated to these carriers (Fig. 3).

Dependence on Haptenation Ratio. The dependence of activation on the haptenation ratio (moles of hapten conjugated per mole protein) was examined. AR$_{23}$BGG and AR$_{68}$BGG elicited equivalent responses, as did AR$_7$OVA, AR$_{10}$OVA, and AR$_{22}$OVA (the subscripts refer to haptenation ratios) (Fig. 4). About 30–100-fold more AR$_4$OVA (or at least 10-fold more in terms of hapten concentration) was required in comparison with more highly haptenated OVA conjugates to elicit a similar response (Fig. 4B). Although lightly haptenated AR-OVA was therefore less effective than expected from its content of hapten, the order of effectiveness of AR, S, C, and SNH$_2$ conjugates was unchanged over a wide range of haptenation ratios. Thus even AR$_4$OVA was somewhat more stimulatory for clones Ar-5, Ar-3 and Ar-7 (two to threefold) than C$_{21}$OVA, and much more stimulatory for clones Ar-3 and Ar-5 than S$_{16}$OVA or (SNH$_2$)$_7$ OVA (>300-fold) (Fig. 2). Quantitative estimates of the relative effectiveness of the different haptens were made using OVA conjugates containing similar amounts of hapten. These experiments showed that C-OVA was 30–100-fold less effective than AR-OVA at stimulating clones Ar-3, Ar-5, and Ar-7, while S-OVA and SNH$_2$-OVA were between 300-fold and 1,000-fold less effective at stimulating clones Ar-3 and Ar-5 (not shown).

MHC Restriction. Activation of each clone by hapten analogues showed the same MHC requirement as did activation by arsonate (Table V). Thus, D2.GD (or B10.GD) spleen cells (which express only I-A^d) presented all the activating analogues to clones Ar-5 and Ar-7 as effectively as did syngeneic CAF$_1$ spleen
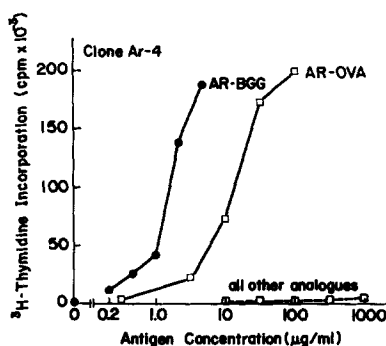

FIGURE 3. DNA synthesis by clone Ar-4 in response to structural analogues of arsonate. [^{3}H]thymidine incorporation by 3 × 10^4 clone Ar-4 cells was measured in response to irradiated CAF$_1$ spleen cells and antigens as indicated. (□) S, C, SHN$_2$, CONH$_2$, NO$_2$, and OAA conjugates of OVA and BGG, and AR(NCS)-KLH; the highest response is shown (<5,000 cpm at 1 mg/ml).
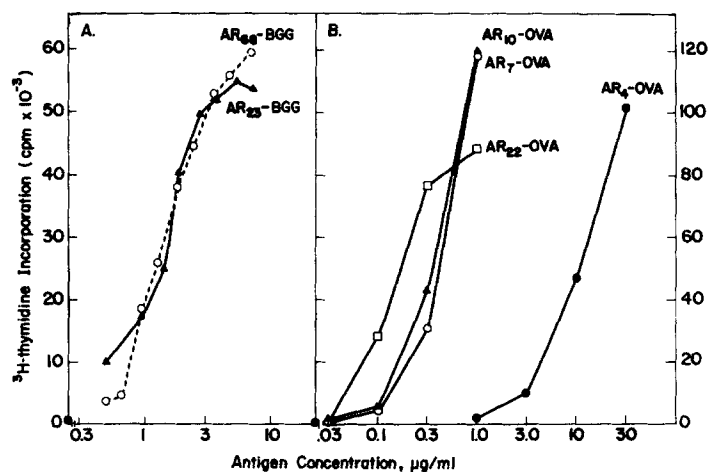
FIGURE 4. Effect of haptenation ratio on DNA synthesis by clone Ar-5 in response to AR-BGG and AR-OVA. [^{3}H]thymidine uptake by 3×10^4 clone Ar-5 cells was measured in response to irradiated CAF$_1$ spleen cells and antigens as indicated. (A) AR-BGG, (B) AR-OVA.

TABLE V

DNA Synthesis by T Cell Clones in Response to Haptenated Ovalbumin

	Antigen	Concentration	[H]thymidine incorporation	
			Splenic APC	
		µg/ml	cpm	
			CAF$_1$	D2.GD
A. Clone Ar-5	AR-OVA	1	314,486	294,108
	C-OVA	10	247,732	198,265
	S-OVA	300	294,864	273,727
	SNH$_2$-OVA	1,000	280,612	331,879
			B10.A	B10.A(4R)
B. Clone Ar-3	AR-OVA	30	92,145	111,237
	C-OVA	100	90,175	111,469
	S-OVA	1,000	10,128	10,894
	SNH$_2$-OVA	1,000	32,275	27,461

Clones Ar-3 and Ar-5 were incubated with irradiated spleen cells and antigens as indicated. Antigen concentrations were chosen to be at or near the maximal stimulatory concentrations, or were 1 mg/ml if maximal stimulation was not attained. [^{3}H]thymidine incorporation (mean of duplicates) was measured between 20 and 40 h after stimulation.

cells (Tables I, V). Conversely, B10.A(4R) APC, which express only I-A^k, presented the activating analogues to clone Ar-3 as effectively as did B10.A APC (Table V). None of the analogues activated a response when presented by inappropriate APC (B10.A for clones Ar-5 and Ar-7; B10.D2 for clones Ar-3 and Ar-4; not shown).

Immunogenicity of Weakly Activating Conjugates. Since T cell activation depends on associative recognition of both I-A and antigen (11, 12), we tested the

possibility that nonactivating or weakly activating conjugates such as S, SNH_2, and $CONH_2$ were poor stimulators because they were not presented by APC in correct association with I-A. We showed that this was not the case: primed lymph node cells or long-term T cell lines from CAF_1 and D2.GD mice immunized with S-BSA, SNH_2-BSA, or $CONH_2$-BSA were activated more vigorously by OVA conjugates of the immunizing haptens than by arsonate (Fig. 5).

Monoclonal Antibodies to Arsonate. We compared the patterns of specificity exhibited by cloned T cells with those exhibited by monoclonal antibodies to arsonate. The dissociation constants for AR-TYR and AR-HIS of 15 monoclonal antiarsonate antibodies were determined by fluorescence quenching (Table VI), and their binding to AR-BSA, S-BSA, and C-BSA was assessed in a solid-phase radioimmunoassay (Fig. 6). Their content of the cross-reactive idiotype for arsonate (31) was determined in a solution radioimmunoassay (31, 32). All antibodies showed a slight preference for AR-HIS over AR-TYR (generally two to fivefold); the preference was striking in IIIG6, which bound AR-TYR very poorly, and in VA12, which did not bind AR-TYR detectably (Table VI). A majority of antibodies (9/15) showed a strong preference for arsonate over sulphonate and carboxylate (Fig. 6*A*, Table VI); the remainder (6/15) discriminated only poorly among these three haptens (Fig. 6*B*, Table VI). Binding

FIGURE 5. Response of primed lymph node cells and long-term hapten-reactive T cell lines to the immunizing haptens and arsonate. Draining lymph node cells from D2.GD mice primed with S-BSA (*A*) or $CONH_2$-BSA (*B*), and long-term cell lines derived from draining lymph nodes of D2.GD mice primed with SNH_2-BSA (*C*) or $CONH_2$-BSA (*D*) were assayed for DNA synthesis in response to AR-OVA, S-OVA, SNH_2-OVA, or $CONH_2$-OVA as indicated (see Materials and Methods). Similar results were obtained using lymph node cells and long-term cell lines from primed CAF_1 mice.

TABLE VI

Characteristics of Monoclonal Antibodies to Arsonate

Antibody	CRI[‡]	Subclass	K_d^{app}*[ ]		Category[§]
			AR-HIS	AR-TYR	
			μM		
IF6	+	IgG1,κ	1.1	3.2	Selective
IIIG6	−	IgG1,κ	1.2	I	
IVA8	−	IgG1,κ	2.0	8.6	
VA12	−	IgG2a,κ	0.9	¶	
VC7	+	IgG1,κ	0.6	1.8	
VD2	+	IgG1,κ	0.9	2.7	
VD3	Int	IgG1,κ	0.8	1.9	
VIC1	Int	IgG1,κ	1.0	3.0	
IXG5	Int	IgG1,κ	1.5	4.0	
IIC4	−	IgG1,κ	0.04	0.1	Nonselective
VIA3	Int	IgG2b,κ	1.3	4.6	
VIA10	+	IgG2b,κ	1.0	1.6	
VIIIG10	+	IgG2b,κ	0.9	1.9	
IXE2	+	IgG2b,κ	0.7	1.3	
IXE6	+	IgG1,κ	0.7	2.0	

* Determined by quenching of tryptophan fluorescence.

‡ CRI, cross-reactive idiotype, determined as in refs. 31, 32. Int, intermediate, i.e., slope of inhibition curve and maximal inhibition at 2,000 ng (<60%) in a solution radioimmunoassay for the CRI (31) were both less than the corresponding values using purified serum antibodies (see 32).

§ Selectivity of the antibodies was monitored by comparing their binding to AR-BSA vs. S-BSA and C-BSA, or to AR-KLH vs. AR(NCS)KLH, in a solid-phase radioimmunoassay (Materials and Methods). Selective antibodies showed a 30-fold or greater preference for conjugates of arsonate vs. its analogues.

I Low affinity binding that did not saturate in the concentration range used (0–30 μM).

¶ No detectable binding ($K_d^{app} > 10^{-4}$ M).

constants for AR-HIS were $\sim 10^{-6}$ M for most antibodies, except IIC4 (4×10^{-8} M; Table VI). The poorly selective class of antibodies also bound well to AR(NCS)KLH, whereas the highly selective class did not (Table VI).

Discussion

Early work using heterogeneous hapten-reactive T cells showed that T cells, like antibodies, could discriminate between related haptens (1, 2, 4–6). Studies with clones of helper and cytolytic T cells have confirmed this conclusion (7–9). The premise of our work was that by using hapten-reactive T cell clones and hapten analogues, the structural features of a hapten that were important for activation might be identified. Further, the structure and dimensions of hapten recognition sites involved in T cell activation might be mapped (1, 2) in much the same way that binding sites of antihapten antibodies were mapped using hapten analogues (13–17).

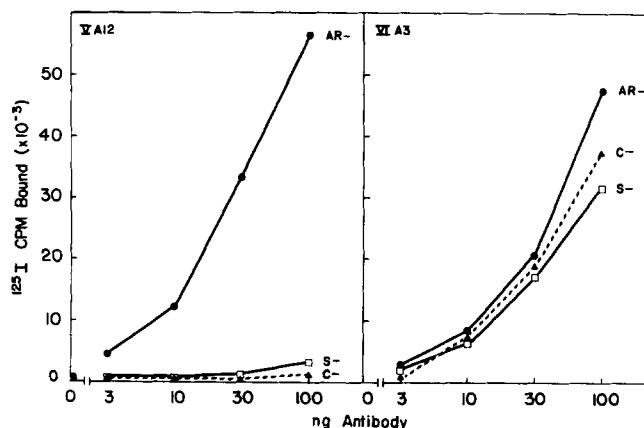We characterize in detail a small number of hapten-specific inducer T cell

FIGURE 6.   Selective binding by monoclonal antibodies to arsonate and related haptens. Binding of monoclonal antiarsonate antibodies VA12 and VIA3 to AR-BSA, S-BSA, and C-BSA was determined in a solid-phase radioimmunoassay using iodinated, affinity-purified, rabbit anti-mouse antibodies as the developing reagent. Other monoclonal antibodies were tested for selectivity in the same way.

clones that recognize the p-azobenzenearsonate hapten in any of several contexts, i.e., conjugated to different carrier proteins or directly conjugated to I-A-bearing APC (Tables II, III; Fig. 4). Such hapten-specific T cells are more easily generated to arsonate (9; this study) than to other haptens such as nitrophenyl haptens (7, 8). We used a series of structural analogues of arsonate (4, 34) to investigate in detail the activation specificities of these clones. The analogues used may be grouped into two series: In the first the most distal portion of the hapten is systematically altered, leaving the azobenzene portion intact; in the second the distal benzenearsonate portion is unaltered, but its linkage to carrier proteins is changed (Table IV).

The clones provided examples of three different patterns of activation by arsonate and its analogues. Clone Ar-4 was completely specific for arsonate conjugates (Fig. 3). Three of four clones (Ar-3, Ar-5, Ar-7) were also activated by p-azobenzoate conjugates, although more weakly, and two of these (Ar-3, Ar-5) were activated by p-azobenzenesulphonate and -sulphonamide conjugates as well (Figs. 1, 2). Activation by the latter two conjugates was 100–1,000-fold less efficient than activation by arsonate (Figs. 1, 2A).

The order of activation of a clone by arsonate and related conjugates was not determined by the MHC protein corecognized. Clone Ar-3, specific for I-A^k, and clone Ar-5, specific for I-A^d, were very similar in their cross-reactive responses to C, S, and SNH$_2$ conjugates (Figs. 1, 2A). Clone Ar-4, also specific for I-A^k, differed from the cross-reactive clone Ar-3 in being highly selective for the arsonate hapten (Figs. 2A, 3). Clones Ar-5 and Ar-7, both specific for I-A^d, differed (although less strikingly) in their activation specificities (Figs. 1, 2B). The results suggest that recognition of arsonate and of MHC proteins by these clones can be considered separable properties, at least for experimental purposes.

By comparing activating and nonactivating haptens, the structural features important for activation may be identified. These include features necessary for

binding, as well as those required to trigger any additional process important for activation, such as a conformational change in the receptor.

Charge. The negative charge on AR, S, and C conjugates may be important for activation. This is most apparent when C conjugates, which activate clones Ar-3, Ar-5, and Ar-7, are compared with the structurally very similar but uncharged and nonactivating NO_2 and $CONH_2$ conjugates (Figs. 1, 2). This may indicate that a positively charged group that interacts electrostatically with hapten is present in the arsonate-binding site, as suggested for antibodies to arsonate and other negatively charged haptens (41, 42).

Shape. The relatively strong cross-reaction between AR and C conjugates for three of four clones (Figs. 1, 2) indicates that they are similar in structure as well as charge. The benzoate hapten is planar; singly charged benzenearsonate may assume a trigonal bipyramidal configuration (43) in which the negative charge is distributed between two oxygens coplanar with the benzene ring. The AR hapten can make additional interactions, such as a hydrogen bond involving the axial OH group and coordination via a second axially oriented bond with a group in the binding site. Such additional interactions may account for the relatively stronger activation of clones Ar-3, Ar-5, and Ar-7 by AR than by C (Figs. 1, 2) and for the stringent specificity for arsonate shown by clone Ar-4 (Fig. 3). S and SNH_2 haptens may also assume the trigonal bipyramidal configuration (43); alternatively, the amide group of SNH_2 may hydrogen bond to the same group as the OH group of singly charged arsonate, thus causing the weak cross-reactions seen with clones Ar-3 and Ar-5 (Figs. 1, 2).

Linkage. The azo-linked benzenearsonate hapten (AR) activated all clones, while the thiourea-linked analogue [AR(NCS)] did not (Figs. 1–3). The linkage itself (—N=N— vs. —N—C—N—) or the amino acid to which the hapten is

$$\overset{\displaystyle \|}{S}$$

coupled (lysine for AR(NCS) vs. primarily tyrosine/histidine for AR) may be recognized. Benzenearsonate linked via an *o*-azo group (OAA) did not activate the clones (Figs. 1–3). The OAA hapten is also not recognized by antiarsonate antibodies (34, 36), suggesting that the *o*-azo group may interfere sterically with recognition of the arsonate function.

For comparison, we examined the selectivities of hapten-binding sites in a panel of 15 monoclonal antiarsonate antibodies derived from A/J mice immunized with AR-KLH (Fig. 6, Table VI). Some antibodies were strongly selective for the arsonate hapten compared with its carboxylate and sulphonate analogues, while others were nonselective (Table VI). Both selective and nonselective antibodies had similar dissociation constants for AR-TYR and AR-HIS, ~1 μM (Table VI); hence the affinity of antiarsonate antibodies for arsonate did not necessarily predict their selectivities for related haptens. In fact, the antibody with highest affinity (IIC4) was nonselective and discriminated poorly between AR, S, and C-BSA, while the antibody with lowest affinity (IVA8) was selective (Table VI). Similarly, the presence or absence of the cross-reactive idiotypes for arsonate (37, 38) does not appear to predict antibody selectivity (Table VI). A previous study of monoclonal antibodies to arsonate also showed that selectivity did not correlate with affinity for AR-(*N*-acetyl-TYR) or presence of the cross-

reactive idiotype; the relative inhibition of antibody binding to AR-BSA by *p*-arsanilic acid versus *p*-aminobenzoic acid ranged from 4.4-fold (which would be considered nonselective by our criteria; see Table VI) to 120-fold (selective) (35).

Unlike the binding of antibodies to monovalent haptens in solution (Table VI), activation of T cells by APC-bound antigen is not a direct measure of antigen recognition. Despite this important reservation, the concentration of antigen required for half-maximal activation may provide a crude estimate of the strength of interaction of a T cell clone with antigen. All four arsonate-reactive clones were half-maximally activated by equivalent concentrations of AR-OVA presented on syngeneic CAF_1 APC (10–30 μg/ml AR_4OVA or 0.3–1 μg/ml $AR_{10}OVA$; Figs. 2–4 and unpublished results); however, as discussed above, their selectivities for related conjugates are not the same. Thus, our results suggest that, as in the case of antiarsonate antibodies (Table VI), the selectivity of a T cell clone and its apparent affinity for arsonate may not be related.

Our results indicate that some T cell clones and some antibodies are extremely selective for AR compared with S and C, while others are less selective (Figs. 1–3, 6). The most selective T cells show at least the ability of antibodies to make fine discriminations between arsonate and its analogues. Certain immunization regimens may elicit primarily the selective antibodies (34, 36) or the selective T cells (4, 9), while others elicit both classes of response (this study).

We have examined the participation of MHC determinants in activation by arsonate and its analogues. T cell activation requires recognition of both antigen and MHC determinants (11, 12, 40; Tables I–III), and differences in the response to related antigens may be due to changes in the structure of either class of determinant (7–10, 12). However, since the arsonate-reactive T cell clones recognize all activating analogues in association with the same MHC protein as arsonate (Table V), differences in the corecognized MHC protein are not responsible for differences in activation by the analogues. Moreover, clones with completely different MHC specificities, such as Ar-3 and Ar-5, can show similar patterns of selectivity (Figs. 1, 2A). In addition, inefficient presentation by APC does not account for the weak activation of arsonate-reactive clones by S-OVA and SNH_2-OVA, and their lack of activation by $CONH_2$-OVA (Figs. 1–3), since primed lymph node cells and long-term hapten-reactive cell lines responded to these conjugates more vigorously than to AR-OVA (Fig. 5). We conclude that in this system, where significant changes in antigen presentation and MHC recognition appear unlikely, small alterations in hapten structure can influence the level of the response. We therefore postulate a site (or subsite) on arsonate-reactive T cell clones that interacts with hapten and may be experimentally separable from the site (or subsite) for MHC determinants.

Summary

We describe clones of hapten-specific inducer T cells from (BALB/c × A/J) F_1 mice that respond to the *p*-azobenzenearsonate hapten conjugated to carrier proteins or directly conjugated to antigen-presenting cells. Some of the clones are also activated by haptens structurally related to arsonate. All activating analogues are recognized by each clone in association with the same major

histocompatibility complex (MHC) protein as is arsonate. Weakly activating and nonactivating analogues are immunogenic in D2.GD and (BALB/c × A/J)F$_1$ mice, since they can effectively activate primed lymph node cells or long-term hapten-reactive cell lines. Hence the specificities of these clones may reflect their intrinsic recognition of arsonate and its analogues, rather than more efficient presentation of certain analogues than of others by antigen-presenting cells, or differential recognition of associated MHC epitopes by the clones. We compare the activation specificities of the clones with the binding specificities of monoclonal antibodies to arsonate, and discuss structural features of the analogues that may be important for activation and binding. Our results suggest that a site (or subsite) on arsonate-reactive T cell clones may interact directly with hapten, and may be experimentally separable from the site (or subsite) for MHC determinants.

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