T CELL CLONES SPECIFIC FOR HYBRID I-A MOLECULES

Discrimination with Monoclonal Anti-I-A^k Antibodies*

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During the past several years, this laboratory has been involved in the study of the unique mixed-lymphocyte reaction (MLR)[1] stimulating determinants and restriction elements for antigen presentation, which are associated with (C57BL/6 × A/J)F_1 [(B6A)F_1] spleen cells when used as stimulator cells or antigen-presenting cells (1). A considerable amount of data has been accumulated strongly suggesting that these F_1-specific MLR-stimulating determinants and restriction elements are in fact hybrid Ia molecules. Because each Ia molecule is composed of one alpha and one beta chain, hybrid molecules can be formed in heterozygous cells by the random association of alpha and beta chains encoded by the two parental haplotypes (2). Thus, in (B6A)F_1 cells there are four possible I-A molecules: $A^k_\alpha A^k_\beta$, $A^b_\alpha A^b_\beta$, $A^k_\alpha A^b_\beta$,$A^b_\alpha A^k_\beta$. (If there is more than one gene each for A_α and A_β chains, then the number of possible hybrid molecules increases accordingly.) Using alloreactive T cell clones of A strain (I-A^k) origin, it should be possible to identify clones that react with three of these four combinations of A region alpha and beta chains; the $A^k_\alpha A^k_\beta$ molecule will not be recognized because it is "self". On the other hand, it is expected that antigen-reactive T cell clones of (B6A)F_1 or (B6 × C3H)F_1 origin will be able to use all four combinations as restriction elements in antigen presentation.

In the experiments that follow, we will show that we can discriminate two different classes of A anti-(B6A)F_1 alloreactive and two different classes of (B6A)F_1 or (B6 × C3H)F_1 antigen-reactive T cell clones with specificity for the two hybrid I-A molecules discussed above. This discrimination is based on experiments using monoclonal anti-I-A antibodies to block the MLR and experiments using spleen cells from the I-A^b mutant mouse strain B6.C-H-2^{bm12} (bm12) as stimulator cells. Previously (3, 4), we have shown that there exist antigen-reactive T cell clones that use either I-A^b, I-A^k, or hybrid I-A^b/I-A^k molecules as restriction elements in antigen recognition. In this report, we will provide evidence that among the hybrid I-A^b/I-A^k-specific clones, we can discriminate those recognizing $A^k_\alpha A^b_\beta$ and $A^b_\alpha A^k_\beta$.

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; KLH, keyhole limpet hemocyanin; MLR, mixed lymphocyte reaction.

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Materials and Methods

Mice. Adult mice of either sex from 6 wk to 6 mo of age were used in all experiments. All mice were bred in our facilities at the Mayo Clinic or Stanford University or purchased from The Jackson Laboratory (Bar Harbor, ME). The recombinant and mutant mice used were generously provided by Dr. Chella David, Mayo Medical School, Rochester, MN.

Culture Medium. Complete culture medium consisted of RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 10 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin. L-glutamine was added to a final concentration of 2×10^{-3} M before use. The alloreactive clones in the 11 series, e.g., 11.17, were maintained and assayed in medium containing 10% horse serum instead of 10% FCS.

Cell Lines. The alloreactive cell lines were maintained as has been described previously (5), except that cloned lines usually required the addition of 0.5–1% rat concanavalin A (Con A) supernatant factor when recultured every 10–14 d. The rat Con A supernatant factor was prepared as described (5). The myoglobin-reactive clone 26.17 and the keyhole limpet hemocyanin (KLH) -reactive clones 9 and 11 have been described elsewhere (3, 4).

Proliferation Assay. The [^{3}H]thymidine incorporation proliferation assay was performed as described previously (5). To test the ability of various antibody preparations to block the proliferative response, a small amount of antibody (≤2.0 μg/well) was added to the appropriate wells in the standard assay system. Specificity of blocking was controlled by testing the same antibody against an inappropriate responder clone.

Antibodies. The monoclonal anti-I-A^k antibodies H150.13, H116.32, and H118.49 were described previously (6, 7). Antibody 10-2.16 was obtained from Dr. Vernon Oi, Stanford University (8). Monoclonal antibodies were purified by affinity chromatography on protein A sepharose and brought to 1.0 mg/ml in phosphate-buffered saline. Antiserum D-33 [(A × B10.D2)F_1 anti-B10.A(5R)] was a contract antiserum obtained from the National Institutes of Health. This antiserum contains antibodies reactive with both K^b and I-A^b molecules.

Two-dimensional Gel Analyses of Ia Molecules. Ia molecules were biosynthetically labeled and isolated as described previously (9). Briefly, spleen cells were teased into a single-cell suspension and incubated with ^{35}S-methionine (1 mCi/ml) for 5 h at 37°C in a solution consisting of methionine-free Dulbecco's modified Eagle's medium, 5% dialyzed FCS, and 10 mM Hepes. Cells were solubilized at ~2×10^7 cells/ml in 0.5% Triton X-100, and cell debris removed by centrifugation. Extracts (usually 100 μl) were precipitated with either monoclonal antibodies or antisera, and the complexes were adsorbed to fixed *Staphylococcus aureus* bacteria. Precipitates were eluted directly into lysis buffer (9.5 M urea, 2% Triton X-100, 1.6% ampholines, pH 5–7, 0.4% ampholines, pH 3.5–10, and 5% 2-mercaptoethanol) and then analyzed by two-dimensional gel analysis as described (9).

Results

Monoclonal Anti-I-A^k Antibodies Block Proliferation of Alloreactive T Cell Clones. We have previously described (1, 10) several alloreactive A anti-(B6A)F_1 T cell clones with different spectra of reactivities against stimulator cells of various haplotypes. Among these is a group of clones that appear, by mapping experiments, to be specific for alloantigens that are controlled by the I-A region of the H-2 complex. Using monoclonal anti-Ia antibodies, we have studied the relationship between the I-A determinants recognized by the T cell clones and the I-A determinants recognized by alloantibodies.

In Table I we present data on the ability of four different anti-I-A^k monoclonal antibodies to block the proliferative response of four alloreactive T cell clones to (B6A)F_1 splenic stimulator cells. All of these alloreactive clones were obtained from A anti-(B6A)F_1 bulk cultures by cloning either in soft agar or by limiting dilution (5). Clones 11.17, 11.19, 10.39.2, and 23AB1.2 are specific for I-A^k/I-A^b hybrid molecules, which can be seen from the data presented in Table II. A similar clone, 1.8.16, has

TABLE I

Inhibition of MLR Stimulation by Monoclonal Anti-I-A^k Antibodies

		Clones			
		11.17	11.19	10.39.2	23 AB1.2
No antibody		14,482	5,393	6,727	15,588
H150.13	αIa.17	178	63	5,403	12,299
10-2.16	αIa.17	108	75	4,141	10,569
H116.32	αIa.19	14,326	4,424	517	4,852
H118.49	αIa.2	14,591	6,270	946	NT

The response, as cpm of [^{3}H]thymidine incorporated, on day 2 of 1×10^4 cloned responder cells to 1×10^6 irradiated (B6A)F_1 stimulator cells in the presence of various monoclonal antibodies.

TABLE II

Stimulation of Alloreactive Clones by [bm12 × B10.A(4R)]F_1 Cells

Stimulator cells	Haplotypes				Clones			
	K	A	E	D	11.17	11.19	10.39.2	23AB1.2
A	k	k	k	d	117	120	302	639
B6	b	b	b	b	161	232	737	1,693
(B6 × A)F_1	b/k	b/k	b/k	b/d	24,821	8,180	11,120	19,975
[B6 × B10.A(4R)]F_1	b/k	b/k	b/b	b/b	21,927	9,708	11,541	17,504
[bm12 × B10.A(4R)]F_1	b/k	b*/k	b/b	b/b	12,761	6,612	1,603	1,788

The response, as cpm of [^{3}H]thymidine incorporation, on day 2 of 2×10^4 cloned responder cells to 1×10^6 irradiated (3,300 rad) stimulator cells.

* The bm12 mouse has a mutation in the A_β chain (16).

TABLE III

Inhibition of Antigen Recognition by Monoclonal Anti-I-A^k Antibodies

		Clones		
		26.17 myoglobin	9 KLH	11 KLH
No antibody		37,410	18,089	24,284
H150.13	αIa.17	20,237	NT*	NT
10-2.16	αIa.17	17,700	0	28,312
H116.32	αIa.19	658	15,941	7,252
H118.49	αIa.2	703	NT	NT

The response, as cpm of [^{3}H]thymidine incorporated, on day 3 of 1×10^4 cloned responder cells to 3 μM myoglobin or 50 μg/ml KLH in the presence of 1×10^6 irradiated (3,300 rad) syngeneic spleen cells.

* Not tested.

been described previously (1, 11). The antibody-blocking data presented in Table I indicate that these four clones represent two different types. Clones 11.17 and 11.19 are blocked by antibodies H150.13 and 10-2.16, which are Ia.17 reagents, and are not blocked by H116.32 and H118.49, which are Ia.19 and Ia.2 reagents, respectively.

Clones 10.39.2 and 23AB1.2 display the reciprocal pattern of blockade. We have found that all other alloreactive F_1-specific clones restricted to I-A tested to date follow one or the other of these two patterns.

Monoclonal Anti-I-A^k Antibodies Block Proliferation of Antigen-reactive T Cell Clones. We have also used these monoclonal anti-I-A^k antibodies to block the proliferative response of soluble antigen-reactive T cell clones. Myoglobin-reactive clone 26.17, which is of (B6A)F_1 origin, recognizes myoglobin when this antigen is presented by spleen cells from mice that are I-A^k/I-A^b heterozygotes (3). As is demonstrated in Table III, antigen recognition by this clone can be blocked by antibodies H116.32 and H118.49, but not by H150.13 or 10-2.16. KLH-reactive clone 11 also can be blocked by H116.32, but not by 10-2.16, whereas clone 9 displays the reciprocal pattern. These KLH-reactive clones are of (B6 × C3H)F_1 origin and recognize antigen when presented by I-A^b/I-A^k heterozygous cells (4). Thus, myoglobin-reactive clone 26.17 and KLH-reactive clone 11 display the same pattern of antibody blockade as the alloreactive clones 10.39.2 and 23AB1.2, whereas KLH-reactive clone 9 displays the same pattern as alloreactive clones 11.17 and 11.19.

Biochemical Evidence that Monoclonal Anti-I-A^k Antibody 10-2.16 Recognizes a Determinant of A^k_β Chains, whereas Antibody H116.32 Recognizes A^k_α Chains. Antibody 10-2.16 appears to recognize a determinant on the A^k_β chain (12). The ability of this antibody to block alloreactive clones 11.17 and 11.19 and KLH-reactive clone 9 suggests that these clones (and antibody 10-2.16) recognize the $A^b_\alpha A^k_\beta$ hybrid molecule. Because alloreactive clones 10.39.2 and 23AB1.2, myoglobin-reactive clone 26.17, and KLH-reactive clone 11 are not blocked by 10-2.16, but are inhibited by H116.32, these clones (and antibody H116.32) must recognize a different determinant on a hybrid I-$A^{b/k}$ molecule. This second determinant might be a different site on the $A^b_\alpha A^k_\beta$ molecule or a site on the reciprocal hybrid I-A molecule, $A^k_\alpha A^b_\beta$.

We examined the I-A molecules precipitated by antibodies 10-2.16 and H116.32. Both of these monoclonal antibodies recognize spleen cells of haplotype *k* but not haplotype *b* (6, 8). Assuming that the I-A α and β polypeptides can freely associate in (B6A)F_1 (b × k) cells and that antibodies reacting with one chain (e.g., α) will precipitate the corresponding chain (β) through noncovalent association, we sought to distinguish whether antibody H116.32 recognized the same molecular complex as 10-2.16 ($A^b_\alpha A^k_\beta$) or the reciprocal complex ($A^k_\alpha A^b_\beta$). Our results are shown in Fig. 1. Antibody H116.32 (Fig. 1 d) precipitates A^k_α, A^k_β, and A^b_β chains from (B6A)F_1 spleen cells, as seen by comparison with molecules precipitated from the parental strain cells (Fig. 1 a, b, c). This pattern indicates recognition of $A^k_\alpha A^k_\beta$ and $A^k_\alpha A^b_\beta$ molecules via a determinant on the A^k_α chain. Antibody 10-2.16, as previously reported (12), precipitates A^k_α, A^b_α, and A^k_β chains from (B6A)F_1 spleen cells (Fig. 1 e), which indicates recognition of $A^k_\alpha A^k_\beta$ and $A^b_\alpha A^k_\beta$ molecules via the A^k_β chain. A mixture of I-A^b and I-A^k molecules precipitated separately is shown for comparison (Fig. 1 c). In summary, our two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis data are most consistent with the interpretation that antibodies 10-2.16 and H116.32 recognize determinants on reciprocal, hybrid I-A molecules $A^k_\alpha A^b_\beta$ (H116.32) and $A^b_\alpha A^k_\beta$ (10-2.16). These data do not imply that either antibody recognizes determinants present on isolated I-A chains.

Identification of Hybrid $A^k_\alpha A^b_\beta$ and $A^b_\alpha A^k_\beta$ Molecules Using bm12 (A^b_β Mutant) Strain Cells. Considerable evidence has been published recently (13–15) documenting the

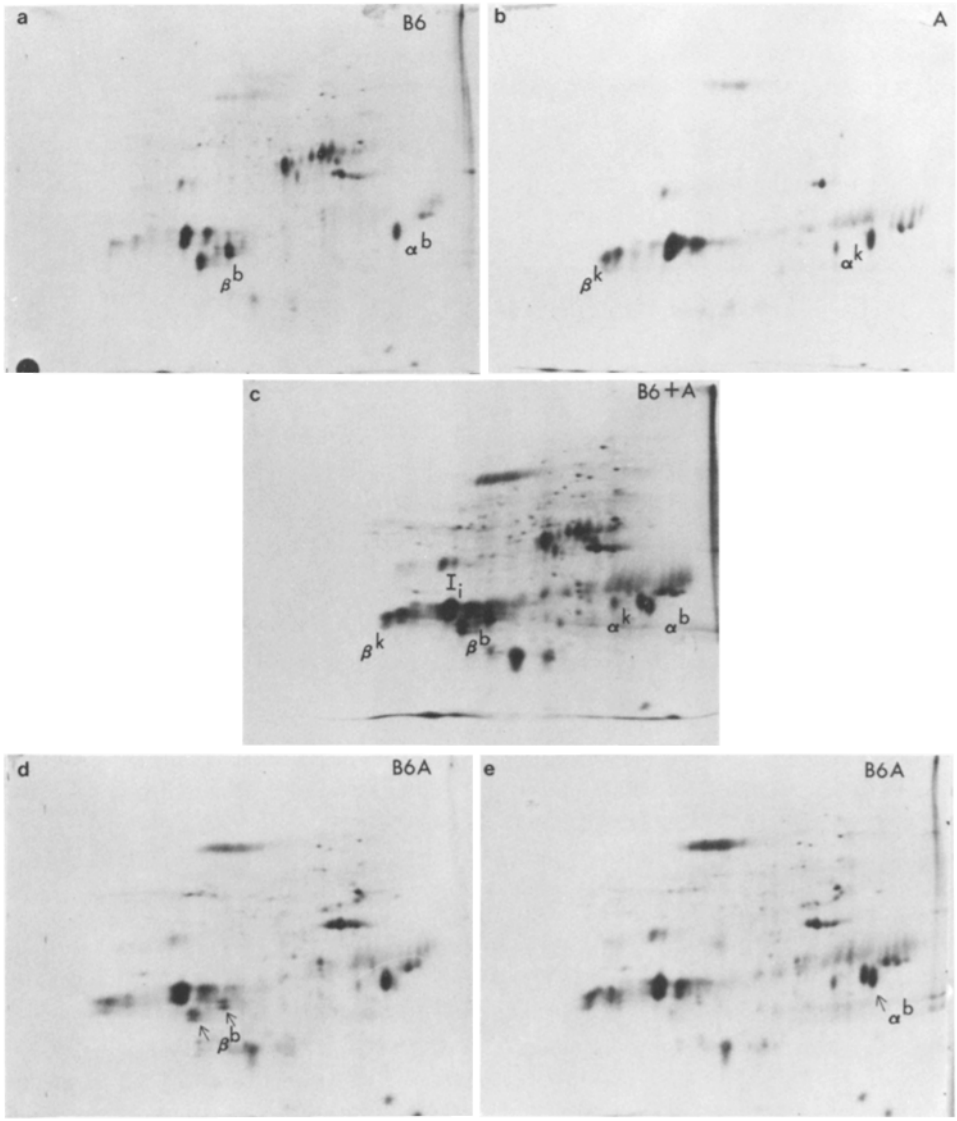

FIG. 1. Two-dimensional analysis of I-A molecules. (a) Strain B6 spleen cell extract precipitated with antiserum D-33; (b) strain A spleen cell extract precipitated with monoclonal antibody 10-2.16; (c) a mixture of the extracts from strain B6 and A (a and b above); (d) (B6 × A)F_1 spleen cell extract precipitated with monoclonal antibody H116.32. The arrows indicate characteristic β^b polypeptides; (e) (B6 × A)F_1 spleen cell extract precipitated with monoclonal antibody 10-2.16. The arrow indicates a characteristic α^b polypeptide. The mixture of parental I-A polypeptides is included for ease of comparison. The basic end of the gels is to the left, the acidic end to the right.

defect in I-A^b antigens of the mutant mouse strain B6.C-H-2^{bm12} (bm12). Tryptic peptide map comparisons of biosynthetically radiolabeled I-A molecules from B6 and bm12 mice have revealed a small number of peptide changes in the beta chain, but none in the alpha chain from bm12 mice as compared with B6 mice (16). If, indeed, only the A_β^{bm12} chain is altered, then we would predict that certain clones recognizing

TABLE IV

Antigen Presentation by (B6 × A)F_1 and [bm12 × B10.A(4R)]F_1 Spleen Cells

Antigen-presenting cell	Haplotype				Clones		
	K	A	E	D	26.17 myoglobin	9 KLH	11 KLH
(B6 × A)F_1	b/k	b/k	b/k	b/k	35,053	18,363	15,203
[bm12 × B10.A(4R)]F_1	b/k	b*/k	b/b	b/b	2,428	18,573	260

The response, as cpm of [^{3}H]thymidine incorporated, on day 3 of 1×10^4 cloned responder cells to 3 μM myoglobin or 50 μg/ml KLH in the presence of 1×10^6 irradiated (3,300 rad) spleen cells.

* bm12 has a mutation in the A_β chain (16).

the $A^k_\alpha A^b_\beta$ hybrid molecule would fail to be stimulated by [bm12 × B10.A(4R)]F_1 spleen cells, whereas clones specific for the $A^b_\alpha A^k_\beta$ hybrid molecule would recognize [bm12 × B10.A(4R)]F_1 spleen cells as effectively as they do [B6 × B10.A(4R)]F_1 spleen cells. We inferred that clones 11.17 and 11.19 recognized the $A^b_\alpha A^k_\beta$ hybrid molecule based on the antibody-blocking experiment shown in Table I, and that clones 10.39.2 and 23AB1.2 recognized the $A^k_\alpha A^b_\beta$ hybrid molecule. If this is correct, then clones 11.17 and 11.19 should be stimulated by [bm12 × B10.A(4R)]F_1 spleen cells, whereas clones 10.39.2 and 23AB1.2 might fail to be stimulated. Similarly, we would predict that [bm12 × B10.A(4R)]F_1 spleen cells could present antigen to KLH-reactive clone 9, but might not to KLH-reactive clone 11 or to Mb-reactive clone 26.17. As shown in Tables I and II, alloreactive clones that are blocked by 10-2.16, but not H116.32, respond to determinants on [bm12 × B10.A(4R)]F_1 cells. Similarly, antigen-reactive clones (Tables III and IV) recognizing hybrid I-A molecules on antigen-presenting cells exhibited the same pattern: those clones inhibited by antibody H116.32 failed to respond to antigen presented by [bm12 × B10.A(4R)]F_1 spleen cells, whereas clones inhibited by 10-2.16 responded to antigen and [bm12 × B10.A(4R)]F_1 cells. Thus, all of the data are consistent with the interpretation that we have distinguished clones that recognize each of the two hybrid molecules controlled by the I-A region in *H-2^a/H-2^b* heterozygous mice, $A^k_\alpha A^b_\beta$ and $A^b_\alpha A^k_\beta$, and furthermore, that in [bm12 × B10.A(4R)]F_1 mice one of the molecules, $A^k_\alpha A^b_\beta$, is not expressed in a form recognizable by certain alloreactive and antigen-reactive T cells.

Discussion

We have previously (1, 3, 4, 17) presented data demonstrating that among the alloreactive and antigen-reactive T cell clones, we can differentiate those that recognize I-A and I-E region Ia molecules by mapping the loci controlling expression of the molecules. Although it was suspected that there were two possible hybrid molecules expressed in I-A region heterozygotes, on the basis of the two-chain nature of Ia molecules, the lack of recombinants within the I-A region did not permit their discrimination by mapping experiments. We therefore turned to using monoclonal anti-Ia antibodies to differentially block stimulation of the cloned cell lines.

In the past, there have been several reports on the ability of anti-Ia alloantisera to block stimulation of appropriate MLR (18, 19). The interpretation of these experiments has been that the Ia molecules detected by the alloantisera are the determinants

that stimulate the MLR. However, there always remained the possibility that the alloantisera contained antibodies for MLR-stimulating determinants that were distinct from Ia antigens, but were closely linked to them genetically. The recent production of monoclonal anti-Ia antibodies and isolation of T cell clones makes it possible to do similar experiments in a more clearly interpretable manner. We used several monoclonal anti-I-A^k antibodies that had been produced and characterized previously (6–8). We found that the ability of these antibodies to block the stimulation of I-A^k/I-A^b-reactive clones divided the clones into two groups. Based on the biochemical analysis, which showed that antibody 10-2.16 reacts with a determinant on the A^k_β chain (12), and our two-dimensional gel analysis, which confirmed this and showed that antibody H116.32 reacts with the A^k_α polypeptide, we concluded that the F_1-recognizing clones blocked by antibody 10-2.16 are reactive with the $A^b_\alpha A^k_\beta$ hybrid molecule, whereas clones blocked by H116.32 are reactive with $A^k_\alpha A^b_\beta$ molecules. Furthermore, we would argue that other antibodies that block proliferation of T cell clones in a pattern identical to that of 10-2.16 also recognize determinants on A^k_β, whereas an H116.32-like blocking pattern characterizes antibodies reactive with the A^k_α polypeptide. We have preliminary data to suggest that this argument is correct. An independently derived anti-I-A^k monoclonal antibody (provided by M. Pierres, Centre d'Immunologie, INSERM-CNRS, Marseilles, France), which blocks proliferation of clones exactly as 10-2.16 does, also immunoprecipitates the same I-A products. Also, we can infer that Ia specificity 17 (10-2.16) and the Ia.17-like specificity of H150.13 are A^k_β determinants, whereas specificities 19 (H116.32) and 2 (H118.49) are related to the A^k_α chain. The functional data presented in this paper are in complete agreement with competitive antibody-binding studies (7) showing close proximity of binding sites for antibodies 10-2.16 and H150.13. These sites are distant from sites recognized by antibodies H116.32 and H118.49, which are, conversely, close to each other.

The mutant mouse strain B6.C-H-2^{bm12} provides us with an additional line of evidence that MLR-stimulating determinants and restriction elements for antigen presentation are Ia molecules (11). The bm12 mutant was originally discovered as a gain-and-loss spontaneous mutant by skin grafting (13). By complementation studies, the mutation mapped to the I-A region, and later serological analysis demonstrated the absence of specificity Ia.8 and the reduction of specificities Ia.3, 9, 15, and 20 on spleen cells of bm12 mice (14, 15). McKean et al.(16), using tryptic peptide analysis, have recently found that the A^{bm12}_β chain isolated from bm12 spleen cells differs from the normal B6 A^b_β chain. We examined whether cells from [bm12 × B10.A(4R)]F_1 mice would express the MLR-stimulating determinants or antigen-presenting determinants recognized by the alloreactive or antigen-specific T cell clones used in this report. We predicted that certain clones that recognized the $A^k_\alpha A^b_\beta$ molecule, as defined by the antibody-blocking experiments, would not respond to stimulation or antigen presentation by [bm12 × B10.A(4R)]F_1 spleen cells. However, [bm12 × B10.A(4R)]F_1 spleen cells should retain the ability to stimulate clones recognizing the $A^b_\alpha A^k_\beta$ hybrid molecule. This prediction was fulfilled (Tables III and IV).

We have provided biochemical, genetic, and functional evidence for discrimination of $A^k_\alpha A^b_\beta$ vs. $A^b_\alpha A^k_\beta$ hybrid I-A molecules by T cell recognition. Although the results of any one line of evidence are insufficient to unambiguously document this discrimination, the antibody-blocking data taken together with the biochemical data strongly

suggest that these two separate hybrid molecules can serve as stimulating determinants or restriction elements for separate sets of T cell clones. These data and the additional line of evidence presented by studying antigen presentation using cells of the bm12 mouse unequivocally demonstrate that the two sets of clones are recognizing reciprocal combinations of alpha and beta A region polypeptides.

Summary

Alloreactive and soluble antigen-reactive, I-A-restricted T cell clones were examined for their ability to recognize hybrid I-A antigens. Several clones that recognized hybrid I-A^b/I-A^k molecules on (C57BL/6 × A/J)F_1 [(B6A)F_1] spleen cells were studied. We were able to distinguish clones that recognized hybrid I-A molecules of the $A^b_\alpha A^k_\beta$ type from those that recognized $A^k_\alpha A^b_\beta$ molecules. We reached this conclusion by considering data from three independent types of experiments. (*a*) Monoclonal antibodies were used to inhibit T cell stimulation. Antibodies 10.2.16 and H116.32 distinguished two mutually exclusive "families" of T cell clones. One group of clones was inhibited by 10-2.16 and not H116.32, the other group exhibited reciprocal inhibition. (*b*) T cell proliferation was assayed using antigen-presenting cells from B6.C-H-2^{bm12} (bm12) and [bm12 × B10.A(4R)]F_1 mice. Because the bm12 strain has a mutation that results in an altered A^b_β polypeptide chain (A^{bm12}_β), we reasoned that clones that could recognize the [bm12 × B10.A(4R)]F_1 cells were recognizing $A^b_\alpha A^k_\beta$ molecules. Alternatively, clones not recognizing [bm12 × B10.A(4R)]F_1 cells had specificity for $A^k_\alpha A^b_\beta$ molecules. (*c*) I-A molecules immunoprecipitated from radiolabeled (B6A)F_1 splenocyte extracts were analyzed by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These experiments confirmed an earlier report that antibody 10.2.16 recognized determinants on the A^k_β chain (12). Antibody H116.32 immunoprecipitated products consistent with recognition of A^k_α determinants. Taken together, these three types of results offer conclusive evidence that T cell clones recognizing "hybrid" I-A molecules use either $A^b_\alpha A^k_\beta$ or $A^k_\alpha A^b_\beta$ molecules as recognition or restriction sites. Clones whose proliferation was supported by [bm12 × B10.A(4R)]F_1 cells and blocked by anti-I-A^k antibody 10-2.16 recognized $A^b_\alpha A^k_\beta$ molecules. Clones that were blocked by antibody H116.32 and did not recognize [bm12 × B10.A(4R)]F_1 cells use a recognition site(s) on $A^k_\alpha A^b_\beta$ molecules. Thus, we can demonstrate both functionally and biochemically that hybrid F_1 I-A molecules of the structure $A^k_\alpha A^b_\beta$ and $A^b_\alpha A^k_\beta$ both exist on (B6A)F_1 splenocytes and that both configurations are used in immune recognition phenomena.

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