**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 Aα:ββ heterodimers are, on average, more polymorphic than their Eα:ββ 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 allosresponse. 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skin grafts differing only at the I-A locus, they do not reject those that are dissimilar only at I-E (5, and our own unpublished results).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 Aα 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 Eα16 line, which carries an Eα transgene on a non-MHC chromosome (12), and the Aγ line, which carries a drastically mutated Aβ 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 Eα16 and Aγ lines have both been described (12, 13). Eα16 mice from the 18th backcross to C57Bl/6 (B6) were mated with...
A"1 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 hemagglutinating 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 x 10^-5 M 2-ME, with 1.5 x 10^5 responder and 3 x 10^5 virus-infected stimulator spleen cells from the same mouse. Cultures were incubated at 37°C, 5% CO2 for 5 d, and tested in a standard 51Cr-release assay with 8 x 10^3 51Cr-labeled EL4 target cells for well either uninfected or infected with 1,000 HAU A/X31 virus/10^6 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 51Cr-release in the supernatant was assessed in a beta-counter (Beckman Instruments, Fullerton, CA). Results are expressed as (experimental release–spontaneous release)/total release x 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 VB6* sequences. More than 50 clones were sequenced from each of two A-only and two E-only mice. Repeat sequences 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. (Top) CD4/CD8 profiles of representative mice of each genotype. (Bottom) Percent usage of individual VB segments in CD4 + cells. (B) Relative CD4 cell frequency was calculated in littermate sets as 100 x (% CD4 + T cells in LN of E + mouse/ % CD4 + T cells in LN of A + littermate). (Dot) Individual animal.
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 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^+ T 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 a 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 under-represented. As indicated in Fig. 4A, 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 VB6+ TCRs from lymph node cells isolated from E-only and A-only littermates. VB6+ 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 noticed 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 VB17 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 alloreactions...
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 sophisticated 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.

We thank M. Lemeur, A. Dierich, C. Waltzinger, P. Gerber, P. Bohn-Marchal, C. Ebel, P. Michel, N. Zinck, and S. Metz for their contributions.

This work was supported by institute funds from the INSERM and the CNRS, and by grants to D. Mathis and C. Benoist from the Association pour la Recherche sur le Cancer and the National Institutes of Health (NIH). D. Cosgrove received fellowships from NATO and the Fondation pour la Recherche Médicale Française; H. Bodmer from the Medical Research Council/CNRS, and the Wellcome Trust; and M. Bogue from the NIH.

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Received for publication 2 April 1992 and in revised form 11 May 1992.

Note Added in Proof: All of the experiments described in this paper were performed on mice bred in a conventional animal facility, where effects on the general health status of A~ /A~ homozygotes can sometimes be observed, e.g., runting. CTL responses in these mice were consistently deficient, but fully corrected by expression of E or A molecules. Recently, we have repeated the CTL experiments on mice bred in an SPF facility, where A~ /A~ homozygotes and littermates are indistinguishable in their general health. SPF A~ /A~ mice are consistently able to recall a quite efficient CTL response against influenza virus.

References

tion; dissimilar CDR3s in CD4+ versus CD8+ cells. J. Exp. Med. 174:989.


