Brief Definitive Report

Evaluation of the Functional Equivalence of Major Histocompatibility Complex Class II A and E Complexes

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Summary

Most mice display two conventional major histocompatibility complex class II isotypes, A and E. Several A^+E^- strains have been observed, but never any that are A^-E^+. Because of this and because of hints from several lines of functional analysis, it has been proposed that the two isotypes might not operate equivalently. This proposition has not been directly testable until now because of the lack of an E-only strain. We report the production of such mice, exploiting previously created class II-transgenic and class II-"knock-out" lines. A^+E^-, A^-E^-, and A^-E^+ littermates have been compared by a number of parameters. We find that E and A molecules are, for the most part, functionally equivalent. However, subtle differences are seen in their ability to engage CD4 molecules on immature thymocytes, and in the profile of receptors on T cells selected into the periphery.

The majority of mouse strains express both of the conventional MHC class II isotypes: A and E. Strains that display A but not E complexes exist in the laboratory and in the wild, and by numerous criteria, they seem to exhibit normal immunological competence (1). This phenotype has arisen several times during murine evolution, the result of at least five independent mutations (for review see reference 2). It is intriguing, then, that strains of the reciprocal phenotype, displaying E but not A complexes, have never been observed.

One explanation for the existence of A^+E^- but not A^-E^+ mice might be that the two isotypes are not functionally equivalent. Indeed, several points can be made in favor of such a notion. First, many more antibody responses to foreign proteins seem to be restricted by A rather than E molecules. This might reflect the fact that $A\alpha:A\beta$ heterodimers are, on average, more polymorphic than their $E\alpha:E\beta$ counterparts (3). It might also result from the preferential association of endogenous superantigens with E complexes, which could cause many T cells potentially restricted to this isotype to be deleted in the thymus (for review, see reference 4). Second, A and E molecules appear not to have the same capacity to elicit an alloresponse. Although mice quite readily reject skin grafts differing only at the I-A locus, they do not reject those that are dissimilar only at I-E (5, and our own unpublished results). This is probably not due to the above-mentioned difference in degree of polymorphism, because grafts with just an $A\alpha$ discordance are readily attacked. Third, immune suppression seems more associated with E than with A molecules. This concept has been expounded most heartily by Mitchison and Oliveira (6, 7) and is rooted in the observation that almost all dampening responses that have been described so far, for example those characteristic of the lactate dehydrogenase and F liver protein systems, are E restricted. In addition, mice which express E molecules are generally more susceptible to certain parasitic infections, provoking the speculation that a response restricted by this isotype can suppress the "clearing" response normally restricted by A molecules (8). Fourth and finally, there is some evidence that A and E molecules differentially mediate B cell interactions, perhaps because of variant signaling properties (9–11).

Until now, the proposition that the two class II isotypes are not functionally equivalent has been impossible to test because of the lack of an E-only strain. We report here the production of such mice, exploiting the $E\alpha16$ line, which carries an $E\alpha$ transgene on a non-MHC chromosome (12), and the A^0_β line, which carries a drastically mutated $A\beta$ gene created by homologous recombination in embryonic stem cells (13). Our initial perspective has been to pose the question: are E and A molecules equally able to complement the immunological defects characteristic of class II-deficient animals?

Materials and Methods

The $E\alpha16$ and A^0_β lines have both been described (12, 13). $E\alpha16$ mice from the 18th backcross to C57Bl/6 (B6) were mated with

A_β^0 animals at the second backcross to B6. The resulting double heterozygotes were intercrossed and resulted in three types of offspring: A^+E^-, A^-E^-, and A^-E^+.

To test for CTL generation, mice were infected with seven hemagluttinating units (HAU) of influenza A/X31 virus intranasally, spleens were removed 2 wk later, and bulk cultures established in 20 ml RPMI supplemented with 10% FCS, antibiotics, and 5×10^{-5} M 2-ME, with 1.5×10^7 responder and 3 $\times 10^6$ virus-infected stimulator spleen cells from the same mouse. Cultures were incubated at 37°C, 5% CO_2 for 5 d, and tested in a standard ^{51}Cr-release assay with 8×10^3 ^{51}Cr-labeled EL-4 target cells per well either uninfected or infected with 1,000 HAU A/X31 virus/10^7 cells. Responder and target cells were incubated at the killer to target (K/T) ratios indicated in Fig. 3 for 5 h, at which time ^{51}Cr-release in the supernatant was assessed in a β-counter (Beckman Instruments, Fullerton, CA). Results are expressed as (experimental release – spontaneous release)/(total release – spontaneous release) $\times$ 100%. Spontaneous release was always <11% of the total release in 2.5% Triton-X 100.

The PCR-based sequencing protocol has been described in detail (14, 15). Briefly, lymph nodes were removed, the CD4$^+$ T cell population isolated by electronic sorting, RNA purified, and TCR cDNA synthesized and amplified by two rounds of PCR. The amplified fragments were cloned into an M13 vector, and the clones screened for Vβ6$^+$ sequences. More than 50 clones were sequenced from each of two A-only and two E-only mice. Repeat sequences were eliminated from consideration. The amplification primers have been reported (14). The Vβ6 screening oligo was: ACATCTGCC-CAGAGAACGAGATGGCCGTT.

All other materials (including antibodies, antigens, etc.) and methods (including cytofluorimetry, Ab titering, etc.) have been detailed in reference 13.

Results and Discussion

We recently employed homologous recombination in embryonic stem cells to produce mice lacking expression of the MHC class II E and A isotypes (20). Display of E molecules was avoided by starting with an ES cell line derived from a strain 129 mouse. Such animals are b-haplotype at the H-2 complex, and thus fail to assemble E complexes because of a deletion in the promoter region of the Eα gene. Display of A molecules was abrogated by introducing a drastic mutation into one allele of the ES cell line, generating offspring that carry the mutation in the heterozygous state ($A_\beta^{0/+}$) and intermating them to produce offspring that carry the muta-

tion as homozygotes ($A_\beta^{0/0}$). These mice were not expected to express mixed isotype $A_\alpha^b E_\beta^b$ molecules, and do not detectably do so (see lengthy discussion in reference 20).
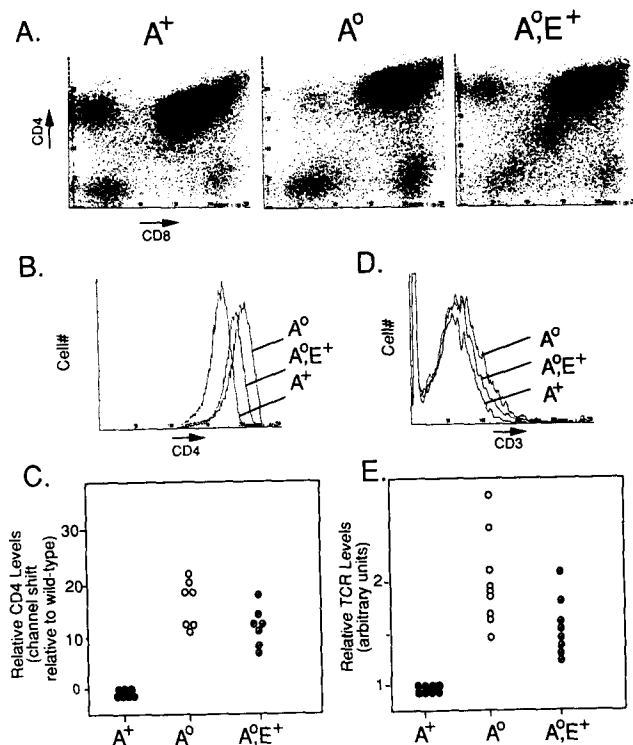
The class II-deficient mice exhibit several immune system irregularities, most notably: (a) They have drastically reduced numbers of CD4$^+$ T cells in the peripheral lymphoid organs. A few CD4$^+$ cells are present, ~3–7% the usual numbers, but these are a peculiar T cell subset according to surface marker expression and tissue localization. (b) They appear to lack fully mature CD4$^+$CD8$^-$ thymocytes. Some CD4$^+$CD8$^{-/lo}$ cells with rather high TCR levels are observed, but these are not mature single-positive cells by several criteria. The mutant animals have normal numbers of CD4$^+$CD8$^+$ thymocytes, but these double-positive cells are unusual in their high CD4 and TCR levels. (c) They have increased numbers of peripheral CD8$^+$ T cells, but are not able to recall a normal CTL response after infection with influenza/A virus. (H. Bodmer et al., manuscript in preparation). (d) They are capable of producing terminally differentiated plasma cells, but are inefficient producers of natural antibodies of the IgG1 isotype and appear incapable of making antibody responses to T-dependent antigens.

These immunodeficient mice seemed to provide a new opportunity for assessing the functional equivalence of A and E molecules. Thus, $A_\beta^{0/+}$ heterozygotes were mated to heterozygotes of the Eα 16 line, which carries a perfectly functioning Eα transgene on a non-MHC chromosome (12, and reference 16 for review). Double heterozygotes ($A_\beta^{0/+}$,$E_\alpha^{+/-}$) were identified by Southern blotting and were intercrossed to produce three types of useful offspring: A^+E^-, A^-E^-, and A^-E^+. In each experiment described below, sets of littermates were analyzed to determine whether E and A molecules are equally capable of correcting the different irregularities exhibited by class II-deficient mice.

Peripheral T Cells. Representative dot plots of lymph node cells double-stained with anti-CD4 and -CD8 mAbs are presented in Fig. 1 A and a plot of the relative numbers of CD4$^+$ lymph node T cells in several individuals is shown in Fig. 1 B. Clearly, expression of the E molecule is capable of restoring the peripheral CD4$^+$ T cell compartment, greatly depleted in class II-deficient animals. Other parameters of peripheral CD4$^+$ T cells, perturbed in A$^-$ mice, are fully normalized (size, CD3 or CD44 levels, etc; data not shown).
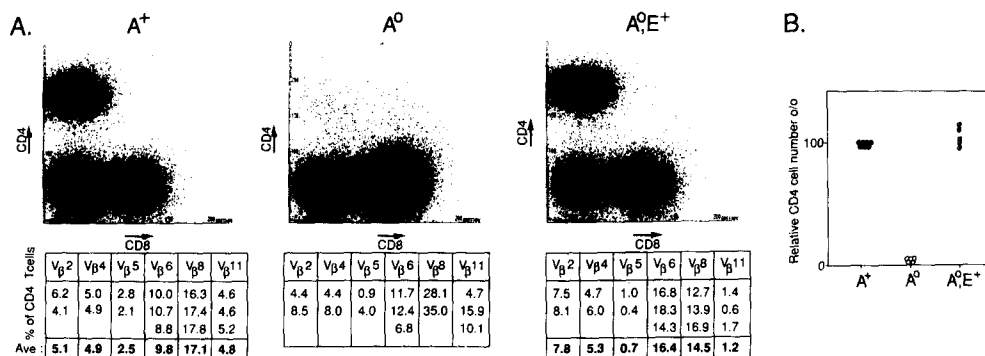
Figure 1. The E molecule restores the CD4$^+$ lymphocyte population in peripheral lymph nodes. (A) Lymph node cells were analyzed by three-color flow cytometry after staining with anti-CD4, anti-CD8 and Vβ-specific mAbs. (Top) CD4/CD8 profiles of representative mice of each genotype. (Bottom) Percent usage of individual Vβ segments in CD4$^+$ cells. (B) Relative CD4 cell frequency was calculated in littermate sets as 100 $\times$ (% CD4$^+$ T cells in LN of E$^+$ mouse/% CD4$^+$ T cells in LN of A$^+$ littermate). (Dot) Individual animal.

A. A^+

V$_\beta$2	V$_\beta$4	V$_\beta$5	V$_\beta$6	V$_\beta$8	V$_\beta$11
6.2	5.0	2.8	10.0	16.3	4.6
4.1	4.9	2.1	10.7	17.4	4.6
			8.8	17.8	5.2
5.1	**4.9**	**2.5**	**9.8**	**17.1**	**4.8**

A^0

V$_\beta$2	V$_\beta$4	V$_\beta$5	V$_\beta$6	V$_\beta$8	V$_\beta$11
4.4	4.4	0.9	11.7	28.1	4.7
8.5	8.0	4.0	12.4	35.0	15.9
			6.8		10.1

$A_\beta^0 E^+$

| V$_\beta$2	V$_\beta$4	V$_\beta$5	V$_\beta$6	V$_\beta$8	V$_\beta$11
7.5	4.7	1.0	16.8	12.7	1.4
8.1	6.0	0.4	18.3	13.9	0.6
			14.3	16.9	1.7
7.8	**5.3**	**0.7**	**16.4**	**14.5**	**1.2**

Figure 2. The E molecule restores normal CD4+ thymocyte populations. Thymocytes were analyzed by three-color flow cytometry after staining with anti-CD4, -CD8, and -CD3. (*A*) CD4/CD8 profiles. (*B*) Histograms of CD4 staining intensity for double-positive cells. (*C*) Data grouped from several such experiments, comparing the mean CD4 fluorescence intensity in DP cells of A° and A°E+ mice relative to A+ littermates (expressed as channel shifts between mean fluorescence intensities, on a log scale where a 32-channel shift corresponds to doubled intensity). (*D*) CD3 profiles of DP cells are superimposed. (*E*) Relative TCR levels in several experiments. As it is difficult to assess CD3 or TCR levels that range from essentially negative to dull, we evaluated the overall shifts in CD3 or αβ-TCR spectra by quantitating the proportion of cells with staining intensities above an arbitrary level (positioned so that 10–13% of DP cells in A+ mice would be brighter than that level). Relative TCR levels in A° or A°E+ littermates were then calculated as: Proportion of bright DP cells in E+ mouse/Proportion of bright DP cells in A+ littermate. This gating excluded the DP cells with very bright CD3 or αβ-TCR staining, equivalent to that of single-positive cells. (*Dot*) One mouse.

To determine whether the repertoire of CD4+ T cells in E-only mice is normal, we triple-stained some littermates with anti-CD4, anti-CD8, and specific anti-Vβ antibodies. The percentages of CD4+ cells displaying various Vβs are tabulated for individual mice in Fig. 1 *A*. All Vβs tested are expressed in A⁻E+ mice and, when percentages are compared with those from A+E⁻ or A⁻E⁻ individuals, the expected negative selection of Vβs 5 and 11 (17–20) and positive selection of Vβ6 (21, 22) is observed.

The large scatter in Vβ percentages for the class II-deficient mice has been noted before and may be due to pauciclonality (13, and M. Bogue, unpublished observations).

Thymic T Cells. Fig. 2 *A* presents representative dot plots of thymocytes stained with anti-CD4 and -CD8 mAbs. Display of either A or E complexes promotes the development
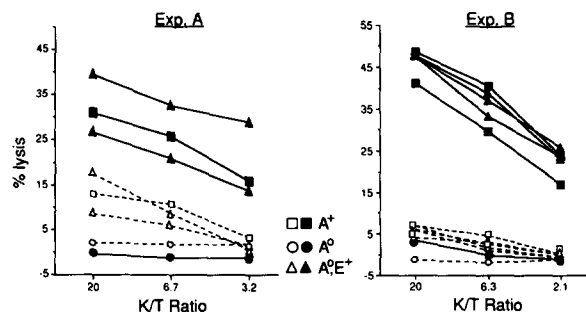
Figure 3. The E molecule restores CTL responses. CTL responses to influenza A virus in individual mice, in two experiments. 2 wk after infection, mice were killed and spleen cells restimulated with virus in vitro. 5 d later, bulk cultures were tested for CTL activity in a 5-h ⁵¹Cr-release assay against EL-4 cells, either uninfected (*open symbols*) or infected (*closed symbols*) with virus.

of a fully mature CD4+8⁻ compartment. These cells are present in normal numbers, express high levels of TCR (not shown), display no, as opposed to low levels of CD8, and have no or low levels of heat-stable antigen (not shown).

Fig. 2 also illustrates the one irregularity not fully corrected by the display of E molecules. As mentioned above, double-positive thymocytes in class II-deficient mice express aberrantly high levels of TCR and CD4 compared with thymocytes from wild-type mice. This was also observed after anti-CD4 mAb treatment in in vivo or in thymic organ cultures, and was interpreted as evidence that in the normal, unmanipulated animals the CD4 molecules on double-positive thymocytes are already engaged, leading to a downregulation of CD4 and the TCR (23–25). Surprisingly, E-only mice also have unusually high levels of CD4 (Fig. 2, *A–C*) and of TCR (Fig. 2, *A*, *D*, and *E*). Although expression, especially of the TCR, is reduced compared with that in class II-negative littermates, it does not drop to the level of littermates that display A molecules.

Cytotoxic T Cell Responses. It has been reported that mice treated with an anti-CD4 mAb, and assumed to be depleted of CD4+Th cells, exhibit a slightly reduced, but still easily detectable, CTL response to influenza A virus (26, 27). However, we have observed greatly diminished memory CTL responses with splenocytes derived from class II-deficient animals recently infected by influenza (H. Bodmer et al., manuscript in preparation). This recall assay is quite sensitive, requiring that there was efficient in vivo priming of CTLs during infection, and that the in vitro stimulus is sufficient to regenerate the specific CTL activity.

Fig. 3 presents results from two independent experiments where A+E⁻, A⁻E⁻, and A⁻E+ littermates were infected intranasally with influenza A/X31 virus, and 2 wk later spleen cells were removed and challenged in vitro by infection with A/X31. Class II-deficient animals, as mentioned above, make little or no response. Expression of A or E molecules equivalently restores the ability to generate virus-specific CTLs.

B Cell Responses. Although class II-deficient mice were found to have defects in the B cell compartment, they do host terminal differentiation to plasma cells, as evidenced by the efficient production of serum antibodies and a normal
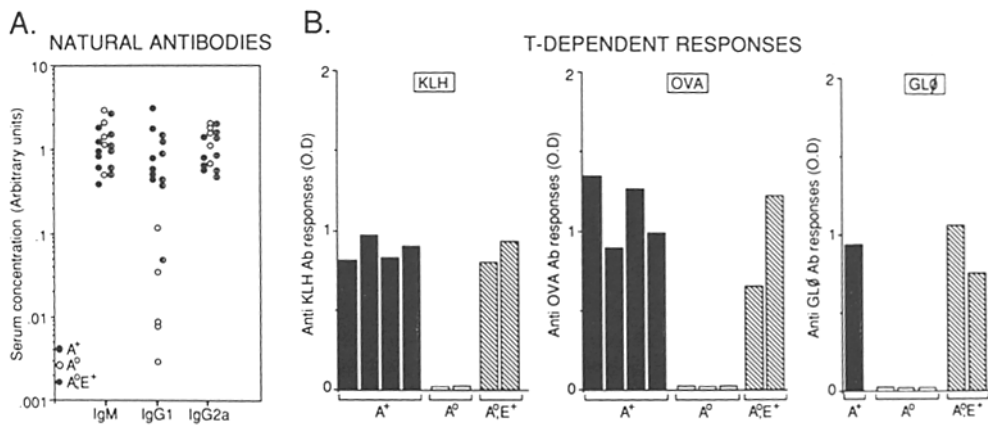
capacity to respond to T-independent antigens (13). Yet, an abnormal profile of Ig isotypes is found in sera from these mice. In particular, the IgG1 isotype is drastically underrepresented. As indicated in Fig. 4 *A*, expression of either A or E molecules promotes efficient production of IgG1 antibodies.
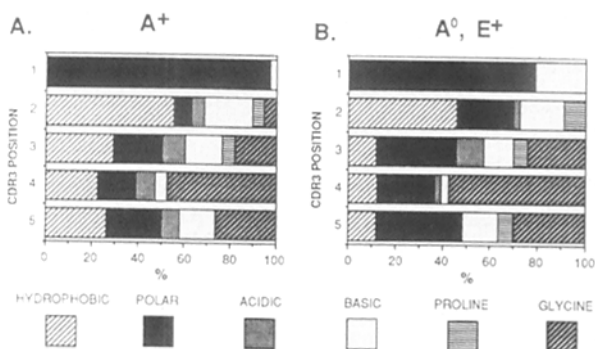
A major and expected defect in the class II-deficient animals is the inability to respond to T-dependent antigens (13). We tested whether E expression alone can complement this defect by quantitating Ab production after injection of two large multi-epitope proteins (KLH and OVA) and one E-restricted polypeptide (GLØ). E-only animals responded as well as A-only littermates to the two proteins, and also mounted an efficient response to the polypeptide.

T Cell Receptor Junctional Regions. The majority of TCR diversity resides in the V-D-J junctional region, where choice of D segment, choice of J segment, exonucleolytic nibbling and N nucleotide addition all make a contribution to sequence variation. We wondered whether E and A molecules might select populations of T cells expressing TCRs with structurally distinct junctional regions (or CDR3s). Therefore, we sequenced randomly selected Vβ6⁺ TCRs from lymph node cells isolated from E-only and A-only littermates. Vβ6⁺

receptors were chosen because, of all the TCRs expressed by these mice, we already possess the largest data base on this variable region (unpublished data).

Because of space considerations, the sequences are not presented, but are available on request. By general criteria, including D region usage, J region usage, and CDR3 length, the two sets appear indistinguishable. We did notice some differences in amino acid composition between positions 1 and 5 of the CDR3s, as illustrated in Fig. 5. In the sequence set from A-only mice, and in a much larger set of previously published Vβ17 sequences (14), position 1 of CDR3 is almost always occupied by a germline-encoded serine. Only about 2–5% of sequences have another residue at this position. However, in the set from E-only animals, >20% of the sequences carry a basic residue at CDR3 position 1. More subtle differences can be seen at other positions: a general increase in the frequency of polar at the expense of hydrophobic residues, and differences in charged amino acids at positions 4 and 5.

The differences could be due to some structural feature of the MHC molecules themselves, or to some feature of a peptide (or other ligand) involved in positive selection of T cells in the thymus or their later expansion in the periphery.

Conclusions

By almost all criteria, A and E molecules are equally capable of correcting the irregularities previously documented in class II-deficient mice. Expression of either isotype leads to restoration of the CD4⁺ T cell compartment in the periphery, promotes differentiation of fully mature CD4⁺CD8⁻ cells in the thymus, permits efficient CTL generation, and completely reestablishes normal antibody production. The last two criteria are perhaps the most revealing, as they imply normal operation of multiple class II-mediated events.

Nonetheless, E-only mice do differ subtly in certain parameters. Perhaps most interesting, the CD4 on CD4⁺CD8⁺ thymocytes can be engaged efficiently by A, but not by E complexes. This could be because the two isotypes have different affinities for this coreceptor molecule, and this explanation would fit nicely with their capacities to elicit skin graft rejection, given the fact that primary class II alloresponses

Figure 5. Amino acid composition of junctional regions of Vβ6⁺ TCRs from A⁺ (*A*) and A°E⁺ (*B*) mice. Amino acid frequencies for positions 1–5 of the CDR3 region. Amino acids except glycine and proline are grouped as follows: hydrophobic: LIFMVAW; polar: QNTSY; acidic: DE; basic: RKH.

are known to be CD4 dependent. No matter what the explanation, our results suggest that efficient engagement of CD4 on double-positive thymocytes and the resulting down-regulation of CD4 and TCR levels is not required for the terminal differentiation of thymocytes, nor for their export to the periphery.

Viewed in its ensemble, our results do not provide much explanation for the E/A dichotomies mentioned in the introductory section. It remains possible that more sophisti-cated comparisons are required: an assessment of TH1/TH2 phenotypes in the CD4 T cell compartment; a measure of the relative CD4 dependence of responses to different antigens coupled with some evaluation of the affinity of the T cells elicited; and a direct measure of signaling properties through the E and A molecules on B cells and other APCs. With the mice described in this report, such comparisons are now possible.

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