

SEROLOGIC AND FUNCTIONAL CHARACTERIZATION OF A PANEL OF ANTIGEN-PRESENTING CELL LINES EXPRESSING MUTANT I-A CLASS II MOLECULES*

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In contrast to the large number of spontaneous mutations that have been detected in the *H-2K* and *H-2D* genes by reciprocal skin-grafting studies (1, 2), only one spontaneous mutation of a gene for an Ia (class II) molecule, that in the B6.C-H-2^{bm12} (bm12), has been identified (3). Results of studies using the T cells and antigen-presenting cells (APC) from this mutant mouse strain and from the strain from which the mutant arose (C57BL/6) have provided some of the strongest evidence that Ia molecules act as restriction elements for the presentation of antigens to T cells and that Ia molecules are the products of *Ir* genes (4, 5). The further analysis of the mechanisms by which Ia molecules mediate "restriction" in cellular interactions and *Ir* gene function would be aided by the development of a larger number of *I* region mutants.

We have recently described (6) a method for the selection of mutant cells with altered Ia antigenic determinants from a cloned population of B lymphoma-B cell hybridoma cells that possess potent antigen-presenting activity. Lipopolysaccharide (LPS)¹-stimulated B cells from (BALB/c × A/J)F₁ (CAF₁) donors were fused with cells from the M12.4.1 BALB/c B lymphoma cell line. The resulting hybridoma, TA3, retained the antigen-presenting activity of the parental B lymphoma line and expressed Ia antigens and *Ir* gene-determined antigen-presenting properties of the A/J type. Mutants of TA3 were obtained by exposing them to ethylmethane sulfonate and subjecting the mutagenized cells to cytotoxic elimination with a monoclonal anti-(α)-I-A^k antibody, followed by selection of cells that continued to express other I^k-encoded determinants by electronic cell sorting using a different monoclonal antibody (positive immunoselection). We obtained mutants of two distinct types. One appeared to have undergone a fairly limited alteration since it lost only some of the I-A^k antigenic determinants. Only a single example of this type was obtained (A8). The second type, represented

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¹ *Abbreviations used in this paper:* APC, antigen-presenting cell; C, complement; ECS, electronic cell sorting; EMS, ethylmethane sulfonate; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FMF, flow microfluorometry; GαMlg, goat anti-mouse γ₂ Fc fragment; GAT, poly(L-Glu⁶⁰,L-Ala³⁰,L-Tyr¹⁰); OVA, ovalbumin; PPD, purified protein derivative; HEL, hen egg lysozyme; HGPRT, hypoxanthine guanosine phosphoribosyl transferase; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; mAb, monoclonal antibody; OVA, ovalbumin; PPD, purified protein derivative.

by No. 90, appeared to have lost expression of the entire I-A^k molecule but to have retained the I-E molecule. Functional studies with A8 demonstrated that the loss of a limited number of determinants on the I-A^k molecules of this cell occurred together with the loss of a specific I-A^k-encoded restriction element. These results provided further support for the identification of Ia molecules as restriction elements in antigen recognition by T cells and as the products of *Ir* genes.

In this paper, we present the serologic and functional characterization of a panel of mutants prepared from TA3 by a minor modification of the previously described methods. The serologic phenotype of these mutants was investigated with a large panel of monoclonal anti-I-A^k antibodies. The mutants were then screened for their ability to activate a panel of cloned antigen-specific and autoreactive T cell hybridomas. The data generated from these studies enable us to begin to correlate changes in function with changes in the antigenic structure of the Ia molecule.

Materials and Methods

Mice. B10.A mice were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB/cAnN and CAF₁ mice were obtained from the Division of Research Services, National Institutes of Health.

Antigens. Hen egg lysozyme (HEL), purified by chromatography on the weak-cation-exchange resin Biorex 70 (7), was the kind gift of Dr. Eli Sercarz, University of California at Los Angeles and was used at 10 µg/ml in vitro. The random polymer of L-Glu⁶⁰, L-Ala³⁰, and L-Tyr¹⁰ (GAT), purchased from Vega Biochemicals, Tucson, AZ, was provided by Dr. J. Ashwell, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases and was used at 50 µg/ml. Ovalbumin (OVA), purchased from Miles-Yeda, Ltd., Rehovoth, Israel or from Sigma Chemical Co., St. Louis, MO was used at 100 µg/mouse for immunization and at 100–500 µg/ml in culture. Purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Connaught Medical Research Laboratory, Willowdale, Ontario, Canada) was used in culture at 20 µg/ml. Keyhole limpet hemocyanin (KLH) (Calbichem-Behring Corp., La Jolla, CA) was used at 100 µg/ml.

Monoclonal Anti-Ia Antibodies. The following monoclonal antibodies (mAb) were used in these studies: 26.7.11 (anti-I-A^k) and 17.3.3 (anti-I-E^k) were produced by Ozato et al. and have been described (8, 9); 10.2.16 (anti-I-A^k) was produced by Oi et al. (10); a panel of mAb (8C, 39A, 39E, 39B, 39J, 39C, 40A, 40J, and 40N) specific for I-A^k-encoded determinants was the kind gift of M. Pierres (11).

B Lymphomas and B Cell-B Lymphoma Hybridomas. M12.4 is an Ia-positive BALB/c B lymphoma originally transferred into tissue culture and cloned by Kim et al. (12). M12.4.1 is a hypoxanthine guanosine phosphoribosyl transferase (HGPRT)-deficient variant of the M12.4 line prepared by Hamano et al. (13) by selection in the presence of 6-thioguanine after mutagenesis with ethylmethane sulfonate (EMS). The production of the TA3 B cell-B lymphoma hybridoma by fusion of LPS-activated spleen cells from CAF₁ mice with M12.4.1 has been described (6).

Selection of Ia Mutants from the TA3 Hybridoma. 5×10^6 TA3 cells were cultured in 25 ml of complete media with 5 µl of EMS (Sigma Chemical Co.); 16 h later the cells were harvested and washed three times to remove the EMS. The dose of EMS used killed between 30 and 50% of the cells. The remaining cells were allowed to expand; 4–10 d later, they were subjected to immune lysis with the 10.2.16 or 26.7.11 anti-I-A^k mAb by incubating the cells at 10^7 /ml with a 1:10 dilution of 10.2.16 culture supernatant or a 1:100 dilution of 26.7.11 ascites for 30 min at 4°C followed by a 30 min incubation at 37°C with complement (C) (Cedarlane Laboratories, Hornby, Ontario). This resulted in the death of >99% of the cells. The few remaining cells were passed over a Ficoll gradient to eliminate dead cells, and allowed to expand. These cells were then subjected to a

positive selection by electronic cell sorting (ECS) with 26.7.11 (for cells initially treated with 10.2.16 and C) or 10.2.16 (for cells initially treated with 26.7.11 and C) as described below. The selected cells were then expanded and subjected to a second round of positive selection by ECS with 26.7.11 or 10.2.16, followed by a second negative selection with 10.2.16 or 26.7.11 and C to eliminate wild-type cells before cloning. Cells were cloned by limiting dilution in 96-well microtiter plates on a bed of thymocytes as feeder cells. After 7–10 d, cells were transferred to 24-well Costar plates (Costar, Cambridge, MA), expanded, and analyzed by flow microfluorometry (FMF).

FMF Analysis and ECS. FMF analysis and ECS were performed as previously described (14, 15) using a FACS II Dual Laser System (B-D FACS Systems, Sunnyvale, CA). Cells to be analyzed by FMF were electronically selected for viability on the basis of forward light scatter intensity as well as by propidium iodide exclusion as measured in the red fluorescence detector of the FACS II after laser excitation at 568 nm. All fluorescence data were obtained using logarithmic amplification; information on $2\text{--}4 \times 10^5$ viable cells were collected in the green fluorescence detector after excitation at 488 nm. Data on individual cells were stored and analyzed using a PDP 11/34 computer (Digital Equipment Corporation, Maynard, MA) interfaced to the FACS II (16). Data are plotted as number of cells (y axis) versus log fluorescence intensity (x axis).

For FMF analysis, $50 \mu\text{l}$ ($\sim 10^6$) of cells were either incubated with $20 \mu\text{l}$ of a titrated quantity of fluorescein-conjugated 26.7.11 mAb (ammonium sulfate precipitate from ascites) or were incubated with $20 \mu\text{l}$ of 10.2.16 or 17.3.3 culture supernatants or of purified mAb of the group derived by Pierres et al. (11), followed by reaction with a fluorescein-conjugated Fab fragment of an affinity-purified goat anti-mouse γ_2 Fc fragment (FITC-GaMIg) (F/P = 2.6) (gift of Ms. B. J. Fowlkes, Laboratory of Microbial Immunity, NIAID). All incubation steps were carried out on ice for 20–30 min, followed by two washes with Hank's balanced salt solution containing 3% fetal calf serum (FCS) and 0.1% NaN₃. As a specificity control for staining by the directly fluoresceinated 26.7.11 mAb, we initially used a similarly prepared directly fluoresceinated IgM mAb directed at an I-A^b specificity. Since the fluorescence histogram of TA3 cells with this reagent did not differ from the histogram of unstained TA3 cells, subsequent experiments used unstained cells as the control for the 26.7.11 mAb. The specificity control for staining by anti-Ia mAb followed by fluoresceinated GaMIg was the fluorescence histogram obtained with the fluoresceinated GaMIg reagent alone.

For ECS, cells were passed through the FACS II at a rate of 2,000 total light scatter signals per second. Sorted cells were selected on the basis of ranges of fluorescence intensity that included TA3 cells stained with 26.7.11. Cells were also selected for viability on the basis of forward light scatter intensity. The sorted cells were collected in 5-ml tubes containing a 1.5 ml cushion of complete media with 25% FCS. Immediately after sorting, the cells were reanalyzed by FMF to assess purity, which was between 85 and 95%.

Production of T Cell Hybridomas. The procedure for the production of T cell hybridomas was similar to that described by Kappler et al. (17) and has been presented in detail in a previous report (16). Briefly, antigen-stimulated T cell blasts prepared by a 3 d in vitro stimulation with antigen of lymph node cells from immune B10.A mice were fused with the HGPR^T-negative T cell line BW 5147, an AKR thymoma obtained from the Salk Institute Cell Distribution Center, La Jolla, CA. The GAT-specific hybridoma was the gift of Dr. L. Samelson, Laboratory of Immunology, NIAID; the KLH-specific hybridomas SKK 9.11 and SKK 23 and the OVA-specific hybridoma AODH-3.4 were the kind gifts of Dr. Phillipa Marrack, National Jewish Hospital, Denver, CO. The panel of antigen-specific T cell hybridomas used in these studies is shown in Table I.

Assay of T Cell Hybridomas for Activity. 1×10^5 T hybridoma cells were co-cultured with varying numbers of irradiated (10,000 rad from a ¹³⁷Cesium source) B lymphoma or B cell-B lymphoma hybridoma cells or with 2×10^5 irradiated (2,000 rad) spleen cells in the presence or absence of antigen in 0.2 ml of "fusion" medium, containing hypoxanthine, aminopterin and thymidine. After 2 d of culture, supernatants were collected and assayed for interleukin 2 (IL-2) content in a secondary culture using HT-2 cells, an IL-2-dependent

TABLE I
T Cell Hybridomas

Abbreviation	Antigen specificity	Restriction element
HEL(a)	Hen egg lysozyme	I-A ^k
HEL(b)	Hen egg lysozyme	I-E ^k
GAT	Poly (Glu ⁶⁰ Ala ³⁰ Tyr ¹⁰)	I-A ^k
OVA No. 1	Ovalbumin	I-A ^k
OVA No. 2	Ovalbumin	I-A ^k
AODH-3.6	Ovalbumin	I-A ^k
PPD	Purified protein derivative	I-A ^k
Auto No. 1	Autoreactive	I-A ^k
Auto No. 2	Autoreactive	I-A ^k
Auto No. 3	Autoreactive	I-A ^d
SKK-9.11	Keyhole limpet hemocyanin	I-A ^k
SKK-2.3	Keyhole limpet hemocyanin	I-A ^k
SKK-45.10	Keyhole limpet hemocyanin	I-A ^k

T cell line developed by Dr. James Watson, University of Auckland, Auckland, New Zealand and provided to us by Dr. Phillipa Marrack, or CTLL cells, an IL-2-dependent T cell line originally developed by Dr. Kendall Smith, Dartmouth University, Hanover, NH and provided to us by Mr. T. Briner, Massachusetts Institute of Technology, Cambridge, MA. HT-2 cells ($2-4 \times 10^3$) or CTLL cells ($5-10 \times 10^3$) were cultured for 24 h in the presence of 35% primary culture supernatant and the degree of stimulation measured by the incorporation of [³H]thymidine (6.8 Ci/mmol; New England Nuclear, Boston, MA) into DNA 5-20 h later.

Results

Selection of Ia Mutants. We and others (18, 19) have previously shown that the fusion of normal LPS-activated B cells with the M12.4.1 drug-marked BALB/c B lymphoma results in B cell-B lymphoma (B-B) hybridomas that are potent APC. TA3 is one such hybridoma, prepared by the fusion of M12.4.1 with LPS-activated B cells from a CAF₁ mouse. TA3 displays Ia molecules and restriction elements derived from the B cell partner, in that it bears I-A^k and I-E^k specificities, defined by a panel of monoclonal anti-Ia^k antibodies, and is able to present antigen to Ia^k-restricted T cells. To obtain Ia mutant lines from TA3, we followed a protocol similar to that described for the production of H-2K/D mutants (20, 21) and described in detail by us recently (6). Briefly, TA3 cells treated with the mutagen EMS were subjected to negative immunoselection with the α I-A^k mAb 10.2.16 in the presence of C. The few remaining cells (<1%) were allowed to expand in number and then subjected to positive selection by ECS on the FACS using another α I-A^k mAb, 26.7.11, which detects a I-A specificity distinct from that recognized by 10.2.16. It was from this population of positively selected cells that the initial I-A^k mutant cell line A8 was obtained.

However, to obtain this single mutant line it was necessary to screen 6% clones. To improve our yield in isolating mutants of this type, we subjected this positively selected population, which apparently contained <2% of the desired cell type, to a second round of positive selection by ECS with the 26.7.11 mAb. Analysis of the resulting population revealed that ~80% of the 26.7.11-positive cells were

also positive with 10.2.16 (i.e., appeared to be wild-type cells). This population was therefore subjected to a second round of immune lysis with 10.2.16 and C to eliminate wild-type cells. The fluorescence profile of the resulting cell population is shown in Fig. 1, *left* panels. This population does not appear to contain cells positive for the 10.2.16-defined determinant. ~20% of the cells are positive with the 26.7.11 antibody.

Cells from this doubly selected population were then cloned by limiting dilution. Of 100 clones tested, 19 stained positively with 26.7.11 and 17.3.3 and were weakly reactive with 10.2.16. Examples of such clones, A19 and B18, are shown in Fig. 2 as is the parental cell line, TA3. As we have previously observed in the study of our first such mutant, A8, the 19 lines of this type, upon repeated FMF examination, were often weakly or moderately positive with 10.2.16, suggesting that this determinant had not been completely extinguished. Among the remaining 81 clones, only B3 appeared to be of the wild-type phenotype since it reacted strongly with both the 10.2.16 and 26.7.11 mAb. The other 80 clones reacted with neither the 10.2.16 or 26.7.11 mAb. Further serologic analysis of the 19 mutants that stained strongly with 26.7.11 and weakly with 10.2.16 is presented below.

A second group of mutants was selected by the same approach except that the mAb used for the negative and positive selection steps were reversed. Thus, 26.7.11 was used as the negative immunoselecting agent and 10.2.16 as the positive selecting agent. After two rounds of negative and positive immunoselection, ~90% of the resulting cells stained positively with 10.2.16 and <5% reacted with 26.7.11. The fluorescence histograms of this population are shown in Fig. 1, *right* panels. 19 of 22 clones obtained by cloning this population bore the I-A^k determinant defined by 10.2.16 and lacked the determinant defined by 26.7.11. In contrast to the weak positive reaction detected with the negative selecting

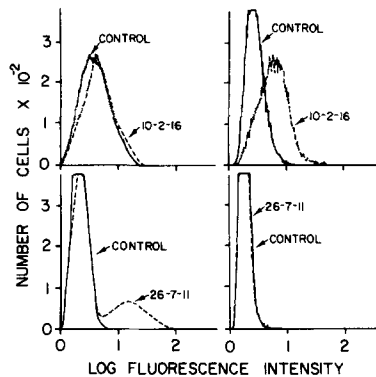


FIGURE 1. FMF analysis of EMS-treated TA3 cells after double negative selection with 10.2.16 plus C and double positive selection with 26.7.11 (*left*) or double negative selection with 26.7.11 plus C and double positive selection with 10.2.16 (*right*). 1×10^6 cells were incubated with 10.2.16 mAb and then with the Fab fragment of FITC-G α M1g and were analyzed by FMF. The 26.7.11 staining was performed with directly fluoresceinated (FITC) 26.7.11 mAb. Control cells were incubated with the Fab fragment of FITC-G α M1g only, or, for comparison with FITC-26.7.11 staining, were unstained. Preliminary experiments comparing cells stained with a directly fluoresceinated α 1-A^b mAb revealed no difference in fluorescence profiles from unstained cells.

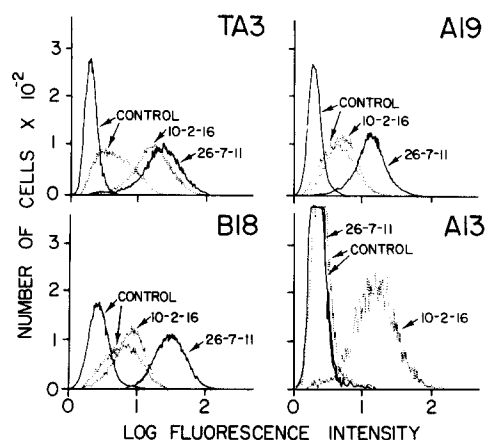


FIGURE 2. FMF analysis of TA3 cells, type A mutants A19 and B18, and type B mutant A13. Solid lines show results of cells stained with FITC-26.7.11 or of unstained cells. Dotted lines show results of cells stained with 10.2.16 followed by FITC-G α MIg or of control cells stained with FITC-G α MIg only.

TABLE II
Reactivity Pattern of TA3 Mutants with a Panel of α I-A^k mAb

Antigen-presenting cell line	α I-A ^k mAb directed at determinants in four epitope groups								
	40A	40N	39B*	39E	39C	39J	40J	39A	8C
TA3	+++	+++	+++	+++	+++	+++	+	+++	+
A8	-	-	++	+/-	+++	+++	+	+++	+
Type A mutants	-	-	++	+/-	+++	+++	+	+++	+
Type B mutants	+++	+++	++	+++	-	-	-	+++	+
No. 90	-	-	-	-	-	-	-	+	-

FMF analysis of the designated APC with the series of α I-A^k mAb described by Pierres et al. (17). Results are presented semiquantitatively: (-) no staining above background, (+) weak staining above background, (++) intermediate staining above background, (+++) strong staining above background, (+/-) weak staining obtained in some but not all experiments.

*Staining with 39B varied in intensity, ranging from +/- to +++.

agent, 10.2.16, on the first group of mutants, this second group of mutants did not display any reactivity with 26.7.11, the mAb used for its negative selection. An example of one of these 19 mutants, A13, is shown in Fig. 2. 2 of the 22 clones resulting from this selection appeared to be of wild-type phenotype and 1 clone was negative for both I-A^k-encoded determinants. The first group of mutants discussed above (represented by A8, A19, and B18) will be referred to as type A mutants while the second group, exemplified by A13, will be referred to as type B mutants.

Further Serologic Characterization of Type A and Type B Mutants. Recently Pierres et al. (11) have produced 17 mAb specific for determinants on the I-A^k molecule. These mAb appear to define five different epitope groups on the I-A^k molecule, as determined by cross-blocking studies. We examined the serologic profile of the 19 type A mutants with representative mAb from four of these five groups. Table II demonstrates that, similar to the results obtained with A8, a determi-

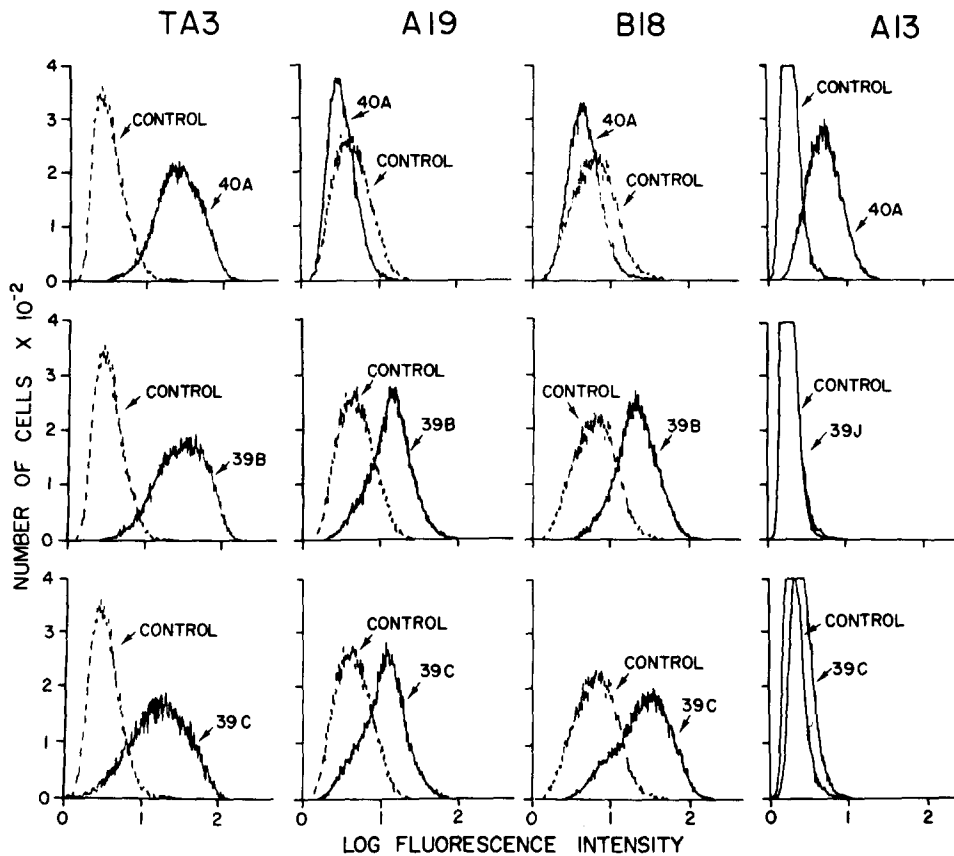


FIGURE 3. FMF analysis of TA3 cells, type A mutants A19 and B18, and type B mutant A13 with selected α -I-A^k mAb. The 40 A and 39B mAb are in Pierres' (11) epitope group I and the 39C and 39J mAb in epitope group II. Staining procedures were the same as used for the 10.2.16 mAb. Control cells were reacted with FITC-G α M1g only.

nant(s) recognized by two mAb (40A and 40N) of the group defined by 40A, 40N, 39B, and 39E, is absent on the surface of all of these mutants. The third antibody of this group, 39B, often reacts strongly with the mutant cells and the fourth, 39E, resembles 10.2.16 in that it reacts weakly with the mutant cells. The antigenic determinants defined by mAb of the remaining three groups appear to be present on the 19 type A mutant cell lines at levels equal to their expression on TA3 cells. Fig. 3 illustrates FMF patterns of two type A mutants with three of these mAb.

We have observed that there is considerable variability in the intensity of the staining of type A mutants with 39B. 39B shows variability in staining of type A mutants both from mutant to mutant and in the same mutant line from day to day, ranging from moderately reactive to strongly positive. As already noted, 10.2.16 also displays variability in its degree of staining of type A mutants, although of a more modest degree. Neither 40A nor 40N, which are in the same epitope group as 39B, display such variability. They uniformly fail to react with type A mutants. Similarly, the mAb of the other groups show quite reproducible

positive reactions with type A mutants.

The serologic profiles of all the 19 type A mutants, therefore, appear to be very similar. The results suggest that the mutation(s) may involve the alteration or loss of a portion of one epitope of the I-A^k class II molecule.

The panel of anti-I-A^k mAb was also used to examine two of the type B mutants (A5 and A13). The serologic profiles of the type B mutants are shown in Table II; Fig. 3 illustrates FMF profiles of A13. The determinant(s) recognized by mAb of the 40A, 40N, 39B, and 39E epitope group as well as the determinants defined by the 8C and 39A mAb are present on A13 at levels equal to their expression on TA3 cells. A determinant(s) defined by the three mAb (39C, 39J, 40J) of the second epitope group is absent on the surface of A5 and A13. The type B mutation(s) thus appear to involve the loss or alteration of a different epitope of the I-A^k class II molecule than is affected in the type A mutants. Furthermore, it appears to involve an entire epitope as opposed to a portion of one.

Mutant 90 has been described previously. Based on its failure to react with both the 10.2.16 and 26.7.11 mAb as well as with any of the Pierres' (11) mAb except 39A, we concluded that it had probably lost expression of the I-A^k class II molecule. The weak reaction of 39A with mutant 90 is not surprising since this mAb has been shown to cross-react weakly on the I-A^d-encoded class II molecule. Mutant 89, used in some experiments, is phenotypically similar to No. 90. Both Nos. 89 and 90 react with 17.3.3, indicating that they express an I-E^k-encoded class II molecule.

Functional Analysis of the Mutants. To test the functional activity of the mutant lines, we assembled a panel of cloned B10.A T cell hybridomas specific for a variety of foreign antigens in the context of I-A^k-encoded restriction elements. A list of these hybridomas is shown in Table I. Selected type A mutants were tested against a panel of antigen-specific and autoreactive I-A^k-restricted T cell hybrids to determine the extent of the loss of APC function that had occurred with the alteration of the Ia molecule. Table III demonstrates that all of these type A mutants are effective in stimulating three such I-A^k-restricted T cell hybridomas, two of which were specific for KLH and one for OVA. In contrast, none of these mutants stimulated responses of five additional I-A^k-restricted antigen-specific T cell hybridomas, including hybridomas specific for HEL, OVA, PPD, and KLH. Each mutant was active in presenting HEL to the I-E^k-restricted T cell hybridoma HEL(b).

Although these results suggested that the type A mutants were similar to one another functionally as well as antigenically, a study of their capacity to stimulate three additional hybridomas raises the possibility that the mutants are different from one another. A8, A19, and B13 stimulated responses by an I-A^k-restricted GAT-specific T cell hybridoma and by two I-A^k-specific autoreactive hybridomas. By contrast, B18 and C13 were either weakly or nonstimulatory for these hybridomas. All of these lines stimulated an autoreactive T cell hybridoma specific for an I-A^d determinant (Table IV).

We next examined the antigen-presenting activity of several of the type B mutants, including A5, A2, and A13. The results are shown in Table V. These mutants failed to present antigen to any of the panel of I-A^k-restricted antigen-

TABLE III
Antigen Presentation by Type A Mutants to a Panel of T Cell Hybridomas

T Cell Hybridoma	Specificity	APC Line					
		TA3	A8	A19	B13	B18	C13
HEL(a)	HEL:I-A ^k	34,400	0	0	0	132	81
OVA No. 1	OVA:I-A ^k	14,316	480	628	276	0	0
OVA No. 2	OVA:I-A ^k	84,586	1,864	374	1,032	985	972
PPD	PPD:I-A ^k	29,727	85	220	135	0	0
SKK 9.11	KLH:I-A ^k	69,798	NT	283	288	155	NT
SKK 2.3	KLH:I-A ^k	26,924	NT	11,805	26,238	8,596	NT
SKK 45.10	KLH:I-A ^k	124,464	NT	101,068	114,068	56,440	NT
AODH 3.4	OVA:I-A ^k	74,779	NT	60,294	23,432	14,213	NT
HEL(b)	HEL:I-E ^k	76,714	104,554	68,841	74,082	84,406	101,012

10⁵ T hybridoma cells were co-cultured with the designated irradiated (10,000 rad from a ¹³⁷Cesium source) APC in the presence or absence of the relevant antigen, HEL (10 µg/ml), OVA (300 µg/ml for OVA Nos. 1 and 2; 500 µg/ml for AODH 3.4), PPD (25 µg/ml), KLH (500 µg/ml), in 0.2 ml of complete medium. After 1–2 d of culture, supernatants were collected and assayed for IL-2 content in a secondary culture of HT-2 or CTLL cells. 2–10 × 10⁵ HT-2 or CTLL cells were cultured for 24 h in the presence of 35% primary culture supernatant and the degree of stimulation measured by the incorporation of [³H]thymidine into DNA. Results are expressed as cpm stimulated by supernatants obtained in the presence of antigen minus that obtained in the absence of antigen. Varying numbers of APC of each type were used. Only the maximum response obtained is shown. These were generally achieved with 10⁴ APC per well. NT, not tested.

TABLE IV
Two Auto-I-A^k-reactive and One Antigen-specific I-A^k-restricted T Cell Hybridoma Can Be Activated by Only Some Type A Mutant APC

APC Line	T cell hybridoma			
	Auto No. 1 (I-A ^k)	Auto No. 2 (I-A ^k)	GAT (GAT:I-A ^k)	Auto No. 3 (I-A ^k)
TA3	52,903	105,668	27,432	35,508
No. 90	4,979	1,533	0	NT
A8	25,124	53,352	17,351	34,073
A19	40,317	73,739	31,459	6,211
B13	32,124	64,514	39,664	35,908
B18	879	13,586	302	18,607
C13	202	265	849	70,899

Assay performed as detailed in legend to Table III. APC were used at several cell numbers in these cultures, generally 10³, 10⁴, and 10⁵. Only the maximum Δcpm is presented here. GAT was used at 50 µg/ml. NT, not tested.

specific T cell hybridomas or to stimulate the autoreactive I-A^k-specific T cell hybridomas over a wide range of numbers of APC (only the 10⁴ dose is shown here, for simplicity). They were able, however, to use their I-E^k-encoded restriction elements to activate the HEL-specific I-E^k-restricted T cell hybridoma HEL(b), indicating that type B mutation(s) had not affected a more generalized antigen-presentation function. The functional results obtained with the type A and B mutants are summarized in Table VI.

TABLE V
Antigen Presentation by Type B Mutants to a Panel of T Cell Hybridomas

T Cell hybridoma	Specificity	APC line			
		TA3	A5	A2	A13
HEL(a)	HEL:I-A ^k	107,524	642	107	90
OVA No. 1	OVA:I-A ^k	89,389	168	216	118
SKK 9.11	KLH:I-A ^k	26,804	146	46	60
SKK 2.3	KLH:I-A ^k	20,050	50	131	35
SKK 45.10	KLH:I-A ^k	64,169	286	407	109
AODH 3.4	OVA:I-A ^k	31,121	329	162	184
GAT	GAT:I-A ^k	75,177	468	417	NT
Auto No. 1	I-A ^k	52,903	704	NT	NT
Auto No. 2	I-A ^k	53,123	206	NT	675
Auto No. 3	I-A ^d	63,523	47,044	68,483	19,421
HEL(b)	HEL:I-E ^k	116,762	111,146	95,039	89,233

Assay performed as detailed in legend to Tables III and IV. Results are expressed as cpm stimulated by supernatants obtained in the presence of antigen minus cpm stimulated by supernatants obtained in the absence of antigen. NT, not tested.

Discussion

We have recently described (6) a protocol that allows the selection and propagation of hybrid cells with Ia mutations from a homogeneous population of functional APC. In this paper we have used a modification of that method to allow us to select more such limited loss mutants. The biochemical nature of the alterations that have occurred in these mutants is not yet known, but we can tentatively attribute the type A mutant(s) to effects on the A_β^k chains and the type B mutation(s) to effects on the A_α^k chain. These assignments are based on knowledge of which I-A^k chain is principally responsible for the expression of specific antigenic determinants. Thus, Frelinger et al.² have assigned specificities recognized by some anti-I-A^k mAb to either A_α^k or A_β^k chains both by using these Ab to inhibit the proliferative responses of a panel of (k × b)F₁ alloreactive and antigen-specific T cell clones as well as by performing two-dimensional gel electrophoresis on the Ia molecules precipitated by these Ab from F₁ cells. Their results indicate that 10.2.16, 40N, 39B, and 39E define determinants on the A_β^k chain while mAb 26.7.11, 39J, and 40J recognize specificities on the A_α^k molecule. Silver et al. (22) and Kupinski et al. (23) had previously demonstrated that the 10.2.16 determinant is on the A_β^k chain. It is most likely then that the type A mutants have sustained an alteration in the A_β^k chain rather than the loss of this molecule since determinants 10.2.16 and 39B are present at some level in these mutants, whereas 40N is not detectable. Recent preliminary results using the 10.2.16 mAb as an immunoprecipitating agent (D. J. McKean and L. Glimcher, unpublished observations) reveal the presence of an A_β^k chain in the type A mutant A19.

However, we cannot exclude the possibility that the type A mutation(s) has occurred in a regulatory gene, resulting in the diminished synthesis of the A_β^k

² Frelinger, J. G., M. Shigeta, A. J. Infante, P. A. Nelson, M. Pierres, and C. G. Fathman. Multiple functional sites on a single Ia molecule defined using T cell clones and chain-specific anti-Ia antibodies. Manuscript submitted for publication.

TABLE VI
Summary of Stimulatory Properties of Mutant APC

APC Line	T Cell Hybridomas (Specificity: restriction element)									
	HEL(a) (HEL:I-A ^b)	OVA Nos. 1 + 2 (OVA:I-A ^b)	SKK9.11 (KLH-I-A ^b)	PPD (PPD-I-A ^b)	GAT (GAT:I-A ^b)	Auto Nos. 1 + 2 (I-A ^b)	AODH3.4 (OVA:I-A ^b)	SKK23 + 45.10 (OVA:I-A ^b)	HEL(b) (HEL:I-E ^b)	Auto No. 3 (I-A ^b)
TA-3	+	+	+	+	+	+	+	;	+	+
Type A mutants										
A19	-	-	-	-	+	+	+	+	+	+
B13	-	-	-	-	+	+	+	+	+	+
B18	-	-	-	-	-	-	+	+	+	+
Type B mutants										
A5	-	-	-	NT	-	-	-	-	+	+
A13	-	-	-	NT	NT	NT	-	-	+	+
No. 90	-	-	-	-	-	-	-	-	;	+

(+) indicates that APC line stimulates a response by the T hybridoma cell line; (-) that it does not; NT, that responsiveness was not tested.

polypeptide chain. If this were so, the unaltered A_β^k chain, produced in smaller than normal amounts, could combine with either the A_α^k chain to form a "parental" I-A class II molecule or with the A_α^k chain to form a hybrid I-A class II molecule (24). If the $A_\beta^k A_\alpha^d$ molecule differs from the $A_\beta^k A_\alpha^b$ and the $A_\beta^k A_\alpha^k$ molecules and exhibits the determinants recognized by 10.2.16, 39B, and 39E but not those recognized by 40N (and presumably 40A), then the phenotype of the type A mutants could be explained without the need to postulate a structural mutation in the A_β^k gene. The variability observed in the intensity of the staining by 39B and 10.2.16 might be attributed to the stage in the cell cycle when these lines were examined since it has been clearly shown that the amount of cell surface Ia molecule varies with the stage in cell cycle (25).

The biochemical event leading to the type B mutation may have been the loss of or greatly diminished synthesis of the A_α^k chain. Absence of expression of this chain might be secondary to a deletion of the entire gene segment encoding the A_α^k chain or could be secondary to a point mutation in a regulatory gene that prevents synthesis. Alternatively, a point mutation may have occurred that has led to a drastic change in the structure of the A_α^k chain and the resulting $A_{\beta A_\alpha}^k$ molecules with consequent loss of reactivity with all the A_α^k chain-specific mAb.

To the extent that the type B mutants have been studied functionally, they resemble each other, just as they do antigenically. In particular, type B mutants have thus far lacked the capacity to present antigen to any I-A^k-restricted T cell hybridoma or line. Since each mutant line was obtained from the same EMS-treated cell population, they could represent clones resulting from a single mutational event. Type A mutants appear serologically like one another but some evidence for functional diversity exists. Thus, two type A mutants, B18 and C13, do not present GAT to an I-A^k-restricted GAT-specific T cell hybridoma and stimulate two auto-I-A^k-specific T cell hybridomas poorly or not at all. By contrast, other type A mutants (A8, A19, and B13) stimulate these three hybridomas quite well. It should also be noted that some I-A^k-restricted T cell clones specific for KLH and OVA are stimulated by each of the type A mutants while other I-A^k-restricted T cell clones for specific for these same antigens are not stimulated by these mutants. This strongly suggests that the antigenic determinants (epitopes) on these complex proteins which different T cell clones recognize differ from one another.

An understanding of the mutational events leading to the altered I-A^k molecules expressed by the type A and type B mutants will require structural analysis of both these Ia antigens and of the genes that specify the chains of these molecules. Such studies are now in progress.

Although we do not yet have evidence concerning the nature of structural changes, if any, of the I-A class II molecules in these mutant cell lines, it is useful to consider the functional properties of Ia molecules in order to appreciate the potential effects of structural changes in them. Ia molecules act as restriction elements, in that they are corecognized, with antigen, by antigen-specific T cells (see references 26 and 27 for a review). Thus, the Ia molecules should possess a site complementary to a combining region of a T cell receptor. This site on the Ia molecule has recently been designated the histotope (28). The number of distinct histotopes a given Ia molecule possesses is unknown. A mutation in the

histotope of an Ia molecule could impair its ability to be corecognized by some, or possibly all, T cells primed against antigens that are presented together with the comparable wild-type Ia molecule. This would be particularly likely if there were but a single histotope on each Ia molecule and if all T cells that corecognized that histotope used structurally similar recognition sites for that purpose. Thus, if type B mutants are not based on a failure to express the A_2^k chain, they might very well represent mutations within the histotope of the $I-A^k$ molecule.

As a result of the study of Ia molecules as Ir gene products, it has been proposed that they possess a second class of sites through which they interact with antigen in the construction of putative antigen-Ia molecule complexes. Although only limited direct evidence has been obtained for the existence of such complexes, detailed study of the fine specificity of T cell responses to peptide antigens (29–31) strongly suggests that antigen and restriction elements interact. A similar conclusion has been reached from the finding that T cell responsiveness is determined by the product of the concentration of antigen and the number of Ia molecules on APC, implying that the activating agent is a complex of antigen and Ia molecule (32). This second class of sites on Ia molecules has been designated desetopes (28). Mutations within a desetope would have obvious effects on the function of Ia molecules as Ir gene products. Whether a given Ia molecule has one or more desetopes, it is likely that a mutation within the domain of a desetope might be selective in terms of its consequences for the capacity of the mutant cells to present distinct antigens, since a given Ia molecule must be able to interact with a wide range of structurally distinct foreign antigens. Changes in the structure of the desetope might impair the capacity of the Ia molecule to interact with antigens of one structural type without effecting interactions with structurally distinct antigens. Since type A mutants retain the capacity to present some but not all antigens that $I-A^k$ molecules of the wild type can present, these mutations would be good candidates to occur within the desetope. On the other hand, since some type A mutants fail to stimulate auto- $I-A^k$ -specific T cell hybridomas, the possibility that the mutation has occurred in the histope, the site recognized by the T cell, remains strong. Furthermore, one must also be aware that either (or both) the type A or type B mutations may occur outside these putative sites but nonetheless lead to structural alterations in the molecule that preclude its function in antigen presentation without reference to the structure of the antigen.

Summary

An improved method is described for selecting mutant cells with an altered pattern of Ia antigenic determinants and antigen-presenting properties from an homogeneous population of functional antigen-presenting cells (APC). The APC line used, TA3, was a somatic cell hybrid obtained by fusing normal heterozygous H-2^{a/d} B cells with a drug-marked variant of a BALB/c B lymphoma line. Two phenotypic groups of mutants were obtained by this method. Serologic analysis with a panel of anti- $I-A^k$ monoclonal antibodies suggested that the change in the first group of mutants (type A mutants) involved the alteration of a portion of one epitope of the $I-A^k$ molecule while in the second group of mutants (type B), an alteration of a different Ia epitope group had occurred. Functional studies

using a panel of cloned antigen-specific and autoreactive T cell hybridomas demonstrated that the loss of a limited number of I-A^k determinants in the type A mutants correlated with the loss of some but not all I-A^k-encoded restriction elements, while the type B mutation(s) resulted in the ablation of all I-A^k-restricted APC functions tested. These mutations may occur in the region of the Ia molecule that interacts with the T cell receptor (the histope) or in a postulated region that interacts with antigen (the desetope). The finding that both type A and B mutations lead to loss in the capacity to be corecognized with many different antigens by I-A^k-restricted T cell hybridomas suggests that the Ia molecule may possess very few distinct histopes and/or desetopes or that the tertiary structure of the Ia molecule is crucial in the formation of these sites. Alternatively, the mutations, particularly the type B mutations, may have led to the failure of expression of an entire α or β chain.

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