

SEROLOGICAL AND BIOCHEMICAL IDENTIFICATION OF HYBRID Ia ANTIGENS*

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Genes within the *I* region of the *H-2* gene complex code for two serologically and biochemically detectable products mapping in the *I-A* and *I-E* subregions (1). The Ia molecules are comprised of two noncovalently associated subunits of ~35,000 mol wt (alpha chain) and 26,000–28,000 mol wt (beta chain). Ia.7 was the first Ia antigen marker mapped to the *I-E* (old *I-C*) subregion (2) and is a public specificity associated with haplotypes *k*, *d*, *j*, *p*, *r*, *u*, *v*, and *w*.³ Later, private Ia specificities mapping in the *I-E* subregion in haplotypes *H-2^p* (Ia.21), *H-2^k* (Ia.22), and *H-2^d* (Ia.23) were identified (1, 3, 4). The two-dimensional gel electrophoresis studies of Jones et al. (5, 6) has suggested that the *I-E* subregion molecule is formed by complementation of genes mapping in the *I-A* and *I-E* subregions. Recent tryptic peptide studies by Cook et al. (7) showed considerable heterogeneity among the beta chains of I-E molecules from recombinants B10.A(*A^kE^k*), B10.A(5R) (*A^bE^k*), and B10.S(9R) (*A^sE^k*), whereas little or no heterogeneity was found among the alpha chains. The above studies suggest that I-E molecules are hybrid molecules with a beta chain being coded for by a gene mapping in the *I-A* subregion (E_{β}) and the alpha chain coded by a gene mapping in the *I-E* subregion (E_{α}).

The first suggestion of the existence of hybrid *I*-region determinants was shown by Fathman et al. (8, 9) in mixed lymphocyte reaction (MLR)¹ studies. They showed unique MLR-stimulating determinant on F₁ cells that were not present on cells from either parent. These unique MLR determinants appear to result from the interaction of genes controlled by two or more loci within the *I* region of the *H-2* gene complex. Recently studies by Schwartz et al. (10) indicate that immune response to poly(Glu⁵⁵Lys³⁶Phe⁹) (GL ϕ) antigen may be mediated by unique hybrid or complementing gene products coded by genes in the *I-A* and the *I-E* subregions. Hence, these complementing or hybrid Ia gene products can function as stimulators in MLR as well as in immune recognition to specific antigens.

Although the MLR, immune response, and biochemical studies have suggested the existence of unique hybrid Ia antigens, no serological evidence has been presented. We became interested in the possibility that Ia specificities 22 (*E^k*) and 23 (*E^d*) may be determinants generated by hybrid Ia molecules, when specific immunizations (B10.D2 \times B10)F₁ anti-B10.A(3R) and [B10.A(5R) \times D2.GD]F₁ anti-B10.D2 failed

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¹ Abbreviations used in this paper: GL ϕ , poly(Glu⁵⁵Lys³⁶Phe⁹); LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction(s); SDS, sodium dodecyl sulfate.

to yield anti-Ia.22 and anti-Ia.23 antibodies after multiple immunizations. In this study we present serological and biochemical evidence that Ia.22 and Ia.23 are present on hybrid Ia molecules from F₁ animals but not expressed in either parent.

Materials and Methods

Animals. All mice used in these studies were produced and maintained in our Immunogenetics mouse colony (Mayo Clinic, Rochester, Minn.). We used (B10 × B10.D2)F₁ animals for the detection of specificity Ia.22 and [D2.GD × B10.A(5R)]F₁ animals for the detection of Ia.23. H-2 haplotypes of recombinant strains used in this study are noted in Table I.

Antisera. The antisera were produced in our laboratory as previously described (11). To detect Ia.22, the antiserum (C3H.Q × B10.D2)F₁ anti-B10.AQR was used in most of these studies. This antiserum, besides anti-Ia.22 activity, also contains activity against the *I-A^k* subregion. However, it is negative with both the parental haplotypes, B10 (*H-2^b*) and B10.D2 (*H-2^d*) from which the F₁ was produced. Specificity and mapping of Ia.22 is defined by the activity of this antiserum with B10.A(3R) (*K^bA^bB^bJ^bE^kC^dS^dD^d*). For Ia.23 we used the antiserum (B10.LG × C3H/Hej)F₁ anti-C3H.OL. This antiserum is specific for Ia.23. Even though this recipient-donor combination has incompatibility for the *I-A* subregion, it has no activity against the *I-A^d*-coded antigens. The antiserum is negative on both the parental strains, D2.GD and B10.A(5R), used to produce the F₁ animals. As an added cautionary measure the anti-Ia.22 was absorbed with B10.A(4R) (*I-A^k*) to make it specific for Ia.22, and anti-Ia.23 was absorbed with D2.GD (*I-A^d*) to make it specific for Ia.23. The following antisera were also used: for anti-Ia.22, (B10 × HTI)F₁ anti-B10.A(5R) absorbed with B10.D2 cells and [B10.A(4R) × B6]F₁ anti-B10.A(2R) absorbed with B10.D2 cells; for anti-Ia.23, (D2.GD × B10)F₁ anti-B10.BDR-1 absorbed with B10.K and (B10 × A)F₁ anti-B10.D2 absorbed with D2.GD.

Cytotoxic Tests. Serological tests for the detection of hybrid Ia antigens were done by a microcytotoxic dye-exclusion assay as previously described (12). The target cells were either fresh spleen cells or 72-h cultures of lipopolysaccharide (LPS)-stimulated spleen blasts. LPS blasts were prepared as previously described (13). In vitro absorptions were performed by incubating 250 μl of 1:10 dilution of the antisera with 100 million fresh spleen cells or LPS spleen blasts for 1 h.

Immunoprecipitation and Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis. F₁ and parental LPS-stimulated blast cells were prepared by culturing 48-h LPS spleen cells in RPMI-1640 media (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 50 μg/ml LPS as previously described (13). The LPS-stimulated blast cells were radiolabeled in RPMI-1640 media without leucine supplemented with glutamine, penicillin (100 U), streptomycin (100 U), 5% fetal calf serum, and 30 μCi/ml [³H]leucine. The LPS blasts were radiolabeled at 37°C in a 5% CO₂ incubator for 12 h. The radiolabeled cells were then solubilized with 0.50% Triton X-100 (Rohm and Haas, Philadelphia, Pa.) in Tris-buffered saline, centrifuged, and the extract was then purified by lentil lectin affinity chromatography. The lentil lectin-purified extracts were tested with anti-Ia.22 and anti-Ia.23 sera with standard immunoprecipitation and SDS-polyacrylamide gel electrophoresis procedures previously described (14).

Sequential immunoprecipitations were done by pretreating radiolabeled extracts overnight with either excess normal mouse serum or antiserum. After removal of immune complexes with *Staphylococcus aureus*, the extracts were divided and subjected to second immunoprecipitations overnight with various test antisera. The antigen-antibody complexes were removed with *S. aureus*, and the Ia molecules were analyzed by SDS gel electrophoresis.

Results and Discussion

Serological Identification. Initial cytotoxic studies carried out with fresh spleen cells from F₁ animals showed only weak reactions. All the cytotoxic tests reported in this manuscript was done with LPS-stimulated spleen blasts to enrich for B lymphocytes as well as to enhance lysis. Viable blast cells from 48- to 72-h cultures were separated from dead cells by layering on Ficoll-Isopaque (Pharmacia Fine Chemicals, Div. of

TABLE I
H-2 Regions of Recombinant Strains Used

Strain	Haplotype	K	A	B	J	E	C	S	D	TL
D2.GD	g2	d	d	b	b	b	b	b	b	b
B10.A(5R)	i5	b	b	b	k	k	d	d	d	a
B10.A(3R)	i3	b	b	b	b	k	d	d	d	a
B10.S(9R)	t4	s	s	?	k	k	d	d	d	a
B10.HTT	t3	s	s	s	s	k	k	k	d	c
B10.LG	ar1	d	f	f	f	f	f	f	?	?
C3H.OL	o1	d	d	d	d	d	d	k	k	b
B10.AQR	y1	q	k	k	k	k	d	d	d	a
B10.BDR-1	g4	d	d	d	d	d	d	d	b	b

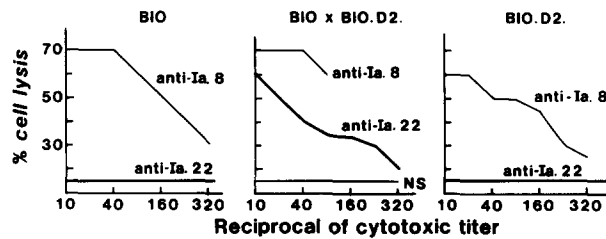


FIG. 1. Microcytotoxic reactions of anti-Ia.22 [(C3H.Q × B10.D2)_{F1} anti-B10.AQR] against B10, (B10 × B10.D2)_{F1}, and B10.D2 LPS-stimulated spleen lymphocytes. Anti-Ia.8 is a positive control [(B10.A × A) anti-B10.A(5R)].

Pharmacia, Inc., Piscataway, N. J.). As shown in Fig. 1, LPS spleen blasts from (B10 × B10.D2)_{F1}, B10, and B10.D2 were tested with anti-22 sera. The antiserum (C3H.Q × B10.D2)_{F1} anti-B10.AQR killed ~50–60% of the _{F1} LPS blasts up to a titer of 1 in 160, whereas no lysis was obtained with the two parental cells. All three targets were uniformly lysed by anti-Ia.8 (*I-A*). These results indicated that Ia.22 is expressed on the hybrid molecules generated by the complementation of *A^b* with *E^d*. Several more _{F1} combinations and antisera were tested to confirm this observation (Table II). A second _{F1} combination involving the same haplotype combination (B6 × DBA/2)_{F1} gave identical results. An _{F1} (D2.GD × B6) failed to show the expression of Ia.22, which suggested no complementation between *A^d*-*E^b*. These results indicate that expression of Ia.22 in B10.A(5R) is a result of *A^b* and *E^k* complementation. B10.S(9R) (*A^sE^k*) as well as (B10.S × B10.D2)_{F1} were positive for Ia.22, which suggested complementation of *A^s*-*E^k* and *A^s*-*E^d*. In the *H-2^k* haplotype the complementation must be between *A^k* and *E^k*. Other combinations, (C3H.Q × B10.D2)_{F1} and (A.CA × B10.D2)_{F1}, were negative for Ia.22, which suggested noncomplementation of *A^q*-*E^d* and *A^f*-*E^d*.

As shown in Fig. 2, both D2.GD and B10.A(5R) LPS spleen blasts are negative for Ia.23. Yet, the _{F1} LPS spleen blasts from [D2.GD × B10.A(5R)] gave strong positive reactions. Hence, Ia.23 can be generated by the complementation of *A^d* with *E^k*. Again, identical expression of Ia.8 (*I-A*) was seen in all three targets. As shown in Table II, absorbed anti-Ia.23 sera gave similar results. (D2.GD × B6)_{F1} and (B10.LG × C3H)_{F1} did not express Ia.23. These results suggest that *A^d*-*E^b* and *A^f*-*E^k* do not

TABLE II
Expression of Ia.22 and 23*

Target cells‡	C3H.Q × B10.D2§, anti- B10.AQR, [Abs.B10. A(4R)]	B6 × B10.A(4R) §, anti- B10.A(2R), [Abs. B10.D2]	B10.LG × C3H , anti- C3H.OL, [Abs.D2.G D]	D2.GD × B10 , anti- B10.BDR- 1, [Abs.B10. K]
B10	0	0	—	—
B10.D2	0	0	—	—
(B10 × B10.D2)F ₁	80	80	—	—
(B6 × DBA/2)F ₁	40	40	—	—
(B10.S × B10.D2)F ₁	80	80	—	—
B10.S	0	0	—	—
B10.S(9R)	80	80	—	—
B10.A(5R)	80	80	0	0
D2.GD	—	—	0	0
[D2.GD × B10.A(5R)]F ₁	—	—	40	40
B10.P	—	—	0	0
(D2.GD × B10.P)F ₁	—	—	20	20

* Reciprocal titers from microcytotoxic test.

‡ Targets are LPS-stimulated spleen lymphocytes.

§ Absorbed (Abs.) antisera made specific for Ia.22.

|| Absorbed antisera made specific for Ia.23.

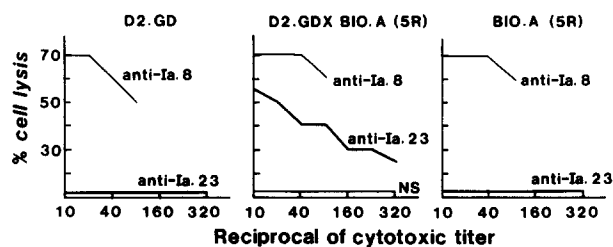


FIG. 2. Microcytotoxic reactions of anti-Ia.23 [(B10.LG × C3H)F₁ anti-C3H.OL] against D2.GD, [D2.GD × B10.A(5R)]F₁, and B10.A(5R) LPS-stimulated spleen lymphocytes.

complement. On the other hand, (D2.GD × B10.P)F₁ was positive for Ia.23, which suggested *A^d-E^p* complementation.

In an attempt to test whether the anti-Ia.22 and anti-Ia.23 sera were detecting the same hybrid Ia determinant in the independent haplotype (homologous ciscomplementation), recombinant (nonhomologous ciscomplementation), and in the F₁ hybrids (transcomplementation), cross-absorptions were done. Anti-Ia.22 was absorbed with B10.K, B10.A(3R), and (B10 × B10.D2)F₁ LPS spleen blasts and then tested with LPS spleen blasts from these strains as targets. Each cell type completely absorbed for each other. Similarly, B10.D2 and [D2.GD × B10.A(5R)]F₁ cells were used to absorb anti-Ia.23 and tested against both the cell types. Again, complete absorption was seen. These results suggest that the antisera were detecting the same determinant in all three types of complementation.

Biochemical Identification. To confirm the expression of Ia.22 on (B10 × B10.D2)F₁ cells and Ia.23 on [D2.GD × B10.A(5R)] cells, F₁ and parental spleen LPS blasts were radiolabeled, and the immunoprecipitated antigens were examined by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3, anti-Ia.22 serum immunoprecipitated

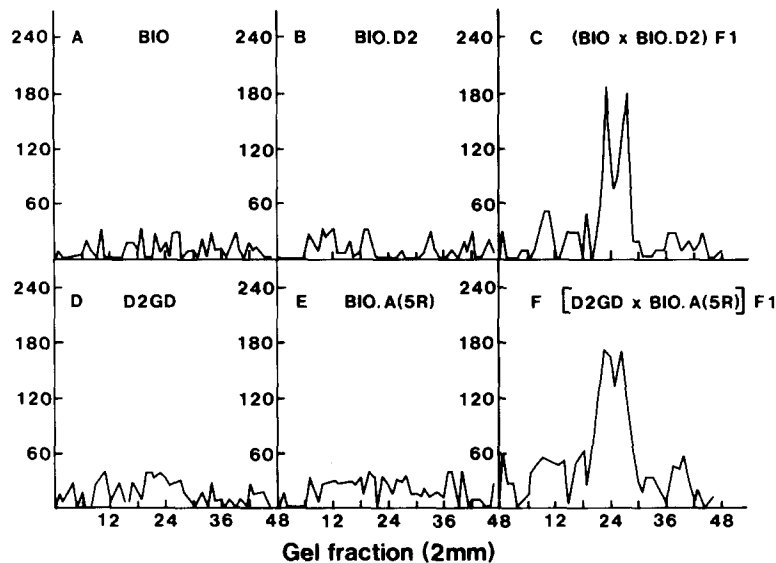


FIG. 3. SDS-polyacrylamide gel patterns of molecules precipitated with anti-Ia.22 serum, (C3H.Q \times B10.D2) F_1 anti-B10.AQR (panels A, B, C) and anti-Ia.23 serum, (B10.LG \times C3H) F_1 anti-C3H.OL (panels D, E, F). In each analysis, the antigen preparation was from radiolabeled LPS-stimulated lymphocytes.

typical Ia peaks from the (B10 \times B10.D2) F_1 extract but not from either B10 or B10.D2 extracts. Similarly anti-Ia.23 serum immunoprecipitated Ia molecules from [D2.GD \times B10.A(5R)] F_1 extracts but not from D2.GD or B10.A(5R) extracts. These results indicate that the cytotoxic activity of anti-Ia.22 against (B10 \times B10.D2) F_1 LPS blasts and anti-Ia.23 against [D2.GD \times B10.A(5R)] F_1 LPS blasts is due to the expression of hybrid Ia molecules on these cells.

With F_1 mice, two types of I-E molecules are possible. For example, with (B10 \times B10.D2) F_1 , A^dE^d (Ia.23) molecules generated by ciscomplementation and A^bE^d (Ia.22) molecules generated by transcomplementation are possible. Sequential immunoprecipitations were done to see if both are present. As shown in Fig. 4, pretreatment of (B10 \times B10.D2) F_1 extracts with excess anti-Ia.23 serum cleared the extract of Ia molecules immunoprecipitated by anti-Ia.23 serum, but had no effect on molecules immunoprecipitated by anti-Ia.22 serum. This shows that there are two separate I-E molecules in (B10 \times B10.D2) F_1 . Both of these molecules are Ia.7 positive, because pretreatment with anti-Ia.7 serum cleared for molecules immunoprecipitated by anti-Ia.22 and anti-Ia.23 sera.

The expression of Ia.22 on (B10 \times B10.D2) F_1 cells most probably results from complementation of beta chain from A^b and alpha chain from E^d . This is a transcomplementation in contrast to the ciscomplementation seen in B10.A(3R) and B10.A(5R). Specificity Ia.22 in B10.A(3R) and B10.A(5R) is generated by a complementation between $A_e^b(E_\beta)$ and E_α^k rather than $A_e^b(E_\beta)$ and E_α^d shown here. Ia.22 determinant can also be generated by transcomplementation between $A_e^s(E_\beta)$ and E_α^d (B10.S \times B10.D2) F_1 or by ciscomplementation between $A_e^s(E_\beta)$ and E_α^k [B10.S(9R) and B10.HTT]. Ia.23 can be generated by complementation between the beta chain of A^d and alpha chain of E^k [D2.GD \times B10.A(5R)] F_1 . Schwartz et al. (10) showed

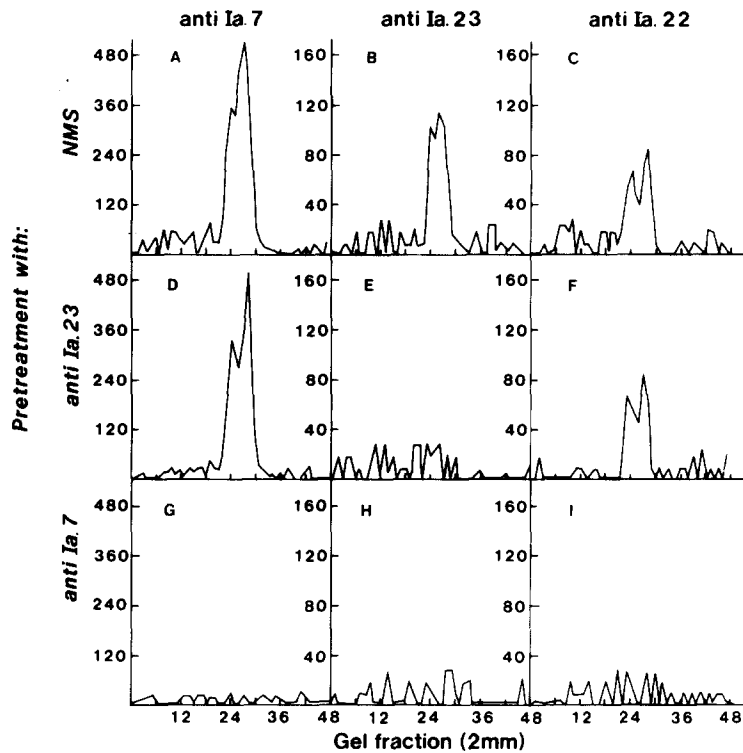


FIG. 4. Sequential immunoprecipitation analysis of Ia antigens from a (B10 × B10.D2) F_1 antigen preparation. Aliquots were pretreated with normal mouse serum (NMS) (panels A, B, C); anti-Ia.23 serum, (B10.LG × C3H) F_1 anti-C3H.OL (panels D, E, F); and anti-Ia.7 serum (D2.GD × B6) F_1 anti-B10.HTG (panels G, H, I). The immune complexes were removed with protein-A-bearing *S. aureus*. The supernates were then divided into thirds, and each third was subjected to a second precipitation with anti-Ia.7 (panels A, D, G), anti-Ia.23, panels (B, E, H), or anti-Ia.22, (panels C, F, I).

that most Ia.7 (*I-E*)-positive strains can complement with *I-A^d* or *I-A^b* for response to $GL\phi$ antigen. We tested a second F_1 combination (D2.GD × B10.P) and found complementation for Ia.23. Studies with other Ia.7-positive strains are underway. Our studies would suggest that Ia.23 is involved in the response to $GL\phi$ antigen. These results suggest that Ia.7 is present on the alpha chain coded by the *I-E* subregion, whereas Ia.22 and Ia.23 are expressed either on the beta chain coded by the *I-A* subregions or generated by the hybrid molecule.

Summary

Ia specificities 22 and 23 were found to be determinants on hybrid Ia molecules by serological and biochemical studies. Lipopolysaccharide-stimulated splenic lymphocytes from (B10 × B10.D2) F_1 expressed Ia.22 although both the parents were negative. Similarly [D2.GD × B10.A(5R)] F_1 cells expressed Ia.23, whereas D2.GD and B10.A(5R) lacked it. Ia.22 can be generated by gene complementation of A^k-E^k , A^b-E^d , A^b-E^k , A^s-E^d , and A^s-E^k , whereas Ia.23 can be generated by A^d-E^d , A^d-E^k , and A^d-E^p . Other possible complementing combinations are under study. The role of Ia.22 and 23 in mixed lymphocyte reactions and immune responses are discussed.

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