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

By Dominic Cosgrove, Helen Bodmer, Molly Bogue, Christophe Benoist, and Diane Mathis

From the Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique et U.184 de l'Institut National de la Santé et de la Recherche Médicale, Institut de Chimie Biologique, 67085 Strasbourg, France

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:Aβ heterodimers are, on average, more polymorphic than their Eα:Eβ 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 Aβ 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 Aβ lines have both been described (12, 13). Eα16 mice from the 18th backcross to C57Bl/6 (B6) were mated with
A4 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 10^7 responder and 3 x 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 31Cr-release assay with 8 x 10^3 S^Cr-labeled EL4 target cells per 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 31Cr-release in the supernatant was assessed in a beta-counter (Beckman Instruments, Fullerton, CA). Results are expressed as (total release-spontaneous release)/(total release-spontaneous 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 V6+ 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 V6 screening oligo was: ACATCTGCC-S6AAGTGCAGATTCGGT.

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 A and E molecules. (20). Display of E molecules was avoided by starting with an ES cell line derived from a strain 129 mouse. Such animals are b-haplotypic at the H-2 complex, and thus fail to assemble E complexes because of a deletion in the promoter region of the Ecr 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+0/+) and intermuting them to produce offspring that carry the mutation as homozygotes (A+/+). These mice were not expected to express mixed isotype A2/E9 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- thymocytes 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+0/0 heterozygotes were mated to heterozygotes of the Ecr 16 line, which carries a perfectly functioning Ecr transgene on a non-MHC chromosome (12, and reference 16 for review). Double heterozygotes (A+E-/++ or A+0/E+/-) 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).
C) Data grouped from several such experiments, comparing the mean CD4 fluorescence intensity in DP cells of A~ and A~ + mice relative to A~ littermates were then calculated as: Proportion of bright DP cells in 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 were shown (not shown), display no, as opposed to low levels of CD4, 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 terminal differentiation to plasma cells, as evidenced by the efficient production of serum antibodies and a normal 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).

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 puercoclinality (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 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 CD4, 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 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. 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 (GL0). 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, exonuclease 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. 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: LIFMVAVW; polar: QNTSY; acidic: DE; basic: RKH.
are known to be CD4 dependent. No matter what the ex-
planation, 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 in-
troductive 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
ciliated; 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.

Address correspondence to Diane Mathis and Christophe Benoist, LGME/CNRS et U.184/INSERM,
Institut de Chimie Biologique, 11, rue Humann, 67085 Strasbourg Cedex, France. Dominic Cosgrove
is currently at Boy's Town Medical Center, Omaha, Nebraska.

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 Aαβ homozygotes can some-
times be observed, e.g., running. CTL responses in these mice were consistently deficient, but fully cor-
rected by expression of E or A molecules. Recently, we have repeated the CTL experiments on mice bred
in an SPF facility, where Aαβ homozygotes and littermates are indistinguishable in their general health.
SPF Aαβ mice are consistently able to recall a quite efficient CTL response against influenza virus.

References

1. Jones, P.P., D.B. Murphy, and H.O. McDevitt. 1981. Variable
synthesis and expression of Eα and Aε (Eβ) Ia polypeptide
chains in mice of different H-2 haplotypes. Immunogenetics.
12:321.
2. Robinson, M.A., and T.J. Kindt. 1989. Major histocompati-
bility complex antigens and genes. In Fundamental Immuno-
ology. W.P. Paul, editor. Raven Press, New York. vol. 2, pg.
489.
3. Mengle-Gaw, L., and H.O. McDevitt. 1985. Genetics and ex-
pression of mouse Ia antigens. Annu. Rev. Immunol. 3:367.
4. Marrack, P., and J. Kappler. 1990. The staphylococcal en-
terotoxins and their relatives. Science (Wash. DC). 248:705.
5. Sachs, D.H., J.L. Cone, and G.W. Humphrey. 1976. The hu-
moral response to an Ia antigen and skin graft rejection: mecanistic possibilities. Transplant. Proc. 3:413.
6. Mitchison, N.A., and D.BG. Oliveira. 1986. Epirestriction and
a specialized subset of T helper cells are key factors in the regu-
lation of T suppressor cells. In Progress in Immunology VI,
Sixth International Congress of Immunology, B. Cinader and
R.G. Miller, editors. pg. 326.
7. Oliveira, D.BG. 1989. F protein and immune suppression genes.
Immunology. 2(Suppl):26.
8. Wassom, D.L., C.J. Krco, and C.S. David. 1987. I-E expres-
sion and susceptibility to parasite infection. Immunol. Today.
8:39.
9. Takahama, Y., S. Ono, K. Ishihara, M. Muramatsu, and T.
Hamaoka. 1989. Disparate functions of I-A and I-E molecules
on B cells as evidenced by the inhibition with anti-I-A and
anti-I-E antibodies of polyclonal B cell activation. Eur. J.
Immunol. 19:2227.
10. Takahama, Y., S. Ono, K. Ishihara, H. Hirano, and T. Hamaoka.
1989. B-B cell interaction involved in polyclonal B cell activa-
tion is restricted by I-A but not by I-E molecules. Int. Immunol.
2:63.
11. Corley, R.B., N.J. LoCascio, M. Ovnic, and G. Haughton.
1985. Two separate functions of class II (Ia) molecules: T-cell
stimulation and B-cell excitation. Proc. Natl. Acad. Sci. USA.
82:516.
12. Lemeur, M., P. Gerlinger, C. Benoist, and D. Mathis. 1985.
Correcting an immune-response deficiency by creating Eα gene
transgenic mice. Nature (Lond.). 316:38.
13. Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur,
C. Benoist, and D. Mathis. 1991. Mice lacking MHC class
II molecules. Cell. 66:1051.
14. Candeias, S., C. Waltzinger, C. Benoist, D. Mathis. 1991. The
Vβ17* T cell repertoire: skewed Jβ usage after thymic selec-
tion; dissimilar CDR3s in CD4+ versus CD8+ cells. *J. Exp.
Med.* 174:989.

15. Candeias, S., J. Katz, C. Benoist, D. Mathis, and K. Haskins.
1991. Islet-specific T cell clones from non-obese diabetic mice
express heterogeneous T cell receptors. *Proc. Natl. Acad. Sci.
USA.* 88:6167.

16. Benoist, C., and D. Mathis. 1989. Positive and negative selec-
tion of the T cell repertoire in MHC class II transgenic mice.
*Semin. in Immunol.* 1:117.

17. Liao, H.S., J. Maltzman, and D.H. Raulet. 1990. Expression
of the Vβ5.1 gene by murine peripheral T cells is controlled
by MHC genes and skewed to the CD8+ subset. *J. Immunol.*
144:844.

18. Bill, J., O. Kanagawa, J. Linter, Y. Urqunomiya, and E. Palmer.
1990. Class I and class II MHC gene products differentially
affect the fate of Vβ5 bearing thymocytes. *J. Mol. Cell.
Immunol.* 4:269.

19. Tomonari, K., and E. Lovering. 1988. T-cell receptor-specific
monoclonal antibodies against a Vβ11-positive mouse T-cell
clone. *Immunogenetics.* 28:445.

20. Bill, J., O. Kanagawa, D.L. Woodland, and E. Palmer. 1989.
The MHC molecule I-E is necessary but not sufficient for the
clonal deletion of Vβ11-bearing T cells. *J. Exp. Med.* 169:1405.

21. MacDonald, H.R., R.K. Lees, R. Schneider, R.M. Zinker-
nagel, and H. Hengartner. 1988. Positive selection of CD4+
thymocytes controlled by MHC class II gene products. *Nature
(Lond.)*. 336:471.

22. Benoist, C., and D. Mathis. 1989. Positive selection of the T
cell repertoire: where and when does it occur? *Cell.* 58:1027.

23. McCarthy, S.A., A.M. Kruisbeek, I.K. Uppenkamp, S.O.
Sharrow, and A. Singer. 1988. Engagement of the CD4 mol-
ecule influences cell surface expression of the T-cell receptor on
thymocytes. *Nature (Lond.)*. 336:76.

24. Zuniga-Pflucker, J.C., S.A. McCarthy, M. Weston, D.L. Longo,
A. Singer, and A.M. Kruisbeek. 1989. Role of CD4 in thymo-
cyte selection and maturation. *J. Exp. Med.* 169:2085.

25. Nakayama, T., C.H. June, T.L. Munitz, M. Sheard, S.A.
McCarthy, S.O. Sharrow, L.E. Samelson, and A. Singer. 1990.
Inhibition of T cell receptor expression and function in immu-
ture CD4+CD8+ cells by CD4. *Science (Wash. DC).* 249:1558.

26. Lightman, S., S. Cobbold, H. Waldmann, and B.A. Askonas.
1987. Do L3T4+ T cells act as effector cells in protection
against influenza virus infection? *Immunology.* 62:139.

27. Allan, W., Z. Tabi, A. Cleary, and P.C. Doherty. 1990. Cel-
lar events in the lymph node and lung of mice with influenza.
*J. Immunol.* 144:3980.