IMMUNE RESPONSE GENE FUNCTION CORRELATES WITH THE EXPRESSION OF AN Ia ANTIGEN

I. Preferential Association of Certain A_e and E_α Chains Results in a Quantitative Deficiency in Expression of an A_e:E_α Complex*

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Ia antigens are highly polymorphic membrane glycoproteins coded for by loci in the *I* region of the murine *H-2* major histocompatibility gene complex (MHC)[1]. They are expressed predominantly on cells involved in the generation of immune responses, and considerable information is available on the genetics and structure of Ia antigens common to B lymphocytes and macrophages. One Ia complex is immunoprecipitated by alloantibodies to the *I-A* subregion and consists of A_α and A_β polypeptide chains, both of which are products of loci in the *I-A* subregion (1–3). Antibodies prepared in *I-E* subregion incompatible strains precipitate a complex of E_α and A_e (E_β) polypeptide chains. Although the E_α chain is encoded by a locus in the *I-E* subregion, the A_e chain is the product of a third *Ia* locus in the *I-A* subregion (4–6). Intracellular A_α:A_β and A_e:E_α complexes appear to associate with an additional polypeptide chain, the Ia-associated invariant chain (I_i) (7). This chain is nonpolymorphic (3, 7, 8) and appears not to be expressed on the cell surface (9); at this time its significance is not known. In addition to Ia antigens the *I* region controls functional traits involved in the induction and regulation of immune responses. Immune response (*Ir*) and immune suppression (*Is*) genes regulate the stimulation and suppression, respectively, of immune responses. The generation or suppression of specific immune responses to some antigens is under the dual control of loci in the *I-A* and *I-E/C* subregions (10).

Due to the similarities in the genetics of Ia antigens and *I*-region-controlled functions and to the expression of Ia antigens on cells involved in immune functions, a direct role for Ia antigens in the induction and regulation of immune responses has been postulated. Within the last several years evidence that Ia antigens are the

* Supported by grants AI-15732, AI-14349, CA-16359, and AI-14579 from the National Institutes of Health. Dr. P. P. Jones acknowledges additional support from Cooper Laboratories, Inc.

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[1] *Abbreviations used in this paper:* APC, antigen-presenting cell; FACS, fluorescence-activated cell sorter; FE, fluorescein equivalents; *Ir*, immune response; MHC, major histocompatibility gene complex; 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis.

products of *Ir* genes has accumulated from a variety of genetic, functional, and biochemical studies (4, 11–13). It is apparent from these and other studies that T lymphocytes recognize nominal (foreign, non-MHC) antigens in the context of self Ia on antigen-presenting cells (APC) (14). Although the precise mechanism(s) by which Ia antigens mediate *Ir* gene function is not known, factors that affect the structure and expression of Ia antigens on the surfaces of APC are certain to modulate immune response potential.

Recent genetic and biochemical investigations have revealed that Ia antigens are an extremely diverse set of proteins. Variability in Ia structure and expression has several sources. First, many mouse strains express two distinct complexes of Ia antigens on their cell membranes, A_α:A_β and A_e:E_α. Second, all four Ia polypeptide chains are genetically polymorphic (1, 3, 15), although the degree and complexity of the polymorphism varies from chain to chain. Third, some alleles at the loci controlling the A_e and E_α chains are not expressed, i.e., they behave as null or silent alleles (4, 16, 17). Finally, additional molecular polymorphism arises from combinatorial association of Ia chains. Cells from *I* region heterozygotes can express mixed haplotype (hybrid) A_α:A_β and A_e:E_α complexes in addition to the parental forms (4, 16, 18); as a result, individual cells can express as many as eight different Ia complexes.

All of the factors mentioned above contribute to the qualitative diversity of Ia antigens and thus may affect immune response potential. The studies presented in this paper describe an additional and novel source of variability in Ia antigen expression, one that affects the quantitative levels of certain Ia antigens. Serological and biochemical studies have revealed that cells from F_1 heterozygotes between the *u* haplotype and the *b*, *k*, and *s* haplotypes have much lower amounts of $A_e^{b,k,s}$:E_α^u complexes on their surfaces than A_e^u:E_α^u complexes, apparently due to the preferential association of the two *u*-haplotype chains with each other. In the accompanying paper (19) we report that the quantitative deficiency in expression of $A_e^{k,s}$:E_α^u in certain strains results in a corresponding defect in antigen presenting-cell function. Thus the ability of cells to carry out *I*-region-controlled functions is dependent on the quantity as well as the quality of Ia antigens expressed.

Materials and Methods

Mice. B10, B10.D2, and B10.A(4R) mice used in the fluorescence-activated cell sorter (FACS) and two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) studies were obtained from Dr. H. O. McDevitt, Stanford University School of Medicine, Stanford, Calif. All other mice were bred in our animal facilities at the National Institutes of Health, Yale University (New Haven, Conn.), and Stanford University.

Antibodies. The (B10.S(7R) × A.CA)F_1 anti-B10.HTT antiserum was generously provided by Dr. H. O. McDevitt. Monoclonal antibody Y-17, resulting from a BALB/c anti-B10.A(5R) immunization, has been shown to react with specificity Ia.m44, a combinatorial or conformational determinant on certain A_e:E_α Ia complexes (13). The hybridoma cell lines 14-4-4 and 17-3-3, producing monoclonal anti-Ia.7 and anti-A_e:E_α antibodies, respectively (20), were gifts from Dr. Keiko Ozato and Dr. David Sachs of the National Cancer Institute. The hybridoma cell line MK-D6 producing an anti-I-A^d monoclonal antibody was generously provided by Dr. Philippa Marrack and Dr. John Kappler of the University of Colorado Medical School, Denver, Colo. The preparation and reactivities of the 10-2.16 and 10-3.6 monoclonal anti-I-A antibodies and of the 10-4.22 anti-Ig 5a(δ) antibody have been previously described (21). The anti-dansyl monoclonal antibody 27-4-4 was a gift of Dr. Vernon T. Oi, Stanford University School of Medicine.

Microcytotoxicity. Details of the dye exclusion microcytotoxic assay have been published

previously (22). Briefly, 2,000 spleen cells were incubated with appropriately diluted antiserum for 15 min at 37°C, washed, and incubated with rabbit complement (1:12) for 30 min at 37°C. The cells were then stained with the vital dye nigrosin, and the living and dead cells were enumerated using an inverted microscope.

Quantitative Absorption. In vitro absorption analysis was performed by absorbing 50 μl of $1:10^4$ diluted Y-17 ascites fluid with graded numbers of spleen cells for 1 h at room temperature. The number of cells required for complete absorption of antibody reactive with B10.A(5R) spleen cells was determined in the dye exclusion microcytotoxic assay.

Quantitative Immunofluorescence. All monoclonal antibodies for these studies were purified from culture supernatant fluids on protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, N. J.) columns; conjugation of the purified antibodies with fluorescein isothiocyanate was performed according to the method of Goding (23). All reagents were centrifuged at 100,000 *g* for 10 min before use. Spleen cells (1×10^6) were stained directly by incubation at 4°C for 30 min with saturating levels of each fluorescein-conjugated monoclonal antibody. The stained cells were analyzed on the modified FACS II (B-D FACS Systems, Becton-Dickinson & Co., Sunnyvale, Calif.) in the laboratory of Dr. L. A. Herzenberg, Dept. of Genetics, Stanford University School of Medicine. This FACS II is equipped with a logarithmic amplifier that shows the fluorescence intensity distribution over a $1-10^4$ range.

The calibration of the FACS system with free fluorescein, which allowed the quantitative measurements to be made, was done by Dr. D. R. Parks, Stanford University School of Medicine. For each stained sample, 10,000 cells were analyzed, and the geometric mean fluorescence of the positive cells was calculated and converted to fluorescein equivalents (FE) as described by Ledbetter et al. (24). The mean numbers of antibody molecules bound to positive cells were determined by the calculation

$$\frac{\text{mean FE of positive cells} - \text{mean FE of unstained cells}}{\text{molar fluorescein/protein (F/P) ratio of conjugated antibody}}.$$

F/P ratios of the monoclonal antibodies used were: 14-4-4, 5.3; Y-17, 4.4; 17-3-3, 4.0; 10-3.6, 5.1; and MK-D6, 6.3. The mean autofluorescence of unstained cells and the fluorescence of cells stained with the control monoclonal antibody 27-4-4 (anti-dansyl) were identical and equivalent to 7.7×10^3–9.7×10^3 fluorescein molecules/cell.

Biochemical Analysis of Ia Antigens. Labeling of mouse splenic lymphocytes with [^{35}S]methionine, immunoprecipitation of Ia antigens, 2-D PAGE were done as previously published (7, 25). Immunoprecipitated proteins were separated by charge in the first dimension using nonequilibrium pH gradient electrophoresis, which resolves proteins with isoelectric points between pH 4.5 and 9. The second dimension size separation was done on 10% acrylamide sodium dodecyl sulfate slab gels. Positions of the separated proteins were determined by fluorography, using sodium salicylate as the fluorogenic compound and Kodak XAR-5 film (Eastman Kodak Co., Rochester, N. Y.).

Results

The studies presented below were prompted by the unexpected observation that cells from F_1 mice produced by crossing the $I\text{-}A^k$ strain B10.A(4R) with Ia.7$^+$ strains present the antigen pigeon cytochrome *c* to primed B10.A T cells for proliferative responses, with the exception of Ia.7$^+$ strains carrying the *u* haplotype (19). Considerable evidence now exists that A_e:E_α Ia complexes on the surface of APC are involved in antigen presentation in this system (13, 26); thus, it seemed likely that the inability of the *u* haplotype to function might be due to an absence or defect in the A_e^k:E_α^u complex. Several approaches have been used to examine this possibility. The first involved serological assays for the presence of Ia complexes with E_α^u chains on cell surfaces. We have taken advantage of the availability of the monoclonal antibody Y-17, which reacts with specificity Ia.m44 (13), a combinatorial or conformational determinant on certain A_e:E_α complexes. Ia.m44 is expressed uniquely on the two-

chain complex, not on either chain by itself; complexes of $A_e^{b,s}$:$E_\alpha^{k,d}$, A_e^k:E_α^k, and A_e^r:E_α^r have been shown to express Ia.m44, while A_e^d:E_α^d and A_e^p:E_α^p complexes do not (13). As the data presented below will show, Y-17 does not react with A_e^u:E_α^u complexes but does react with complexes composed of E_α^u and $A_e^{b,k,s}$ chains. Thus, Y-17 can be used to test serologically for the presence of complexes of $A_e^{b,k,s}$:E_α^u in F_1 hybrids carrying the *u* haplotype.

Failure to Detect $A_e^{b,k,s}$:E_α^u Complexes on the Cell Surface with Y-17 by Direct Cytotoxicity. We have shown previously that $A_e^{b,s}$:$E_\alpha^{k,d}$, A_e^k:E_α^k, and A_e^r:E_α^r complexes can be detected on lymphocyte surfaces with Y-17 by direct cytotoxic tests (13). Data in Table I show that A_e^b:E_α^p, A_e^k:E_α^d, A_e^k:E_α^p, A_e^s:E_α^p, and A_e^v:E_α^v complexes can also be detected by direct cytotoxicity. However, no significant lysis above background was observed with spleen cells from (B10 × B10.PL)F_1 or (B10 × PL/J)F_1 (A_e^b:E_α^u), [B10.A(4R) × B10.PL]F_1 (A_e^k:E_α^u), or (B10.S × B10.PL)F_1 (A_e^s:E_α^u) mice. There are several possible explanations for the failure of Y-17 to kill cells from these hybrids. First, Y-17 may not react with $A_e^{b,k,s}$:E_α^u complexes. Second, Y-17 may react with $A_e^{b,k,s}$:E_α^u complexes but they may not be expressed on the surfaces of cells in the F_1 hybrids tested. Third, these complexes may be expressed and recognized by Y-17 but may not cause lysis in the direct cytotoxic assay. Data in the following sections will show that the third alternative is correct, and that the failure to detect $A_e^{b,k,s}$:E_α^u complexes with Y-17 by direct cytotoxicity is due to the low level of expression of these complexes.

Detection of $A_e^{b,k,s}$:E_α^u Complexes with Y-17 by Quantitative Absorption Analysis. To determine whether Ia.m44 is expressed, quantitative absorption analyses were performed; the results are shown in Fig. 1 and Table I. No discernible difference was observed in the capacity of cells from Ia.m44$^+$ homozygous animals to absorb Y-17 activity; comparative absorption ratios of 1.0–1.4 were obtained, using the number of B10.A(5R) spleen cells required to absorb Y-17 activity as a standard. Comparative absorption ratios for Ia.m44$^+$ F_1 hybrids expressing $A_e^{b,k,s}$:$E_\alpha^{d,k,p}$ complexes range from 1.2–3.3 with a mean of 2.0. Most important, *u*-haplotype heterozygotes were able to absorb Y-17 activity, although comparative absorption ratios for F_1 hybrids carrying *I-A*^{b,s} and *I-E*^{u} subregions revealed seven- to eightfold less expression of the Ia.m44 determinant than for Ia.m44$^+$ homozygotes. Reduced expression of Ia.m44 in F_1 hybrids carrying the *I-A*^{k} and *I-E*^{u} subregions will be shown by quantitative immunofluorescence analyses below. Since the Ia.m44 determinant recognized by Y-17 is uniquely expressed on A_e:E_α complexes (13), it can be concluded that F_1 hybrids carrying the *I-A*^{b,k,s} and *I-E*^{u} subregions do express $A_e^{b,k,s}$:E_α^u complexes on their cell surfaces. However, quantitatively less Ia.m44 antigen is expressed compared to homozygous mice or F_1 mice not carrying the *u* haplotype; this low level of expression of Ia.m44 may be insufficient to promote direct lysis by Y-17 and complement in the microcytotoxic assay.

Quantitation of A_e:E_α Expression on Individual Parental and F_1 Cells by Quantitative Immunofluorescence. The levels of cell surface A_e:E_α complexes on spleen cells from parental and F_1 mice were measured by quantitative FACS analysis of cells stained with fluorescein-conjugated 14-4-4 and Y-17 monoclonal antibodies. The 14-4-4 monoclonal antibody reacts with all Ia.7$^+$ strains (20), and as shown in Fig. 2 a, c, and d, stains 55–70% of spleen cells from homozygous mice of *k*, *d*, and *u* haplotypes. 14-4-4 does not stain B10.A(4R) cells (Fig. 2 b), which are Ia.7$^-$ and do not appear to

TABLE I

Cytotoxicity and Absorption Analyses of Y-17 Reactivity with Different A_e:E_α Complexes

Strain	Haplotype origin of chains*		Cytotoxicity	Absorption analysis‖§	Conclusion
	A_e	E_α	50% titer with Y-17‡§	Normalized number of cells required to absorb Y-17 (B10.A(5R) targets)	Y-17 reacts with A_e:E_α complexes
(B6 × BALB/c)F_1	b/d	d	10^4–10^5	ND¶	b:d
B10.A(5R)	b	k	10^5	1.0, 1.0, 1.0, 1.0	b:k
(B10 × A.TFR5)F_1	b	k	10^4–10^5	1.2	b:k
(B10.A(5R) × B10.T(6R))F_1	b	k	10^4–10^5	1.6	b:k
(B10 × B10.A)F_1	b/k	k	10^4–10^5	1.2	b:k, k:k
(B10 × B10.P)F_1	b/p	p	10^4–10^5	ND	b:p
(B10 × B10.PL)F_1	b/u	u	0	7.3, 7.7	b:u
(B10 × PL/J)F_1	b/u	u	0	7.3, 8.2	b:u
(B10.A(4R) × B10.D2)F_1	k/d	d	10^4	1.7	k:d
B10.A	k	k	10^5	1.0, 1.4, 1.3	k:k
(B10.A(4R) × B10.P)F_1	k/p	p	10^4–10^5	3.3	k:p
(B10.A(4R) × B10.PL)F_1	k/u	u	0	ND	(k:u)**
(B10.S × B10.D2)F_1	s/d	d	10^4–10^5	1.7, 3.0	s:d
B10.S(9R)	s	k	10^4–10^5	1.0, 1.3	s:k
(B10.S × B10.P)F_1	s/p	p	10^4	2.3	s:p
(B10.S × B10.PL)F_1	s/u	u	0	7.7	s:u
B10.PL	u	u	0	—‡‡	
PL/J	u	u	0	—	
B10.SM	v	v	10^5	ND	v:v
SM/J	v	v	10^5	ND	v:v

* The haplotype origins of all A_e and E_α chains synthesized are given. The synthesis of A_e and E_α chains is variable, depending on the haplotype in the *I-A* and *I-E* subregions, respectively, as follows (16, 17): *d, k, p, r, u, v:* synthesize both A_e and E_α chains; *b, s:* synthesize A_e but not E_α chains; *f, q:* synthesize neither A_e nor E_α chains; *ap5* (A^fE^k, strain A.TFR5): synthesizes E^k_α but not A^f_e chains.
When both chains are synthesized (loci in *cis* or *trans* chromosomal position), A_e:E_α complexes are found on the cell surface.

‡ Reciprocal dilution of antibody giving 50% of maximal lysis.

§ Specificity controls: no cytotoxicity or absorption (with 62–125 × 10^6 cells) observed with: B10 (A^b_e:—), A.TFR5 (—:E^k_α), B10.P or B10.F(13R) (A^p_e:E^p_α), B10.PL or PL/J (A^u_e:E^u_α), B10.A(4R) (A^k_e:—), B10.D2 (A^d_e:E^d_α), B10.S (A^s_e:—) B10.S(8R) ($A^{k\text{ or }s}_e$:—), B10.T(6R) (—:—), (B10.D2 × A.TFR5)F_1 ($A^{d,k}_e$:$E^{d,k}_\alpha$), (B10.D2 × B10.F(13R))F_1 ($A^{d,p}_e$:$E^{d,p}_\alpha$) (B10.F(13R) × A.TFR5)F_1 ($A^{p,k}_e$:$E^{p,k}_\alpha$), (B10.F(13R) × PL/J)F_1 ($A^{p,u}_e$:$E^{p,u}_\alpha$), or (PL/J × A.TFR5)F_1 ($A^{u,k}_e$:$E^{u,k}_\alpha$).

‖ Number of spleen cells (×10^6) required to completely absorb Y-17 cytotoxic activity ÷ number of B10.A(5R) spleen cells (×10^6) required to completely absorb Y-17. Y-17 used at 1:10^4 dilution. Denominator range = 3 × 10^6–5 × 10^6 cells.

¶ Not done.

** Quantitative immunofluorescence analysis (Fig. 2, Table II) will show that A^k_e:E^u_α complexes are expressed, but in low amounts.

‡‡ No absorption with 187 × 10^6 cells (strain B10.PL); 61 × 10^6 cells for PL/J.

synthesize or express an E_α chain (4). The 14-4-4 antibody can detect A_e:E_α complexes on the surfaces of cells from heterozygous (4R × B10.D2)F_1 and (4R × B10.PL)F_1 mice; however, it is clear from the FACS curves in Fig. 2 that the positions of the positive peaks of 14-4-4 staining in the F_1 were shifted downward by about 0.30 log units relative to the parental strains on the four-decade log scale (compare 14-4-4 staining in Fig. 2 e with 2 c and 2 f and with 2 d). The 0.30 log unit difference between

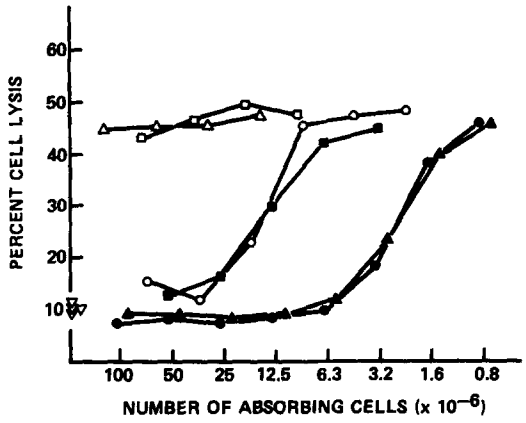

FIG. 1. Quantitative absorption analysis of Y-17 on B10.A(5R) spleen cell targets. The strains used for absorption are indicated. Positive absorption controls: B10.A(5R) (●) (A^b_e:E^k_α) and B10.A (▲) (A^k_e:E^k_α). Negative absorption controls: B10 (△) (A^b_e:—) and (B10.D2 × B10.PL)F_1 (○) ($A^{d,u}_e$:$E^{d,u}_\alpha$). Experimental: (B10.S × B10.PL)F_1 (■) ($A^{s,u}_e$:E^u_α) and (B10 × B10.PL)F_1 (○) ($A^{b,u}_e$:E^u_α). ▽, medium control.

the means of the positive peaks corresponds approximately to a twofold difference in fluorescence intensity between the parental and F_1 cells.

The FACS curves from parental and F_1 cells stained with the monoclonal antibody Y-17 are also presented in Fig. 2. No staining with Y-17 was observed on B10.A(4R), B10.D2, and B10.PL spleen cells, a result that confirms the cytotoxicity and absorption data presented in Table I. Y-17 did stain spleen cells from F_1 mice expressing complexes formed by the association of A^k_e with E^d_α or E^u_α chains; however, striking quantitative differences were detected in the cell surface expression of A^k_e:E^d_α complexes [(4R × B10.D2)F_1, Fig. 2e] compared to A^k_e:E^u_α complexes [(4R × B10.PL)F_1, Fig. 2f] as detected by the Y-17 antibody. The staining of (4R × B10.D2)F_1 cells with Y-17 was significantly brighter in fluorescence intensity (~0.45 log units) than the staining of (4R × B10.PL)F_1 cells. Similar quantitative differences in intensity of Y-17 staining were also observed in indirect staining experiments. Using a fluorescein-conjugated goat anti-mouse $Fc_{\gamma 2}$ second-step antibody to detect Y-17 binding (B10.S × B10.D2)F_1 and (B10 × B10.D2)F_1 cells bound considerably more Y-17 than did (B10.S × B10.PL)F_1 and (B10 × B10.PL)F_1 cells (data not shown). Despite the variable levels of Y-17 staining in the different mouse strains, the same proportions of spleen cells bound 14-4-4 and Y-17 in each strain; these proportions (55–70%) corresponded to the proportion of B cells, as determined by staining with fluorescein-conjugated rabbit anti-mouse Ig antibodies (data not shown).

The use of the calibrated FACS-II for fluorescence analysis enabled us to quantitate directly the number of monoclonal anti-Ia antibodies bound per cell (Table II). Assuming that most antibody binding at saturation is monovalent (27, 28), these numbers reflect the numbers of Ia molecules expressed per cell. The data from staining parental and F_1 spleen cells with monoclonal anti-I-A antibodies (10-3.6, anti-I-A^k; and MK-D6, anti-I-A^d) are included to illustrate gene dosage effects in the expression of A_α:A_β complexes on parental and F_1 spleen cells. As presented in Table II, 31,000–42,000 10-3.6 antibody molecules were bound per positive cell from B10.BR mice and 29,000–34,000 molecules were bound per positive cell from B10.A(4R) mice, both

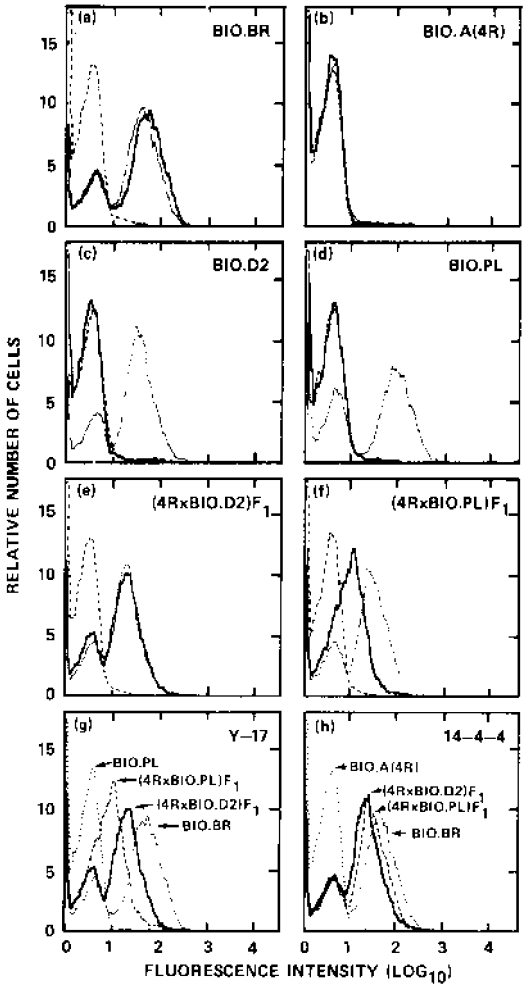

FIG. 2. Immunofluorescence staining of parental and F_1 spleen cells (a–f) with Y-17 (——), 14-4-4 (——) and control (anti-dansyl) monoclonal antibody 27-4-4 (– – –). Cells were stained directly with fluorescein-conjugated antibodies and analyzed on a FACS-II with a logarithmic amplifier. The FACS curves from cells stained with 27-4-4 and those curves from unstained cells (not shown) were identical (superimposed). Panel g shows a composite of the FACS curves from Y-17 stained cells from B10.BR (——), (4R × B10.D2)F_1 (——), (4R × B10.PL)F_1 (– – –), and B10.PL (· · · ·); panel h shows a composite of the FACS curves from 14-4-4 stained cells from B10.BR (——), (4R × B10.D2)F_1 (——), (4R × B10.PL)F_1 (– – –), and B10.A(4R) (· · · ·).

$I\text{-}A^k$ homozygotes, whereas approximately half as many 10-3.6 antibody molecules (19,000–21,000) were bound to heterozygous (4R × B10.D2)F_1 ($I\text{-}A^{k/d}$) cells. Similar gene dosage effects were observed with *d* haplotype cells stained with MK-D6; B10.D2 ($I\text{-}A^d$ homozygous) cells bound 32,000–42,000 molecules/cell while (4R × B10.D2)F_1 cells bound 19,000–20,000 molecules/cell. The results in Table II also show that the 10-3.6 antibody, known to react with specificity Ia.17 common to A_α:A_β complexes of the *k*, *f*, *s*, and *r* haplotypes (21), also reacts with *u* haplotype cells, which had not been tested previously for Ia.17 expression. Thus the (4R × B10.PL)F_1 ($I\text{-}A^{k/u}$) expresses homozygous levels of I-A molecules (30,000–33,000/cell) detectable by 10-3.6.

TABLE II

Levels of Ia Antigen Expression Determined by Quantitative Immunofluorescence

	Haplotype origin of chains*		Number of antibody molecules bound ($\times 10^{-3}$)‡			
	A_e	E_α	Anti-I-A^k 10-3.6	Anti-I-A^d MK-D6	Anti-E_α 14-4-4	Anti-(A_e:E_α) Y-17
B10.BR	k	k	42.3	1.4	19.7	30.2
			31.4	1.4	16.8	22.2
B10.D2	d	d	<0.1	42.4	17.3	0.0
			0.2	32.4	15.4	0.3
B10.PL	u	u	37.5	0.2	36.1	<0.1
			29.2	0.2	26.1	0.1
B10.A(4R)	k	—	34.1	0.9	0.1	0.2
			29.2	0.9	<0.1	<0.1
(4R × B10.D2)F_1	k/d	d	19.0	19.7	10.2	12.2
			20.8	19.5	8.7	11.1
(4R × B10.PL)F_1	k/u	u	33.2	0.7	13.4	3.4
			30.1	0.7	11.8	3.3

* The haplotype origins of all A_e and E_α chains synthesized are given (see Table I).

‡ The upper number of each pair corresponds to the number of antibody molecules bound per cell in the experiment shown in Fig. 2. The lower numbers correspond to results obtained in a duplicate experiment.

The numbers of A_e:E_α molecules per cell as detected by 14-4-4 and Y-17 monoclonal antibodies were also calculated from the FACS curves shown in Fig. 2 and those from a duplicate experiment (curves not shown). For A_e:E_α molecules, as noted for A_α:A_β molecules, gene dosage effects on expression were observed using 14-4-4 with parental and F_1 cells. Homozygous Ia.7^+ cells from B10.BR, B10.D2, and B10.PL stained with 14-4-4 expressed approximately twice as many A_e:E_α molecules per cell as did cells stained with 14-4-4 from heterozygotes. However, gene dosage effects alone cannot explain the quantitative differences between the numbers of A_e^k:E_α^d complexes detected by Y-17 on (4R × B10.D2)F_1 cells (11,000–12,000/cell) and the numbers of A_e^k:E_α^u complexes detected by Y-17 on (4R × B10.PL)F_1 cells (3,300–3,400/cell). This 3.5-fold difference in the number of A_e^k:E_α^d complexes vs. A_e^k:E_α^u complexes in the two F_1 hybrids was confirmed in staining experiments with a second monoclonal antibody, 17-3-3, which appears to have specificity for the same A_e:E_α complexes as those described for Y-17 (29, and our unpublished results). For all parent and F_1 mice in both experiments shown in Table II, the number of 17-3-3 molecules bound per cell was nearly identical to the number of Y-17 molecules bound per cell (data not shown). As with Y-17, the number of 17-3-3 molecules bound to (4R × B10.D2)F_1 cells (11,000–13,500) was three to four times the number of 17-3-3 antibodies bound to (4R × B10.PL)F_1 cells (3,100–3,900).

The seven- to eightfold reduction in expression of A_e^k:E_α^u complexes in F_1 hybrids compared to the expression of A_e^k:E_α^k complexes in homozygous B10.BR cells, as detected by Y-17 staining, is in good agreement with the comparative values obtained from quantitative absorption experiments for A_e^k:E_α^u and A_e^s:E_α^u complexes (Table

I). Seven to eight times more ($b \times u$)F_1 or ($s \times u$)F_1 cells were required to absorb all Y-17 activity compared to homozygous B10.A(5R) ($I\text{-}A^b$, $I\text{-}E^k$) or B10.S(9R) ($I\text{-}A^s$, $I\text{-}E^k$) cells. Thus two independent quantitative methods of analysis revealed unexpectedly low levels of expression of Ia complexes comprised of E^u_α chains and A_e chains of the *b*, *k*, and *s* haplotypes in F_1 hybrids.

The serological approaches described above have shown clearly the quantitative deficiency in expression in $A_e^{b,k,s}$:E^u_α complexes on the surfaces of cells from appropriate heterozygotes. However, these methods provide no information on the molecular basis for this phenomenon. The results of 2-D PAGE analyses presented in the next section suggest that the low levels of $A_e^{b,k,s}$:E^u_α complexes expressed on F_1 cells may be due to the preferential associated of E^u_α chains with A^u_e chains.

Predominant Association of E^u_α Chains with A^u_e Chains over $A_e^{b,k,s}$ Chains in F_1 Hybrids. 2-D PAGE of immunoprecipitated A_e:E_α complexes from heterozygotes clearly show an excess of E^u_α chains associated with A^u_e chains compared with A_e chains of other parental haplotypes. This finding was observed initially in gels of anti-I-E immuno-

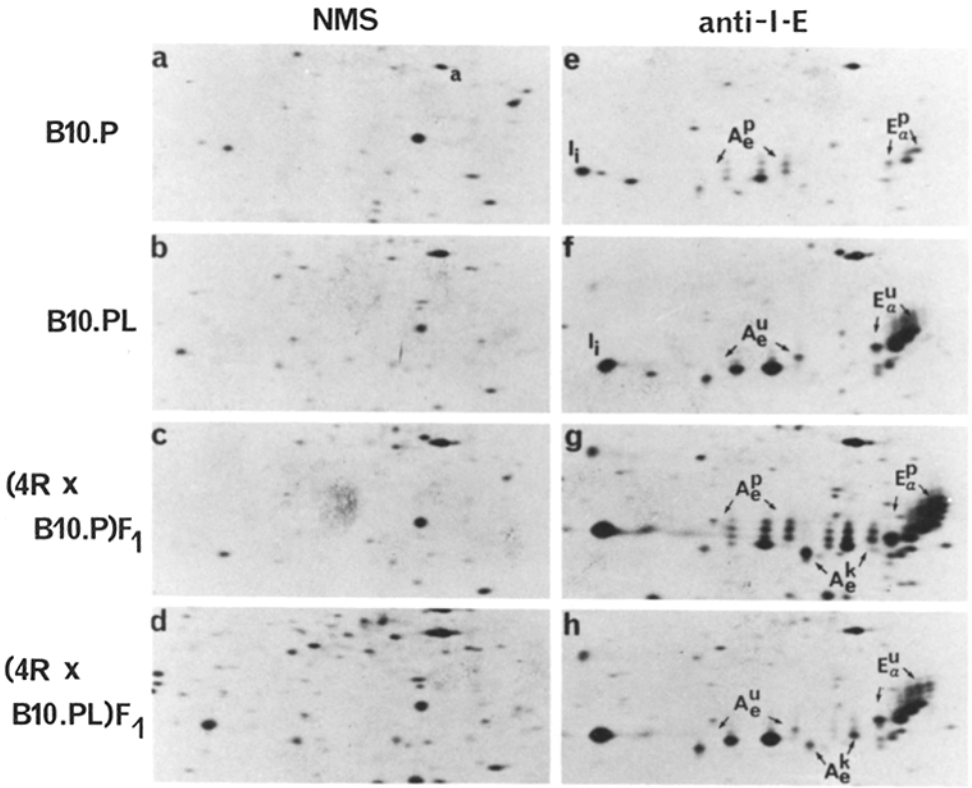

FIG. 3. (A^k_e:E^u_a) complexes are present in lower amounts than (A^u_e:E^u_a) complexes in (4R × B10.PL)F_1 cells: comparison with (4R × B10.P)F_1. Extracts from [^{35}S]methionine-labeled spleen cells were immunoprecipitated with normal mouse serum (a–d) or [(B10.S(7R) × A.CA]F_1 anti-B10.HTT (anti-I-E) antiserum. The panels show portions of fluorograms of 2-D gels of the immunoprecipitated proteins. Panels a, b, e, and f represent the immunoprecipitates from 4×10^6 cells. Panels c, d, g, and h represent the immunoprecipitates from 8×10^6 cells. Fluorographic exposure of the gels was for 7 d. The position of actin (43,000 mol wt) is indicated by the letter a in panel a. Confirmatory results were obtained in a second experiment.

precipitates from (4R × B10.PL)F_1 heterozygotes. As shown in Figure 3h, there is considerably less A_e^k chain than A_e^u chain in this immunoprecipitate. In contrast, (4R × B10.P)F_1, a control heterozygote, has very balanced proportions of A_e^k and A_e^p chains (Fig. 3g).

A second approach to demonstrating biochemically the quantitative differences in A_e^k and A_e^u chains in heterozygotes is through the use of the monoclonal antibody, Y-17. Fig. 4 shows immunoprecipitates prepared with Y-17 and the anti-Ia.7 monoclonal antibody 14-4-4 from (4R × B10.PL)F_1 and the control heterozygote (4R × B10.D2)F_1, as well as from homozygous *k*, *d*, and *u* haplotype strains. From the 14-4-4 precipitates it again appears that A_e^u chains outnumber A_e^k chains in the (4R × B10.PL)F_1 cells (Fig. 4j), whereas A_e^k and A_e^d chains are present in more balanced proportions in (4R × B10.D2)F_1 cells (Fig. 4i). Since Y-17 does not react with A_e^u:E_α^u or A_e^d:E_α^d molecules, it immunoprecipitates only the A_e^k:E_α^u and A_e^k:E_α^d complexes from the two heterozygotes. Comparing the intensities of the gel patterns in Fig. 4n with 4o confirms the abnormally low levels of complexes containing A_e^k chains in (4R × B10.PL)F_1 cells. Only a small proportion of the total E_α^u chains precipitable by 14-4-4 appears to be associated with the A_e^k chains (compare Fig. 4j with 4o), in contrast to the considerable proportion of E_α^d chains associated with A_e^k chains in (4R × B10.D2)F_1 cells (compare Figs. 4i and 4n).

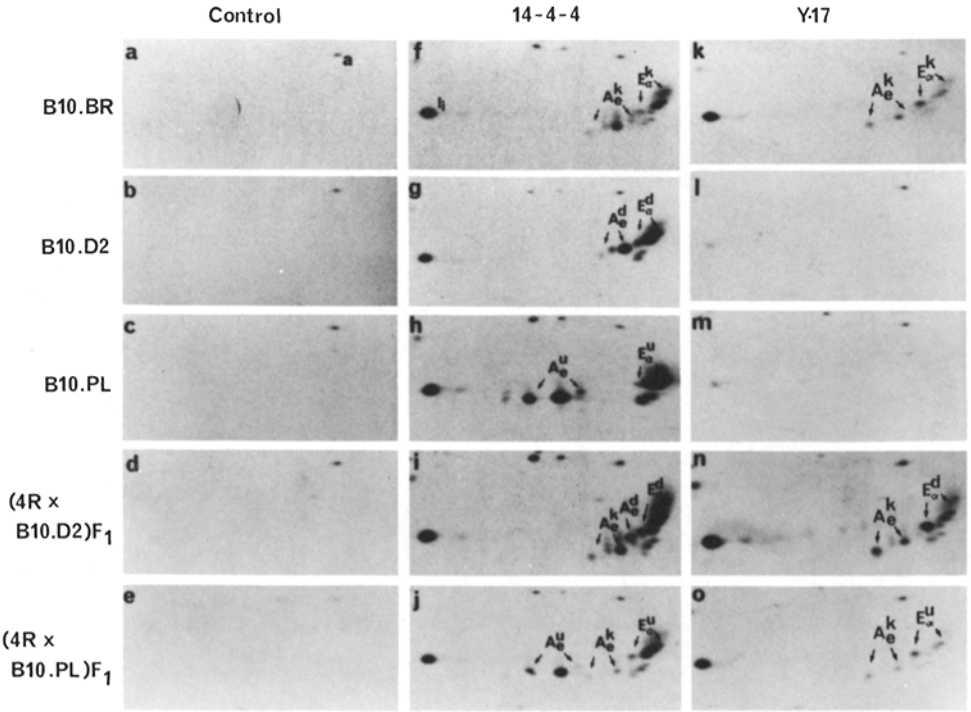

FIG. 4. (A_e^k:E_α^u) complexes are present in lower amounts than (A_e^u:E_α^u) complexes in (4R × B10.PL)F_1 cells: comparison with (4 × B10.D2)F_1. [^{35}S]methionine-labeled Ia antigens were immunoprecipitated with supernatant fluid from hybridomas producing the following monoclonal antibodies: a–e, 10-4.22, (control, anti-Ig-5a heavy chain); f–j, 14-4-4 (anti-Ia.7); k–o, Y-17 (anti-A_e:E_α), see text). All panels represent immunoprecipitates from 4 × 10^6 cells. Fluorographic exposure of the gels was for 7 d.

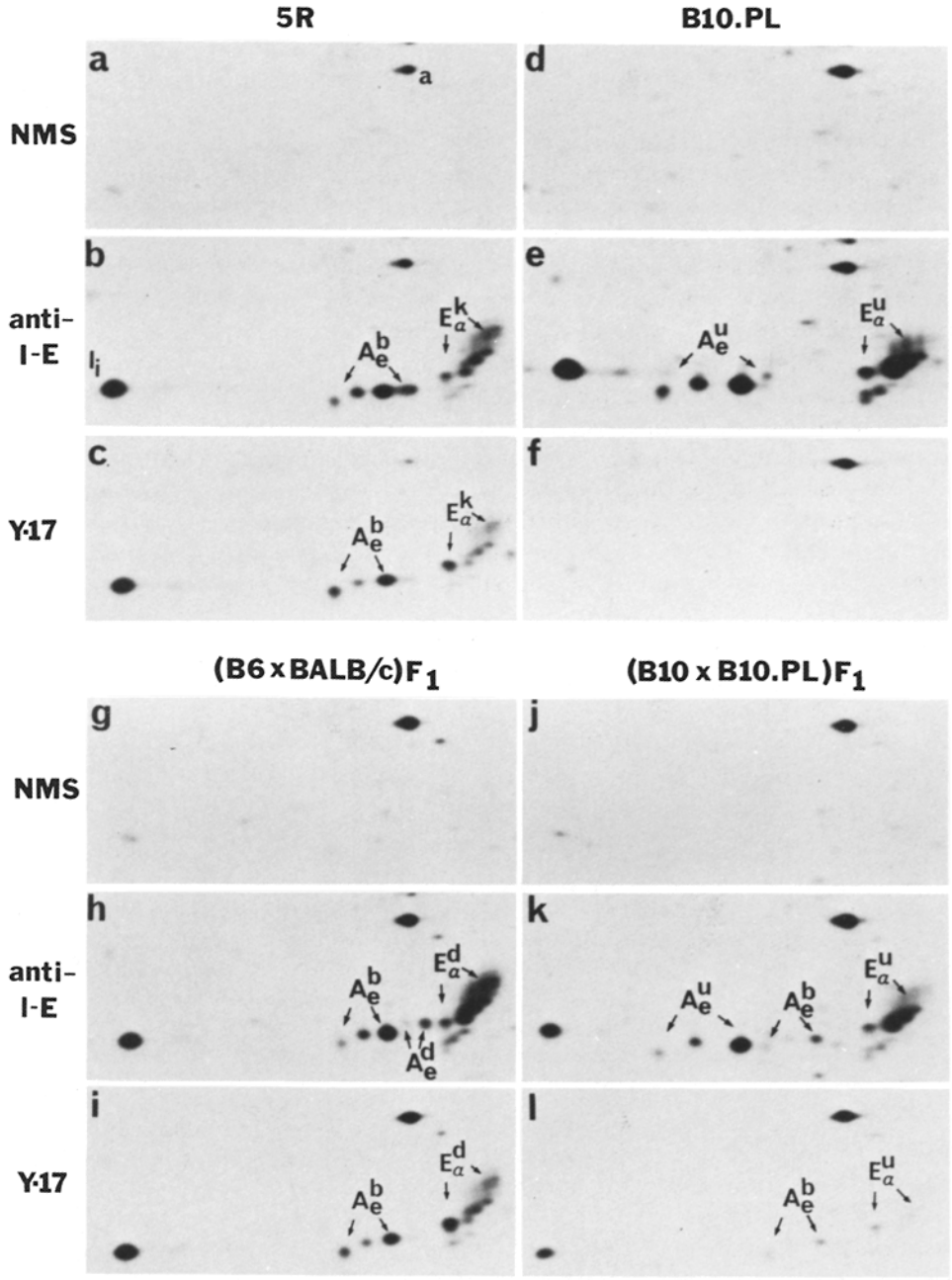

FIG. 5. (A_e^b:E_α^u) complexes are present in lower amounts than (A_e^u:E_α^u) complexes in (B10 × B10.PL)F_1 cells: comparison with (B6 × BALB/c)F_1. [^{35}S]methionine-labeled Ia antigens were precipitated with normal mouse serum (a, d, g, j); [B10.S(7R) × A.CA]F_1 anti-B10.HTT (anti-I-E) antiserum (b, e, h, k); or Y-17 ascites (anti-(A_e:E_α)) (c, f, i, l). All panels represent immunoprecipitates from 4 × 10^6 cells. Fluorographic exposure of the gels was for 6 d. Confirmatory results were obtained in a second experiment.

Similar findings have been obtained for A_e^b:E_α^u complexes in (B10 × B10.PL)F_1 cells. Figure 5k shows the preponderance of A_e^u chains over A_e^b chains in anti-I-E immunoprecipitates from this heterozygote, and the low levels of A_e^b:E_α^u chain complexes are clearly revealed by the faintness of A_e^b and E_α spots from the Y-17 immunoprecipitate (Fig. 5l). In contrast, significantly higher amounts of A_e^b chains are able to associate with E_α^d chains in (B6 × BALB/c)F_1 cells, as indicated by the gels from both the anti-I-E (Fig. 5h) and Y-17 (Fig. 5i) immunoprecipitates. Analogous results were observed in two experiments for A_e^s:E_α^u complexes (data not shown). In summary, biochemical analyses of A_e:E_α molecules from *u*-haplotype heterozygotes have indicated that the E_α^u chains present in cells from these mice are associated predominantly with A_e^u chains and only to a small extent with $A_e^{b,k,s}$ chains.

Discussion

These studies were initiated in an attempt to explain, at the molecular level, the basis for the unique failure of the *u* haplotype among all other Ia.7$^+$ haplotypes to provide an *I-E* subregion capable of interacting with $I\text{-}A^k$ or $I\text{-}A^s$ to allow a response to the antigen pigeon cytochrome *c*. As is shown in the accompanying paper (19), F_1 hybrids between B10.A(4R) ($I\text{-}A^k$) or B10.S ($I\text{-}A^s$) and strains bearing $I\text{-}E^{d,k,p,r}$ are all able to respond to this antigen; the *Ir* genes controlling this response, which map to the *I-A* and *I-E* subregions, appear to act in the APC (30). Our previous studies (4, 13, 26) have suggested that the A_e and E_α Ia polypeptide chains are the product of these *Ir* genes, and that expression of appropriate A_e:E_α complexes is required for responsiveness. Therefore, it seemed likely that the failure of the Ia.7$^+$ *u* haplotype (strain B10.PL) to complement B10.A(4R) or B10.S for responsiveness to pigeon cytochrome *c* resulted from the failure of heterozygous cells to express $A_e^{k,s}$:E_α^u complexes that are qualitatively and/or quantitatively normal.

Our approach to this problem has been to examine a series of F_1 hybrids serologically and biochemically for the expression of A_e:E_α^u complexes. Using Y-17, a monoclonal antibody that reacts with specificity Ia.m44 found on $A_e^{b,k,s}$:E_α^u complexes but not parental A_e^u:E_α^u complexes, we found in quantitative absorption and immunofluorescence studies that $A_e^{b,k,s}$:E_α^u complexes are indeed expressed on the surfaces of cells from appropriate heterozygotes but that they are present at one-fourth to one-eighth of the expected amount. 2-D PAGE analysis of Ia immunoprecipitates revealed a marked predominance of A_e^u:E_α^u complexes over $A_e^{b,k,s}$:E_α^u complexes in the cells.

This dramatic imbalance in the relative amounts of the two combinations of A_e and E_α chains could be due to either a lower level of synthesis of $A_e^{b,k,s}$ chains compared with A_e^u chains or a preferential association of E_α^u chains with A_e^u chains compared with $A_e^{b,k,s}$ chains. To distinguish between these possibilities, the intensities of A_e spots on autoradiograms of total lymphocyte proteins separated on 2-D PAGE were examined. In gels of (4R × B10.PL)F_1 extracts, the A_e^k and A_e^u spots had about the same intensity, similar to the intensities of A_e^k and A_e^d spots from a control heterozygote (4R × B10.D2)F_1 (P. Jones, unpublished results). Similar results were obtained with (*b* × *u*)F_1 and (*s* × *u*)F_1 heterozygotes. These results suggest that $A_e^{b,k,s}$ chain synthesis is normal in *u*-haplotype heterozygotes and that the low level of expression of these chains is probably due to the preferential formation of A_e^u:E_α^u complexes. The small amounts of $A_e^{b,k,s}$:E_α^u complexes expressed apparently are not sufficient to allow immune responses to the antigen pigeon cytochrome *c*.

The quantitative variability in Ia antigen expression shown by these studies thus represents an additional source of Ia heterogeneity with potential effects on immune responsiveness. The dramatic difference in representation of A_e^u:E_α^u and $A_e^{b,k,s}$:E_α^u complexes in *u*-haplotype heterozygotes represents an extreme example of such quantitative variability. However, the immunofluorescence data presented in Table II indicate that smaller differences also exist between *I*-region homozygotes and heterozygotes. As shown by the binding of monoclonal anti-I-A and anti-I-E (specificity Ia.7) antibodies, *I* region heterozygotes express approximately half as much A_α:A_β and A_e:E_α complexes as do the parental homozygous strains. The finding of reduced Ia.7 expression on the (4R × B10.D2)F_1 and (4R × B10.PL)F cells is of particular interest. These F_1 hybrids have only one copy of the gene for an expressed E_α chain; *I-E*^{b} from B10.A(4R) carries a null allele for the E_α chain (4, 16). Because cells from both heterozygotes express approximately half as much Ia.7 as the parental B10.D2 and B10.PL cells, they apparently do not compensate for the presence of only one active E_α gene by increasing the amount of E_α chains synthesized from that one gene. Analogous gene dosage effects on Ia.7 expression were also observed with (B10.S × B10.D2)F_1 and (B10.S × B10.PL)F_1 cells relative to B10.HTT (*I-A*^{s}, *I-E*^{k}) cells and with (B10 × B10.D2)F_1 and (B10 × B10.PL)F_1 cells relative to B10.A(5R) (*I-A*^{b}, *I-E*^{k}) cells (J. McNicholas and P. Jones, unpublished observations). These variations in A_e:E_α complex expression may be involved in previously described gene dosage effects on immune responsiveness to antigens under α-β *Ir* gene control (36, 37).

The quantitative immunofluorescence data in Table II reveal additional unexpected features of A_e:E_α expression. First, Y-17 seems to react with more sites on B10.BR cells than does 14-4-4, a surprising result if these two antibodies simply recognize different sites on the same A_e^k:E_α^k complex. Staining levels similar to those obtained with Y-17 also were obtained with the monoclonal antibody 17-3-3, which appears to recognize the same or a very similar determinant as Y-17 (29). The reason for the lower degree of staining of B10.BR cells by 14-4-4 is not clear; perhaps some of the E_α^k chains expressed on the cell surface are oriented in such a way that the Ia.7 specificity recognized by 14-4-4 is not exposed. Suggestive evidence for the absence of Ia.7 determinants on some Ia.m44$^+$ molecules has come from sequential immunoprecipitation studies. The anti-I-E antiserum (B10.S(7R) × A.CA)F_1 anti-B10.HTT failed to clear all A_e:E_α complexes recognized by Y-17, as determined by 2-D PAGE (P. Jones, unpublished observation).

A second rather surprising finding concerns the level of staining, somewhat higher than anticipated, of (4R × B10.D2)F_1 cells with Y-17. The 14-4-4 staining data indicate that there are approximately half as many E_α (Ia.7) determinants on this F_1 as on homozygous B10.BR and B10.D2 cells, consistent with there being only one copy of the gene for an expressed E_α chain. Of the A_e:E_α complexes expressed by the (4R × B10.D2)F_1 cells, the A_e^k:E_α^d but not the A_e^d:E_α^d complexes should express Ia.m44. If these two forms of A_e:E_α complexes were expressed in equal amounts, Ia.m44 should have been expressed on (4R × B10.D2)F_1 cells at a level one-fourth that on the homozygous B10.BR cells. Instead, however, the F_1 cells express one-half to one-third as many Ia.m44 determinants as B10.BR. One explanation for this result might be that Ia.m44 is exposed on a greater fraction of A_e^k:E_α^d than A_e^k:E_α^k complexes. Alternatively, this finding could reflect preferential chain association analogous to that observed with *u* haplotype chains. If A_e^k:E_α^d complexes formed preferentially compared

to A_e^d:E_α^d complexes, the level of staining with Y-17 would not simply reflect gene dosage effects.

An important concept that has emerged from recent studies of Ia structure and function is the significant role of combinatorial association of Ia polypeptide chains in the generation of Ia molecular polymorphism. Biochemical studies have revealed the presence of mixed haplotype (hybrid) A_e:E_α (4, 16, 38) and A_α:A_β (18) complexes on cells from *I* region heterozygotes. These findings provided a molecular explanation for *Ir* gene complementation. However, the simple formation of complexes of specific Ia α and β chains is not in itself sufficient to explain *Ir* gene complementation; responder F_1 Ia complexes formed from α and β chains derived from two nonresponder parental haplotypes must provide unique structural determinants not expressed by parental Ia molecules. Therefore, T cells involved in the responses to antigens under dual *Ir* gene control must recognize small regions of Ia complexes unique to particular combinations of α and β chains. These regions probably include combinatorial sites at junctions between the two chains as well as conformational determinants in individual chains that require certain allelic forms of the second chain for their expression.

Considerable serological evidence for the existence of unique antigenic determinants on certain A_e:E_α complex has emerged recently. Four distinct determinants of this type have been defined: Ia.m44 recognized by the Y-17 (13) and 17-3-3 (29) monoclonal antibodies, Ia.m47 defined by the A303 monoclonal antibody developed by Harris and Delovitch (39), and Ia.22 and Ia.23 recognized by standard alloantisera (40, 41). It is clear that T cells are also able to recognize unique combinatorial or conformational determinants (13, 42–45). As discussed above, the generation of combinatorial polymorphism through α and β chain joining is critical to the formation of Ia molecules with a wide range of immune response phenotypes. The localization of some of the genetic polymorphisms of Ia antigen polypeptide chains in regions involved in the joining of the two chains may in fact increase the contribution of primary structural polymorphism to Ia antigen diversity. If junctional regions of A_e and E_α chains do tend to vary structurally among haplotypes, occasional effects on the ability of chains from different haplotypes to associate might well be anticipated. The skewed proportions of different A_e:E_α complexes on cells from *u*-haplotype heterozygotes might simply be a consequence of the location and nature of the polymorphism of the A_e and/or E_α chains.

The finding of imbalanced representations of different A_e:E_α complexes in cells from *I* region heterozygotes has significant implications for investigations of analogues of Ia antigens and *Ir* genes in other species. The inability to predict Ia antigen phenotype on the basis of MHC genotype alone may hamper efforts to establish Ia structure-function relationships in species not as well-defined genetically as the mouse. In particular, variability among individuals in the levels of expression of certain HLA-DR antigens might contribute to the difficulty in establishing associations between *HLA-D* and certain disease states in humans.

Summary

These studies were stimulated by the observation, reported in the accompanying paper (19), that $I\text{-}E^u$ failed to interact with $I\text{-}A^k$ or $I\text{-}A^s$ in F_1 mice to allow a response to the antigen, pigeon cytochrome *c*, unlike *I-E* subregions derived from other Ia.7$^+$

haplotypes. Serological and biochemical analyses were performed to determine whether or not cells from these F_1 mice express the $A_e^{k,s}$:E_α complexes that should function as restriction elements for T cell recognition of pigeon cytochrome *c* on antigen-presenting cells. Using the Y-17 monoclonal antibody, which recognizes the combinatorial or conformational determinant Ia.m44 on certain A_e:E_α complexes, we were able to distinguish between A_e^u:E_α^u and $A_e^{b,k,s}$:E_α^u complexes on cell surfaces. Although complement-dependent microcytotoxicity with Y-17 failed to detect $A_e^{b,k,s}$:E_α^u complexes on cells from appropriate F_1 mice, these molecules were detected by both quantitative absorption and quantitative immunofluorescence studies. However, $A_e^{b,k,s}$:E_α^u complexes were found to be present at levels only one-seventh to one-eighth the levels expressed by homozygous *I-A*^{b}, *I-E*^{k}; *I-A*^{k}, *I-E*^{k}; and *I-A*^{s}, *I-E*^{k} cells. The results of two-dimensional polyacrylamide gel electrophoresis analyses suggest that the low levels of expression of $A_e^{b,k,s}$:E_α^u complexes are a consequence of the preferential association of A_e^u and E_α^u chains with each other in the F_1 cells. As will be shown in the following paper (19), the quantitative deficiency in the expression of A_e^k:E_α^u and A_e^s:E_α^u complexes results in a corresponding defect in antigen-presenting cell function, thus providing strong evidence that Ia antigens represent products of *Ir* genes.

The authors thank Patricia Sinnott for help with the 2-D gels, Pamela Ruest, Debbie Hasko, Kevin Lucey, and Ellen Stash for assistance with the microcytotoxicity and absorption analyses, and Gene Filson for help in running the FACS. We would also like to thank the FACS research and development team in the laboratory of Dr. L. A. Herzenberg, especially Dr. David Parks, for assistance with the quantitative fluorescence experiments. We also thank Noreen Spears for excellent secretarial assistance and Dr. Donna P. King for her comments on the manuscript.

Received for publication 7 August 1981 and in revised form 9 October 1981.

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