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THE ASSIGNMENT OF CHAIN SPECIFICITIES FOR ANTI-Ia MONOCLONAL ANTIBODIES USING L CELL TRANSFECTANTS[1]

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The chain specificities of 18 A^k and 26 A^b-reactive anti-Ia monoclonal antibodies have been determined. L cells were transfected with haplotype-matched ($A\alpha^k$:$A\beta^k$, $A\alpha^b$:$A\beta^b$) or haplotype-mismatched ($A\alpha^k$:$A\beta^b$, $A\alpha^b$:$A\beta^k$) cDNA pairs, lines expressing high levels of surface A complex were selected, and antibody reactivity with a panel of reagents was assessed by cytofluorimetric analysis. Most of the antibodies recognized a determinant specified by one chain, either α or (more commonly) β. A few examples of more complex determinants were also observed. A knowledge of the chain specificities of anti-Ia monoclonal antibodies should prove useful for a variety of studies aimed at dissecting Ia structure-function relationships.

The murine Ia antigens are polymorphic cell-surface molecules that regulate the immune response by their influence on cell-cell interactions (1, 2). They play a crucial role in the presentation of antigen by macrophages to T cells, and subsequently in the T cell facilitation of B cell antibody production. Thus far, two Ia antigen complexes have been characterized: A and E, each a heterodimer composed of an α and a β chain.

Experiments aimed at unraveling the complexities of Ia structure and function have been greatly benefited by the existence of anti-Ia monoclonal antibodies (mAb).[3] These reagents have been used to advantage in immunoprecipitation (3), immunofluorescent labeling (4, 5), functional blocking (6–10), and competitive binding (11, 12) experiments. Their value has increased in parallel with our ability to accurately specify the determinants that they recognize on Ia molecules. A precise definition of anti-Ia recognition sites may be even more pressing for contemporary experiments that involve exon shuffling and site-directed mutagenesis (13–18) or anti-Ia therapy (19–21).

Certain of the anti-Ia mAb determinants have been assigned to specific chains on the basis of the reagent's ability to precipitate isolated polypeptides or haplotype mismatched complexes (i.e. $A\alpha^x$:$A\beta^y$ or $A\alpha^y$:$A\beta^x$) (7, 8, 22, 23) or its failure to react with mutant Ia cell lines (24, 25). Both of these approaches involve inherent uncertainties, however. With the former, complications can arise because isolated chains may not fold correctly, or because certain α-β chain associations occur only with difficulty in heterozygous cells (14). With the latter, there can be problems with distinguishing combinatorial or conformational determinants. The recent availability of cloned genes encoding the various Ia antigen polypeptides has permitted chain specificity determinations on the basis of antibody reactivity with cell lines expressing transfected genes (13–15, 17, 26, 27). Here we assign chain specificities for a large panel of anti-Ia mAb by assessing their reactivities on L cells transfected with different Ia gene combinations.

MATERIALS AND METHODS

Expressible cDNA. $A\alpha^k$ and $A\alpha^b$ cDNA were isolated from B10.A and B10 spleen librairies as described (28). Because these clones did not reach to the AUG codon, they were artificially extended in an M13 plasmid by grafting the mature protein-coding portion onto a sufficiently long $A\alpha^d$ cDNA clone (as an AsuI fragment for $A\alpha^b$ and a SacI-StuI fragment for $A\alpha^k$; positions 53 to 608, and 37 to 749, respectively, numbered according to Benoist et al.) (28). The resulting long cDNA (position 1 to 978) were identical to the original $A\alpha^b$ or $A\alpha^k$ clones in the region coding for the mature protein as verified by sequence analysis. The $A\beta^k$ cDNA was kindly supplied by Drs. P. Estess and H. Mc Devitt (Stanford University). This clone originated from a B10.A spleen cDNA library and stretched from position −12 to +848 (numbered from the first base specifying the AUG codon). The $A\beta^b$ cDNA was isolated from a B10 spleen cDNA library and also extended from positions −12 to +848.

The cDNA inserts were cloned into the EcoRI site of the expression vector pKCR3. This vector differs from the original pKCR (29) by deletion of the SV40 polyadelylation site, and of the EcoRI site that occurs between the pBR and SV40 sequences (R. Breathnach, unpublished). The resulting constructs, diagramed in Figure 1 thus consist of the SV40 early promoter; a portion of the rabbit β globin gene including the end of the second exon, the second intron, and the beginning of the third exon; the cDNA to be expressed; and the remaining 3′ region of the β globin gene including the polyadenylation site. Note that the SV40 and globin sequences found upstream of the cDNA after splicing are devoid of translation initiation codons.

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[3] Abbreviation used in this paper: mAb, monoclonal antibody.

The four expressible Aα and Aβ constructs will henceforth be referred to as pKAK, pKAB, pKBK, and pKBB in which the second capital letter denotes the chain and the third indicates the haplotype.

L cell transfectants. The plasmids were introduced into thymidine kinase (tk) negative L cells by calcium-phosphate precipitation (30) in a triple co-transfection of the herpes simplex virus tk gene, an expressible Aα cDNA and an expressible Aβ cDNA. The 20 to 200 independent transformants obtained after hypoxanthine-aminopterin-thymine (HAT) selection were pooled. Indirect immunofluorescent staining (see below) showed that 10 to 50% of the HAT-resistant cells in the bulk population expressed the A complex. The positive cells were sorted after staining with the pan-Ia reagent 40B (12) or with 10-2-16 (31). The haplotype of the transfected α and β chain genes in each sorted population was verified on Southern blots of DNA prepared form these cells, relying on polymorphic HindIII and HinfI (Aα positions 260 and 316) or Rsa I (Aβ position 117) sites. The four L cell transfectant populations will be referred to as: ($A\alpha^k$:$A\beta^k$), ($A\alpha^b$:$A\beta^b$), ($A\alpha^k$:$A\beta^b$), and ($A\alpha^b$:$A\beta^k$).

Fluorescent staining. The mAb were used as ascites fluid, tissue culture supernatant, or protein A Sepharose (Pharmacia) affinity-purified material. The cells were detached from tissue culture dishes with phosphate-buffered saline (PBS) supplemented with 1 mM EDTA. Staining was performed in PBS supplemented with 30 mM HEPES pH 7.4, 3% calf serum, and 0.1% sodium azide by using FITC-labeled goat anti-mouse immunoglobulin (Nordic) as a second step. After washing the cells and fixing them in 1% paraformaldehyde, the fluorescence intensity was measured on an ODAM ATC 3000 flow cytometer equipped with logarithmic amplifiers (2 decades, channels 1 to 255). Staining with the normalization antibody 40B usually resulted in the transfectants ($A\alpha^k$:$A\beta^k$), ($A\alpha^b$:$A\beta^b$), and ($A\alpha^k$:$A\beta^b$) being centered at about channels 150 to 200, whereas ($A\alpha^b$:$A\beta^k$) staining was a bit weaker, around 125. Negative controls (second step only on transfectants, and complete staining on L cells transfected just with the tk gene) appeared between channels 10 and 40.

For all antibodies, a preliminary dose-response analysis was performed on ($A\alpha^k$:$A\beta^k$) and/or ($A\alpha^b$:$A\beta^b$) to determine optimal staining conditions. Test experiments used the lowest saturating dose of antibody, or occasionally, a 10-fold excess; "10 ×" staining did not reveal different reactivity patterns. Each experiment included cell specificity controls (staining with 10-2-16 and 39J) and a normalization standard (staining with 40B).

Data reduction. The peak channel number obtained for each population was normalized by comparison with the positive reference ($A\alpha^k$:$A\beta^k$) or ($A\alpha^b$:$A\beta^b$) stained with the same antibody, and with the test line stained with 40B. The results are represented as: (0), no detectable staining; (+/−), barely detectable, a 10 to 20 channel shift up from negative controls; (+), clearly positive staining, but very weak, a 30 to 60 channel shift up from negative controls; (++), intermediate, a 30 to 60 channel shift down from the positive reference ($A\alpha^k$:$A\beta^k$) or ($A\alpha^b$:$A\beta^b$); (+++), strong staining, but clearly a little below the positive reference level, a 15 to 30 channel shift down; (P), positive reference level, less than a 15 channel difference from ($A\alpha^k$:$A\beta^k$) or ($A\alpha^b$:$A\beta^b$); P^+, stronger than the positive reference level of staining. Although exact channel numbers and shifts varied from one experiment to the next, results normalized in this manner were quite reproducible. The data shown in Table I derived from at least two (usually three or four) independent stainings.

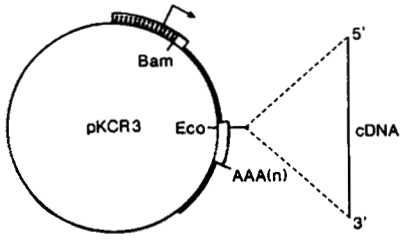

Figure 1. Schematic representation of the cDNA expression vector pKCR3. The *thin line* represents pBR322 derived sequences. The other areas of the plasmid are not drawn to scale. The SV40 early promoter is shown as a *stippled box*, l the β-globin exons as an *open box*. The *thick line* corresponds to portions of the β-globin gene not found in the mature message (intron and 3′ flanking sequences). The polyadenylation site is shown as AAA(n). Bam and Eco are the BamHI and EcoRI unique restriction enzyme sites that can be used for insertion of cDNA to be expressed, either upstream or downstream from the β-globin intron. Here, as in the constructs described in this paper, the cDNA is shown inserted into the EcoRI site.

RESULTS AND DISCUSSION

A relatively simple method to assign chain specificities for anti-Ia mAb is to test their reactivities with haplotype matched and mismatched Ia complexes. To this end, we have developed lines of L cell transfectants that express high levels of cell-surface $A\alpha^k$:$A\beta^k$, $A\alpha^k$:$A\beta^b$, $A\alpha^b$:$A\beta^k$ and $A\alpha^b$:$A\beta^b$. These four lines were stained with 44 mAbs chosen because they discriminate between k and b haplotype A complexes. The reactivities and chain assignments are presented in Table I.

We have analyzed 18 mAb that react with A^k but not A^b and 26 that see A^b but not A^k. Most of these reagents recognize a determinant specified by one chain, either α or β. However, there are indications of more complex determinants. For example, optimum staining by Y-248 requires that both the α and β chains are b haplotype; there is no reactivity with ($A\alpha^k$:$A\beta^b$), and we detect only weak staining of ($A\alpha^b$:$A\beta^k$). Another type of complex determinant is exemplified by Y-8P. Strong staining occurs when $A\alpha^b$ is expressed, regardless of the haplotype of Aβ, suggesting that the determinant resides predominantly on the α chain. Surprisingly though, significant antibody reactivity is also detected on $A\alpha^k$:$A\beta^b$. Finally, the $A\alpha^k$-specific antibodies may see a more complex determinant than is evident at first glance. These antibodies were produced against $A\alpha^k$:$A\beta^k$ but show stronger, heteroclitic, reactivity against $A\alpha^k$:$A\beta^b$, implying an Aβ influence.

The majority of the determinants that could be localized to a single chain mapped to Aβ. It is tempting, then, to speculate on the degree to which the murine immune response sees the polymorphism exhibited by the Aα and Aβ chains. However, any such speculation must be tempered with caution because of the skewed sampling of mAb from different strain combinations and because of potential biases introduced during the hybridoma screenings. As an illustration, none of the mAb derived from the A/J α B10.A (5R) immunization showed pure α chain specificity; however, all three of the antibodies resulting from the BALB/c α C57B1/6 combination recognized determinants primarily on Aα.

Certain of these chain assignments have been postulated on the basis of different experimental designs (7, 8, 22–24). We have included these mAb in our study because of uncertainties intrinsic to the various methods of analysis. All but one of our designations agree with the published information. Kupinski et al. (23) assigned the 11-5-2 determinant to the β chain on the basis of its reactivity with the isolated polypeptide. Our results show that this antibody recognizes a determinant predominantly on the α chain, consistent with the recent observation that 11-5-2 is in fact an anti-Ia.19 reagent (34). Certain other chain assignments have been made on the basis of mAb reactivity with transfectants expressing k:d or b:d hybrid A complexes (13–15, 17, 26, 27). Our data generally confirm the published results, but are at variance in the case of Y-3P and Y-8P, reagents which react with $A\alpha^b$:$A\beta^b$. Cohn et al. (17) observed no staining of $A\alpha^d$:$A\beta^b$ hybrid complexes expressed on B cell hybridomas, a finding which implies that these mAb see essentially α chain determinants. In contrast, we observe clear staining of $A\alpha^k$:$A\beta^b$ complexes, indicative of a more complex determinant that has components specified by both chains. Perhaps the root of this discrepancy is that dif-

TABLE I

Monoclonal antibody reactivity with L cell transfectants

mAb	Chain Assignment	Transfectant Reactivity[a]				Strain[b] Combination	Cross-reactivity[c]									References[d]
		$A^k_\alpha A^k_\beta$	$A^b_\alpha A^k_\beta$	$A^k_\alpha A^b_\beta$	$A^b_\alpha A^b_\beta$		d	f	j	p	q	r	s	u	v	
A^k reactive antibodies																
39F	α	P	O	P^+	O	A.TH α A.TL	–	–	x	–	–	–	–	–	–	8, 12, 13, 24
39J	α	P	O	P^+	O	A.TH α A.TL	–	–	x	–	–	r	–	–	–	8, 12, 13, 32
39C	α	P	O	P^+	O	A.TH α A.TL	–	–	x	–	–	–	–	–	–	12, 32
H116-32	α	P	O	P^+	O	BALB/c α CBA	–	–	x	x	–	r	–	x	–	7, 8, 11, 14, 24, 32, 33
H118-49	α	P	O	P^+	O	BALB/c α CBA	–	–	x	x	–	–	–	x	–	8, 11, 33
11-5-2	α	P	O	P^{++}	O	BALB/c α CKB	–	–	x	–	–	r	–	x	x	23, 31, 34
10-3-6	β	P	P	O	O	CWB α C3H	–	f	x	–	–	r	s	x	x	23, 31
10-2-16	β	P	P	O	O	CWB α C3H	–	f	x	–	–	r	s	u	x	7, 8, 13, 15, 22, 23, 24, 26, 31, 32
K22-203	β	P	P	O	O	B6 α 129	–	f	j	–	–	r	s	x	x	11
A6-61-3	β	P	P	O	O	A.TH α A.TL	d	x	x	x	q	x	x	x	x	35
H 150-13	β	P	P	O	O	BALB/c α CBA	–	f	–	x	q	r	s	x	x	8, 11
HAK 75	β	P	P	±	±	A.TH α A.TL	–	–	–	p	q	–	–	u	v	36
Tu226-25-63	β	P	P	O	O	(A×B10) α Lib18	–	f	j	–	–	r	s	u	–	[e]
F35-52-27	β	P	P	O	O	B10.STC77 α B10.KPA44	–	f	j	–	–	r	s	u	–	37
39E	β	P	+++	O	O	A.TH α A.TL	–	f	x	–	–	r	–	u	–	8, 12, 13, 15, 24, 26, 32
40F	β	P	P	O	O	A.TH α A.TL	–	f	x	–	–	–	–	u	–	8, 12, 13, 15, 24, 32
40L	β	P	P	O	O	A.TH α A.TL	–	f	x	–	–	–	–	u	–	12, 13, 15, 24, 25, 32
40N	β	P	+++	O	O	A.TH α A.TL	–	f	x	–	–	r	–	u	–	8, 12, 13, 26, 32
A^b reactive antibodies																
3B9	α	O*	+++	O*	P	BALB/c α B6	–	f	j	x	q	r	s	u	v	17, 24
4D5	α	O*	+++	O*	P	BALB/c α B6	–	f	–	p	q	r	s	u	v	17, 24
1D9	α	O*	+++	O*	P	BALb/c α B6	–	f	–	x	q	r	s	u	v	24
Y-248	α and β	O*	+	O	P	A/J α B10.A(5R)	–	f	x	p	q	r	s	u	v	[f]
Y-257	β or α	O*	+	+++	P	A/J α B10.A(5R)	–	f	x	p	q	r	s	u	v	[f]
Y-3P	α or β	O*	+++	++	P	[g]	–	f	x	p	q	r	s	u	v	17, 24, 38
Y-8P	α or β	O*	++	±	P	[g]	–	f	x	p	q	r	s	u	v	17, 38
25-9-17S	β	O	O	P	P	C3H α CSW	d	–	x	p	q	–	–	x	x	17, 39
28-8-16S	β	O	O	P	P	C3H α CSW	d	–	x	–	–	–	–	x	x	17, 39
34-5-3S	β	O	O	P	P	C3H α BDF_1	d	–	x	p	q	–	–	x	x	17, 40
B17-263	β	O	O	P	P	AKR α B6	d	–	–	x	q	–	–	x	–	11, 33
B17-123	β	O	O	P	P	AKR α B6	d	–	–	x	q	r	–	x	x	11, 33
BP 107	β	O	O	P	P	SJL α B10.D2	d	–	j	p	q	–	–	u	x	41
9B[h]	β	O	O	P	P	A.TH α A.TL										42
74A[h]	β	O	O	P	P	A.TH α A.TL										43
Y-202	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	–	–	–	–	–	v	[f]
Y-212	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	p	q	r	–	–	v	[f]
Y-214	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	–	–	–	–	–	v	[f]
Y-219	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	–	–	r	–	–	v	[f]
Y-220	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	–	–	r	–	–	v	[f]
Y-222	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	–	–	r	–	–	v	[f]
Y-237	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	p	q	–	–	–	v	[f]
Y-270	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	–	–	r	–	–	v	[f]
Y-271	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	–	–	r	–	–	v	[f]
Y-276	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	p	q	–	–	–	v	[f]
Y-279	β	O	O	P	P	A/J α B10.A(5R)	d	–	x	–	–	r	–	–	v	[f]

[a] Reactivity of the mAb with the four LMTK transfectants. Data was normalized as described in *Materials and Methods*. p corresponds to the positive reference value ($A\alpha^k$:$A\beta^k$ or $A\alpha^b$:$A\beta^b$, depending on the antibody used). O* denotes very weak cross-reactivity, not seen in all experiments.

[b] Strain combination used in the isolation of the mAb. The responder mouse strain is listed before the immunizing strain. B6 and B10 stand for (C57Bl/6 and C57Bl/10, respectively).

[c] Cross reactivity data taken from the references listed, established by typing with congeneic mouse strains. –: no reactivity; x: not reported.

[d] The underlined reference is the initial description of the mAbs. Others present data establishing or giving some indication of chain reactivity.

[e] D. Klein, Z. Zaleska Rutczymska and F. Figueroa, unpublished.

[f] S. DeGraw and D. Murphy, unpublished.

[g] Complex immunization protocol (38).

[h] These mAb are directed against $E\beta^k$, but cross-react with A^b.

ferent hybrid haplotypes have been analyzed. This possibility underlines the importance of studying several haplotype combinations.

A knowledge of the chain specificities of anti-Ia mAb should prove to be useful for a variety of studies aimed at dissecting Ia structure-function relationships. In addition, this information may be important for experiments that involve the injection of anti-Ia-mAb into animals, either to perturb the development of the immune system (32, 33) or to intervene in Ia-associated diseases (19–21).

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