MULTIPLE FUNCTIONAL SITES ON A SINGLE Ia MOLECULE DEFINED USING T CELL CLONES AND ANTIBODIES WITH CHAIN-DETERMINED SPECIFICITY

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Major histocompatibility complex (MHC)[1] restriction is a term used to indicate the obligate recognition of self-MHC products by immune T cells. In the particular case of helper T cells (T_H), immune response–associated (Ia) molecules are recognized in context with foreign antigens on the surface of antigen-presenting cells (APC). This dual recognition of antigen plus Ia is a fundamental aspect of immune response (Ir) gene control of T cell antigen–induced responsiveness (reviewed in reference 1). Earlier reports using anti-Ia antisera to inhibit the proliferation of bulk populations of T cells strongly suggested that Ia antigens were involved in the immune response (2–4). Recent studies have demonstrated that Ia antigens are directly responsible for certain Ir gene effects. First, some antigen-reactive T cell clones, restricted to Ia antigens of responder strain *b*, could not respond to antigen in the context of Ia antigens of the congenic strain B6C-H-2^{bm12}(bm12) (5). The bm12 strain has a mutation in the $A_\beta{}^b$ polypeptide that results in a structurally altered Ia molecule of haplotype *b* (6). Second, there was a direct correlation between the level of expression of an Ia antigen and the ability of APC to present antigen to T cell clones (7, 8). Third, proliferative responses of appropriate T cells could be inhibited by monoclonal anti-Ia antibodies directed toward the APC (9–11). These antibody-blocking studies suggested that monoclonal anti-Ia antibodies could mask and/or alter T cell recog-

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[1] *Abbreviations used in this paper*: APC, antigen-presenting cell; B6, C57B1/6J; (B6A)F_1, (C57B1/6J × A/J)F_1; bm12, B6.C-H-2^{bm12}; 2-D gel, two-dimensional gel electrophoresis; GAT, poly(L-Glu^{60}, L-Ala^{30}, L-Tyr^{10}); [^{3}H]TdR, tritiated thymidine; Ia, immune response–associated; Ir, immune response; KLH, keyhold limpet (*Megathura crenulata*) hemocyanin; Mb, sperm whale muscle myoglobin; mAb, monoclonal antibody; MHC, major histocompatibility complex; 4R, B10.A (4R); SDS PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate–containing buffer; (T, G,)-A-L, multichain poly (L-Try: L-Glu)-poly-D, L-Ala-poly-L-Lys; T_H, helper T lymphocyte.

nition site(s) on APC Ia molecules and that this effect alone was responsible for certain Ir gene functions. The specificity of antibody blocking correlated with haplotype specificity and I-region mapping of the Ir gene effect. Thus, monoclonal T_H cells and monoclonal anti-Ia antibodies were instrumental in showing that recognition of Ia on APC by T cells is the basis for certain Ir gene phenomena.

The combination of monoclonal anti-Ia antibodies and Ia-restricted T cell clones also proved decisive in analyzing T cell recognition of "hybrid" Ia antigens (5, 12, 13). These molecules were found to exist on cells of F_1 animals and to be formed by the free combinatorial association of parental Ia α and β polypeptides that associate noncovalently to form an Ia molecule. For example, in F_1 (b × k) animals, there exist in addition to parental $A_\alpha^b A_\beta^b$ and $A_\alpha^k A_\beta^k$ molecules, hybrid $A_\alpha^k A_\beta^b$ and $A_\alpha^b A_\beta^k$ Ia molecules. It could further be shown that both hybrid combinations ($A_\alpha^b A_\beta^k$ and $A_\alpha^k A_\beta^b$) could be specifically recognized by particular T cell clones. T cell clones that recognized F_1 hybrid Ia antigens (but not parental molecules) were tested for inhibition of proliferation of monoclonal anti-I-A^k antibodies. The clones could be sharply divided into two groups: one group was inhibited by antibody 10-2.16 and not by antibody H116-32, the other group was inhibited by H116-32 but not by 10-2.16. We then showed biochemically that 10-2.16 reacted with A_β^kIa chains, while H116-32 reacted with A_α^k chains. Thus, clones blocked by antibody 10-2.16 recognized $A_\alpha^b A_\beta^k$ molecules, those blocked by H116-32 recognized $A_\alpha^k A_\beta^b$ molecules (14).

In addition to mapping T cell recognition to a particular Ia molecule, it would be instructive to map T cell restriction sites on a single Ia molecule. Current information from gene sequencing and protein studies suggest that Ia molecules may fold into two structural domains (15–18). Similarly, serological studies indicate that Ia molecules have at least three distinct "epitope clusters" (19, 20), which may also be related to protein three-dimensional structure. We would ultimately like to know the nature of the T cell recognition sites, the determinants recognized by antibodies, and the structural basis of these sites. Given the data suggesting multiple structural domains of an Ia molecule, we wanted to know whether there existed more than one functional site on an Ia molecule that could be demonstrated with anti-Ia inhibition of T cell proliferation. Therefore, we utilized a panel of monoclonal anti-I-A^k antibodies to test inhibition of proliferation of a panel of T cell clones that recognized I-$A^{b/k}$ hybrid molecules. A complex blocking pattern would indicate multiple specificities of T cells for a given Ia molecule analogous to the multiple epitopes recognized on I-A molecules by anti-I-A antibodies. Our results indicate that multiple functional sites exist on a given Ia molecule. These data complement and extend previous results from our laboratory that show the existence of more than one functional site per Ia molecule by genetic means (21). An additional important consequence of this study is the mapping of several antibody specificities as being α or β chain determined.

Materials and Methods

Mice. The adult inbred and F_1 hybrid mice were bred in our animal facilities at the Department of Medicine, Stanford University or purchased from The Jackson Laboratory, Bar Harbor, ME. Mice of either sex were used. Mouse strains used were (C57BL 6/JXA/

J)F_1 and B10.A(4R)×57BL10/J.

Long-term Cultured T Cell Clones. Antigen-reactive T cell clones were established from immunized (bxk)F_1 mice. Complete culture media and antigens (GAT, (T,G)-A—L, KLH and myoglobin) were described previously (5, 12, 22, 23). The antigen specificities of the clone are: 26.17:myoglobin, 2eSA12, 2eSA13:TGAL, NAO:KLH, 12.5.a.1, and 12.5.a.31-GAT. All these clones were I-$A^{b/k}$ restricted. The clones were serially restimulated with antigen and irradiated syngeneic spleen cells in fresh media every 10–14 d. The T cell clones were used for assay more than 10 d after stimulation with antigen and APC.

Monoclonal Antibodies. Anti-I-A^k monoclonal antibodies (mAb) 10-2.16 and H116-32 were described previously (24, 25). All other anti-I-A^k mAb were developed and reported by Pierres and co-workers (20, 26). Antibodies were purified using Protein A affinity chromatography and dissolved in phosphate-buffered saline, pH 7.4 (~1 mg/ml). For blocking assay of T cell proliferation, 2.5 μl of antibody solution in 200 μl of medium was used. Preliminary dose response experiments showed that this was the maximum amount of antibody that could be added to the cultures which did not seriously inhibit proliferation nonspecifically (nonspecific inhibition usually <20%).

Blocking Assay of Cloned T Cell Proliferation. T cell clone proliferation assays and assays of T cell blocking of proliferation by anti-I-A^k mAb in vitro were described previously (14). Briefly, 10^4 cloned T cells were cultured with 10^6 irradiated spleen cells (APC) and an appropriate dose of antigen. For the blocking of T cell proliferation, antibodies were added at the beginning of culture and were present throughout. After 2 d incubation, [^{3}H]thymidine (^{3}H-TdR) incorporation was measured by liquid scintillation counting methods. Counts represent the average of three replicates. In most cases, the replicates were within 10% of the mean. The variability between experiments was greater, in particular for those clones that were only partially blocked. We, therefore, present data from one of the experiments in which all the clones were tested simultaneously. Optimal amounts of each antibody for maximal specific blocking of each T cell clone were determined by preliminary dose response experiments.

Two-dimensional Gel Electrophoresis. Methods of analysis of Ia molecules by two-dimensional gel electrophoresis (2-D gels) were as described previously (14). Spleen cells of strains C57BL6 × A/J (B6A)F_1 or C57BL10 × B10.A(4R)F_1 mice were biosynthetically radiolabeled with [^{35}S]methionine and solubilized in buffer containing 0.5% Triton X-100. These F_1 strains were chosen to eliminate the weak reactions with I-E region molecules sometimes seen in our immunoprecipitation assays (J. Frelinger, unpublished data). Cell extracts were precleared with fixed *S. aureus* (Cowan strain I) and then precipitated with monoclonal anti-Ia antibodies. Antigen-antibody complexes were absorbed to fixed *S. aureus* (Cowan strain I), and then eluted directly into isoelectric focusing buffer. Samples were loaded on the acidic end and separated in the first dimension by isoelectric focusing in polyacrylamide gels containing urea and in the second dimension by 10% SDS-PAGE under reducing conditions.

Results

The Experimental System Identifies T Cells and Antibodies Recognizing A_α^k or A_β^k

All the cell lines that are specific for hybrid (B6A)F_1 I-$A^{b/k}$ molecules show a clear division into two groups (10-2.16-group and H116-32 group) based on antibody blocking of proliferation. The F_1 specific T cell clones are blocked by antibody 10-2.16 *or* H116-32, but never both. Since previous biochemical studies showed that 10-2.16 reacts with A_β^k (14, 27, 30) and H116-32 reacts with A_α^k (14), it follows that F_1 restricted T cell clones that are blocked by 10-2.16 recognize $A_\alpha^b A_\beta^k$ molecules; clones blocked by H116-32 recognize $A_\alpha^k A_\beta^b$ molecules. This point is illustrated schematically in Fig. 1. We then examined the

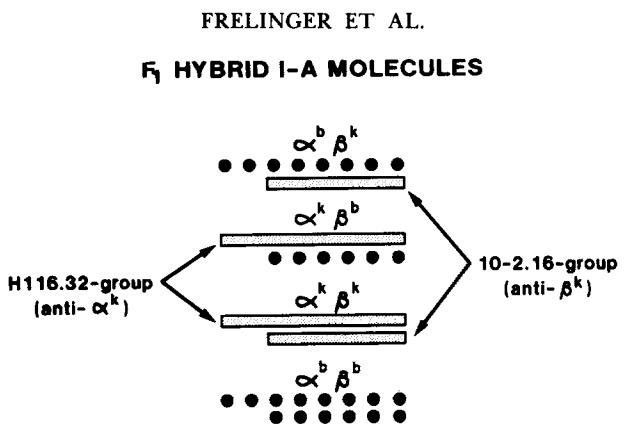

FIGURE 1. Schematic diagram of F_1 hybrid I-$A^{b/k}$ molecules showing immunoprecipitation patterns expected using mcAbs H116–32 and 10-2.16. H116–32 precipitates A_α^k, A_β^k, and A_β^b polypeptides; 10-2.16 precipitates A_α^k, A_α^b, and A_β^k polypeptides.

ability of a number of other anti–I-A^k mAb to inhibit T cell proliferation utilizing clones that were blocked by either 10-2.16 or H116-32. These clones then act as defined reagents to characterize the reactivity of the new mAb for α or β chains. In addition, we have independently characterized the reactivity of many of the mAb by immunoprecipitation. To date, these two methods of assigning the reactivity of the mAb to the α or β chain have agreed.

Several Antibodies Exhibit a Pattern of T Cell Inhibition Similar to 10-2.16. Table I*A* shows the proliferation inhibition caused by five mAb that behave nearly identically to 10-2.16. All of these antibodies potently inhibit proliferation of clones recognizing $A_\alpha^b A_\beta^k$ molecules. Since none of these antibodies recognize Ia molecules of haplotype *b*, they must bind A_β^k chains. No antibodies in the "10-2.16 group" (40N, 39E, 40M, 40F, 39B) block T cell clones reactive with $A_\alpha^k A_\beta^b$ molecules (Table I*A*). They all block a control T cell clone that recognizes $A_\alpha^k A_\beta^k$ (data not presented).

Antibodies that Inhibit T Cell Clones Reactive with $A_\alpha^k A_\beta^b$ Molecules Demonstrate More than One Functional Site on $A_\alpha^k A_\beta^b$. In the same experiment, we tested mAb blocking of T cell clones reactive with $A_\alpha^k A_\beta^b$ molecules. As was previously demonstrated (14), antibody H116-32 clearly distinguishes the reactivity pattern of I-$A^{k/b}$-restricted T cell clones from those that are blocked by the 10.2.16 group of antibodies. Thus, using antibody H116-32 as the prototype monoclonal anti-I-A_α^k, we selected a group of antibodies from the panel to use to test inhibition of clones whose proliferation could be blocked by antibody H116-32 (Table I*B*). Several antibodies (39J, 40J, and 39F) blocked only $A_\alpha^k A_\beta^b$-reactive clones. These antibodies ("H116-32–like group"), therefore, recognize A_α^k. The pattern of inhibition by the H116-32 group was quite different from the results for the 10-2.16 group. In contrast to the "10-2.16–like" antibodies, the antibodies in the H116-32 group were more restricted in their ability to block the panel of T cell clones recognizing $A_\alpha^k A_\beta^b$ complexes. Although all three antibodies (39J, 40J, 39F) which are "H116-32–like" block only $A_\alpha^k A_\beta^b$-reactive clones, they do not block every such clone tested and block less effectively than the "10.2.16 group". Antibody 39J reacts very much like H116-32 in that it blocks prolifera-

TABLE I

Blocking of T Cell Clones by Monoclonal Antibodies

	Uptake of [^{3}H]Thymidine		
	NAO	12.5a.1	12.5.a.31
A. *Blocking of antigen-specific $A_\alpha^b A_\beta^k$-restricted T cell clones by monoclonal anti-I-A^k antibodies.*			
No antibody	20,104	7,882	19,552
10.2.16	270	565	386
39.E	316	951	1,239
40.N	271	1,307	835
40.M	238	592	756
40.F	383	1,955	8,493
39.B	4,408	2,573	6,880
No antibody	20,104	7,882	19,522
116.32	15,644	8,186	10,481
39.J	13,202	10,205	18,251
40.J	14,008	8,449	16,548
39.F	12,182	8,181	15,538
B. *Blocking of $A_\alpha^k A_\beta^b$-restricted T cell clones by monoclonal anti-I-A^k antibodies.*			
	2eSA13	2eSA12	26.17
No antibody	15,419	33,471	7,235
116.32	6,213	2,262	3,026
39.J	4,101	10,274	1,674
40.J	4,113	8,173	6,171
39.F	7,181	19,006	2,682
No antibody	15,419	33,471	7,235
10.2.16	13,494	29,776	8,936
39.E	13,671	32,079	10,291
40.N	12,178	33,247	9,857
40.M	10,664	29,404	9,811
40.F	12,137	31,434	11,333
39.B	12,243	32,498	9,444

TABLE II

Differential Blocking of Two $A_\alpha^k A_\beta^b$ Clones

	No antibody	10.2.16	116.32	39F	40J	39J
2eSA12	20,249	19,487	4,534	17,289	2,936	4,424
26.17	19,653	26,640	4,337	4,502	15,574	2,275

tion of all of the $A_\alpha^k A_\beta^b$ clones tested. Table II presents additional data from another experiment to clearly show the differential blocking of antibodies 40J and 39F on two of the $A_\alpha^k A_\beta^b$-restricted clones (2eSA12 and 26.17). As can be seen from these data, there is quite clearly a difference in the ability of 40J and 39F to inhibit recognition of antigen by these two separate $A_\alpha^k A_\beta^b$ clones. We suggest that these patterns result from multiple T cell "epitopes" (restriction sites) per Ia $A_\alpha^k A_\beta^b$ molecule.

The Reactivity of mAb Reactive with A_α^k or A_β^k Chains Is Determined by 2-D Gel

Analysis. We have localized the binding of mAb to the α or β chains by immunoprecipitation from (b × k)F_1 spleen cells. All of the mAb reported here recognize the *k* haplotype I-A molecules, but not the *b* haplotype molecules. We can take advantage of the combinatorial association of α and β chains in an F_1 animal to determine whether a given monoclonal reacts with an α or β chain. If an antibody reacts with a β chain, it will precipitate 2 α chains and 1 β chain. The 10-2.16–like antibodies, 10-2.16, 40M, 39E, and 40N precipitate 2 α chains and 1 β chain ($A_\alpha^k A_\beta^b A_\beta^k$) from b × k spleen cell extracts. Immunoprecipitates of three of these antibodies are shown in Fig. 2, *a–c*. Hence, we conclude that the 10.2-16–like group of antibodies react with the A_β^k chain. Conversely, the reactivity of the H116-32–like antibodies is determined by the A_α^k chain as determined by the same type of 2-D gel analysis (Fig. 2, *d–f*). These antibodies precipitate 1 α and 2 β chains ($A_\alpha^k A_\beta^k, A_\beta^b$). Further the patterns of the I-A molecules precipitated by these antibodies appear identical. The same pattern is observed for 39F although this is a relatively weak immunoprecipitating antibody (data not shown). These data demonstrate that the H116-32–like group of mAb reacts with a specificity determined by the A_α^k chain.

Discussion

Multiple Functional Sites Recognized by T Cell Clones. The results described above demonstrate that Ia molecules of the configuration $A_\alpha^k A_\beta^b$ have more than one functional site that is used in restricted recognition by T helper cell clones. Previously, we had shown that we could determine which F_1-restricted T cell clones recognized the $A_\alpha^k A_\beta^b$ molecule or the $A_\alpha^b A_\beta^k$ molecule by blocking proliferation with monoclonal anti-I-A^k antibodies (14). Antibody H116-32 inhibits proliferation of $A_\alpha^k A_\beta^b$-reactive T cell clones and reacts with A_α^k chains as shown by immunoprecipitation. In the present report, we found several other antibodies that also inhibited $A_\alpha^k A_\beta^b$-reactive clones. These antibodies, however, could be subdivided into three groups. The first (represented by 39J) behaved identically to H116-32, blocking all of the $A_\alpha^k A_\beta^b$-reactive T cells. The second (40J) blocked only some of the clones, while the third (39F) blocked other clones. The patterns of inhibition of 40J and 39F are not subsets of one another. In the most rigorous analysis, we might argue that the overlapping or subsetting patterns differ only in relative affinity of mAb and T cells for Ia molecules rather than specificity *per se*. However, differences in affinities cannot explain the differential blocking seen with 40J and 39F. There are at least two patterns of T cell recognition of $A_\alpha^k A_\beta^b$ molecules. We believe that these different patterns are the result of the occurrence of different interaction sites for various T cells on the $A_\alpha^k A_\beta^b$ molecule.

In a separate paper, we have argued for the existence of two T cell sites on $A_\alpha^b A_\beta^b$ molecules in studies using the I-A mutant mouse B6.-C-H-2^{bm12} (21). Thus it appears that a given murine Ia molecule can be recognized in several different ways by a group of Ia-restricted T cell clones. This was also suggested recently in the guinea pig, using T cell soft agar colonies (28).

Since some studies have suggested multiple "forms" of Ia α and β chains of certain haplotypes (29), it might be suggested that we have defined multiple forms of $A_\alpha^k A_\beta^b$, each with a single T cell recognition site. By our 2-D gel analysis

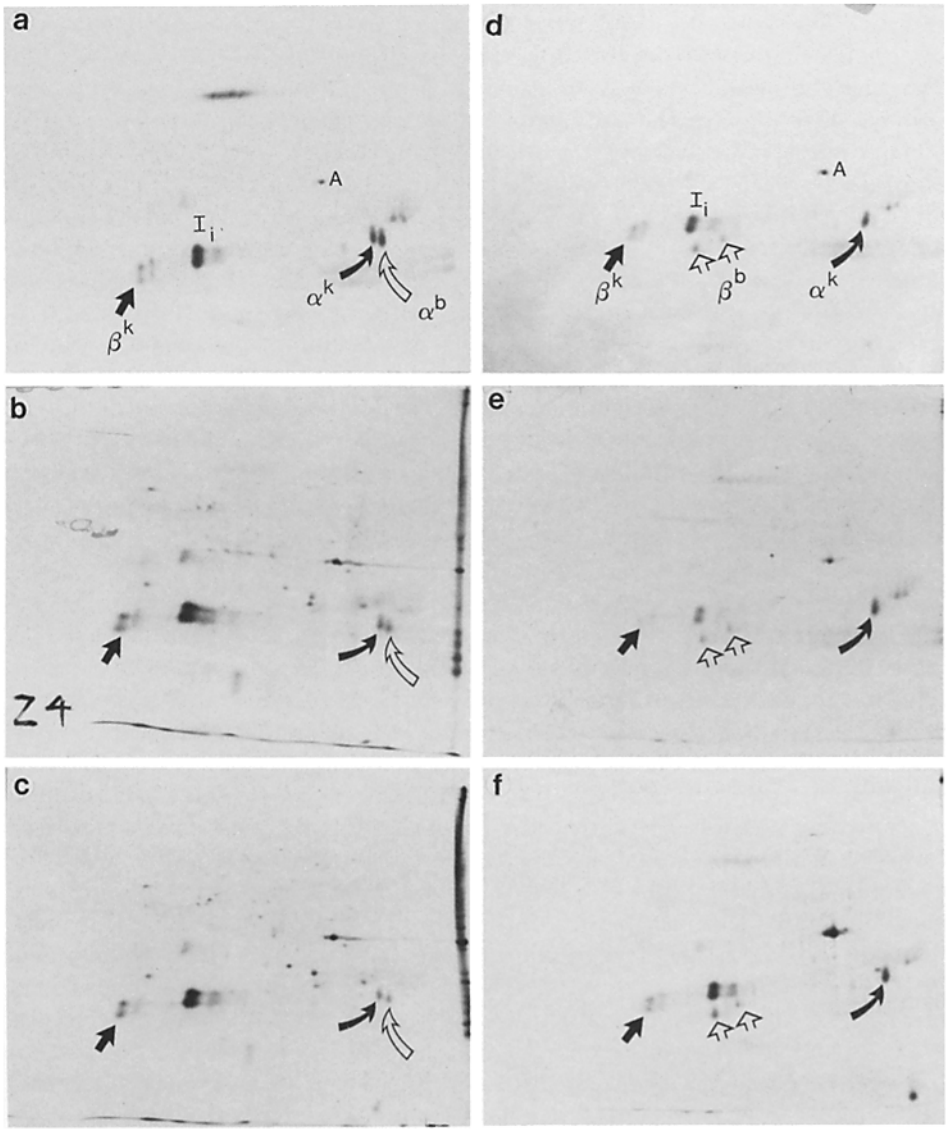

FIGURE 2. Two-dimensional SDS-PAGE of radiolabeled Ia molecules immunoprecipitated from spleen cell extracts of (B6 × A/J)F_1 mice (*a*, *d–f*) or B10 × B10.A(4R) mice (*b* and *c*) immunoprecipitated by various monoclonal antibodies. The different arrows denote different α or β chains: α^k solid curved arrows, β^k solid straight arrows, α^b open curved arrow, β^b open straight arrow; *A* denotes actin, and *Ii* denotes invariant chain. *a*, 10.2.16; *b*, 40 M; *c*, 39E; *d*, H116–32; *e*, 40J; *f*, 39J. Results are displayed as follows: basic pI, left; acidic pI, right; high molecular weight, top; low molecular weight, bottom.

(Fig. 2), we find identical forms of A_α^k precipitated by all the antibodies of the H116-32-group (39J, 40J, 39F). Thus, we believe the T cell sites defined above are present on the same Ia molecule.

Assignment of Alloantigenic Determinants to α or β Chains. As can be seen in Table I*A*, 10-2.16, an anti-A_β^k antibody, potently inhibits certain T cell clones—

those that recognize $A_\alpha^b A_\beta^k$ Ia molecules. Five anti-I-A^k mAb had very similar blocking patterns, although they bound to distinct Ia epitopes (26). We therefore analyzed the material precipitated from (b × k)F_1 spleen cells by these antibodies on 2-D gels. Since the antibodies react with haplotype *k* and not *b* and precipitate $A_\alpha^k A_\beta^b$, and A_β^k, they must bind to a site on A_β^k. As a result, we assign the determinants recognized by these antibodies to the A_β^k chain (Table III). Thus, Ia.1 (40N, 39E, and 40M) and Ia.17 (10-2.16) both map to the A_β^k chain. A recent report (19) has also suggested that these Ia specificities are topographically close to each other.

As shown above, the H116-32 group of antibodies reacts with A_α^k chains of I-A^k. These antibodies have strain reactivities similar to the conventional Ia specificities Ia.2 (40J, 39F) and Ia.19 (39J). These specificities, therefore, should reside on the A_α^k polypetide chain (Table III). The Ia.2 specificity may represent multiple determinants, however, and at least one mAb with Ia.2 specificity (11.5.2) is reported to react with A_β^k. (30) We have not used this particular mAb in our studies. Thus, certain conventional Ia specificities may not map neatly over α or β with mAb. Whether this is a peculiarity of Ia.2 or other private specificities or is a more general phenomenon is unclear. It should be noted that the antibodies in Table I*B* and used in Fig. 2, *d–f* have distinct specificities as demonstrated by competitive binding studies (20). They do, however, react with the same "epitope cluster."

A recent report utilized immunoprecipitation of isolated α and β chains in an attempt to directly localize and unambiguously assign specific alloantigen determinants to the α or β chains (30). Three monoclonals were used in both that study and our current report: 10-2.16, 39F, and 39J. In agreement with these studies and those previously reported by us (14) and Silver et al. (27), 10.2-16 maps to the β chain. In addition, their unpublished results in accord with our results indicate that 40.F and 40.M react with the β chain (J. Freed, personal communication). Kupinski et al. (30) were unable to map either 39F or 39J. However, since they were unable to map any determinant to the A_α chain, their results are not consistent with the ones presented here.

mAb Specificities Are Determined by the α or β Chain, But the Binding Site of the Antibody Need Not Be on the Determining Chain. We wish to emphasize that while the simplest interpretation of our studies is that the mAb react with the α or β chains alone, an alternative possibility is that the determinants defined by mAb are combinatorial or conformational, i.e., involve both α and β chains. In that

TABLE III

Assignment of Ia Specificities to Aα or Aβ Chains

Chain recognized	Monoclonal antibody	Presumed specificity
β	40N, 39E, 40M	Ia 1
β	10.2.16, H150-13*	Ia 17
β	40F*	??
α	40J, 39F, H118-49*	Ia 2
α	39J, H116-32	Ia 19

* Determined by blocking of T cell proliferation only. H118-49 and H150-13 were assigned by blocking in our previous paper (14).

case, antibodies such as H116–32 would precipitate A_α^k, A_β^b, and A_β^k by recognizing a determinants shared by the $A_\alpha^k A_\beta^k$ and $A_\alpha^k A_\beta^b$ complexes, but absent from $A_\alpha^b A_\beta^b$ and $A_\alpha^b A_\beta^k$ complexes. Thus, a major contribution would still be made by A_α^k. To be concise, in some instances we have utilized the phrases "chain specific" or "reacting with the α or β chains" in place of the more accurate phrase "antibodies with α or β chain determined specificity." We wish to reiterate that while the reactions with the antibodies are determined by the α or β chain, the actual physical binding site of the antibody may not be on that chain.

T Cell Clone Recognition May Involve Both α and β Chains. The blocking of T cell clones by anti-α or anti-β reacting antibodies does not imply that the T cell recognition site is located only on one chain or the other. Indeed, since the T cell clones studied here are all reactive with only the hybrid I-$A^{b/k}$ molecules, it appears that the T cells recognize combinational determinants influenced by both α and β chains. Whether the T cells can recognize A_α or A_β chains like the mAb may be addressed by DNA-mediated transfection or by using heterozygous Ia mutant cell lines (31). Finally, in view of data presented above concerning the variable patterns of blocking of A_α^k-reactive mAb, it may be questioned why the Anti-A_β^k antibodies show such a uniformly potent effect on all susceptible clones. There are at least four possible reasons: (*a*) many of the A_β^k mAb are directed to a single region of A_β^k that is recognized by all T cell clones using this molecule in Ia restriction-recognition; (*b*) the A_β^k chain is exquisitely sensitive to conformational changes induced upon antibody binding, such that binding of any appropriate mAb alters T cell recognition of $A_\alpha^b A_\beta^k$; (*c*) the anti-I-A^k mAb tested were made via haplotype *s* and anti-*k* immunization, which may give only a limited repertoire of particularly high affinity anti-A_β^k antibodies; and (*d*) the presence of the two disulfide bonds in the β chain constrain its structure more than the one disulfide in the α chain and thus make it a better ligand for the mAb. At present, there is no clear choice among these possibilities. We have presented data in a previous report (21) that demonstrates that the second possibility does occur: certain antibodies do inhibit T cell proliferation by binding to sites that are distant from the T cell recognition site.

Speculations on the Location of Restriction Sites. It seems likely from protein structural studies and nucleotide sequence analysis that both Ia α and β chains fold into two structural domains (15–18). As a consequence of our results presented here and elsewhere showing multiple functional sites per Ia molecule, it will be of interest to precisely define where the T cell recognition sites are on Ia molecules. One alternative is that there are T cell recognition sites on each of the Ia structural domains. Another possibility is that there are multiple T cell recognition sites on a single Ia domain. At this point, we cannot clearly choose between these alternatives. However, recent DNA sequence data of the A_α genes of the *d*, *b*, *f*, *u*, and *q* haplotypes demonstrate that the sequence differences are relatively limited and clustered (32). Because the mAb used in our studies were derived from an *s* anti *k* immunization, once the A_α^s gene sequence is known, one can make more precise suggestions as to the exact amino acids involved in the binding of the monoclonal antibodies. However, even with the sequence data available, one can suggest that the regions that determine reactivity of the

monoclonals are around amino acids 57 and 75. This is based on the fact that the monoclonal antibodies 40J, 39C, 39F, and 39J react with *k* haplotype strains but not with *d*, *b*, *f*, *u*, and *q* haplotype strains. Among these haplotypes, only these residues are unique to *k* haplotype strains. These sites therefore may also represent T cell functional sites. Our studies presented here, combined with the recent structural studies, represent the beginning steps in characterizing the sites recognized by antibodies and the functional sites of Ia molecules used by T cells.

Summary

Monoclonal antibodies (mAb) were used to inhibit the proliferation of antigen-reactive (C57BL6/J × A/J)F_1 restricted T cell clones. We have been able to subdivide these F_1 restricted T cell clones into two groups: one of which recognizes the $A_\alpha^k A_\beta^b$ molecule and the other group which recognizes the $A_\alpha^b A_\beta^k$ molecule. Using clones with defined reactivities, we could assign the reactivities of monoclonals to the A_α or A_β chains. By immunoprecipitation and two-dimensional analysis of Ia molecules from F_1 spleen cells, we could independently map the reactivities of the mAb as being determined by the A_α or A_β chain. To date, these two methods of chain localization of the antibody reactivity have agreed. Further, the differential blocking of the $A_\alpha^k A_\beta^b$ restricted T cell clones suggests that there exists more than one restriction site per Ia molecule. Increasing the number of possible functional Ia restriction sites, either through combinatorial association of α and β chains or by using more than one site per molecule, should increase the number of ways Ia molecules can function in antigen presentation.

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