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FUNCTIONAL CONSEQUENCES OF OVEREXPRESSED Ia ANTIGENS IN A^K_α/A^K_β TRANSGENIC MICE[1]

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We report the creation and characterization of several transgenic mouse lines that carry genes coding for the A^k_α or A^k_β MHC class II (or Ia) molecules. In all these lines, the transgenes are expressed at the RNA and protein level with correct tissue and cell type specificity. Crosses between certain of them yield progeny displaying very high surface levels of class II protein—roughly five times the normal amount—allowing us to evaluate the consequences of quantitative variation in Ia molecule density on the organization and function of the immune system. The effects appear rather limited: we detect subtle changes in thymic lymphocyte subpopulations, as well as an enhanced Ag presentation capacity in vitro. Yet, in vivo responses are largely unaffected, and Ia overexpression to such levels does not provoke lymphoproliferation, immunodeficiency, or autoimmunity.

Ag-dependent activation of T cells requires the co-recognition of an Ag and a specific MHC molecule (for review, see Ref. 1). For helper T cells, it is the MHC class II, or Ia, molecules that are involved. There are two of these cell surface glycocomplexes in the mouse—A and E—and each is heterodimeric, composed of an α- and a β-chain (A_α:A_β and E_α:E_β; for review, see Ref. 2).

T cell activation is influenced by qualitative as well as quantitative variation in the Ia molecules displayed on APC. Qualitative variation directly affects the compatibility of Ia molecules, antigenic peptides, and the TCR; i.e., allelic polymorphism of Ia molecules conditions the molecular interactions necessary for the formation of an Ia molecule-Ag-TCR ternary complex. Ultimately, the ability of an animal to respond to different Ag—its Ir phenotype—is determined by these qualitative variations (3), and so Ia structure:function relationships have been the object of intense study (for review, see Ref. 4).

Quantitative variation also markedly influences T cell activation. This is most clearly demonstrated in vitro, where the cell-surface density of Ia Ag was found to dictate the efficiency with which T cell clones can be activated (5–9), a finding substantiated in studies using L cell transfectants (10). The influence of Ia Ag levels on immune function in vivo has been harder to demonstrate. Matis et al. showed that reduced amounts of E^k_α E^u_β complexes in F_1 hybrids led to inefficient E-restricted responses (5, 6). A number of other in vivo phenomena were also interpreted as reflecting variation in Ia expression levels, although the interpretation of the data was rather complex (see Ref. 11 for review and references). Finally, Janeway et al. (11) have suggested that certain lymphoproliferative disorders, immunodeficiencies and autoimmune diseases might be due to over- or under-expression of Ia molecules.

We are in a position to evaluate more directly the in vivo effect of quantitative variation in Ia Ag levels. By injecting cloned genomic A^k_α and A^k_β fragments into mouse embryos, we have created several transgenic lines, some of which carry quite high transgene copy numbers. Matings between certain of these mice engender animals that express about five times the normal amount of Ia Ag on all relevant cells. We have studied the consequences of this Ia "overdose" on the composition and operation of the immune system; they appear, perhaps surprisingly, very limited.

MATERIALS AND METHODS

Ag. KLH[3] was obtained from Calbiochem, the copolymers Poly(Tyr-Glu)Poly-Ala:Poly-Lys and HGAL from ICN, bovine insulin and BSA from Sigma. The polymer Glu-Lys-Phe (GLØ) was a kind gift from Dr. H. McDevitt, Stanford University. CFA was purchased from DIFCO.

mAb. Anti-Ia mAb included the anti-A^k_α reagents H-116-32, 39J and 39C, the anti-A^k_β reagents 39E, 10-2-6 and F35-52-27, the anti $A^{b\,or\,s}_\alpha$:$A^{b\,or\,s}_\beta$ reagents 4D5 and Y-219, and the public anti-A mAbs 40B, 3F12 and 2A2 (kind gifts from Drs. G. Hammerling, M. Pierres, D. McKean, D. Klein and D. Murphy; see Ref. 12 for references). T cells and their subpopulations were distinguished by anti-Thy1, anti-CD5, anti-CD8 and anti-CD4 staining with mAb 59AD22, 53.7.3, 53.6.7, and H129.19, respectively (13, 14). KJ16 and 44.2.1 recognize determinants on $V_\beta8$ and $V_\beta6$ of the TCR (15, 16) (kind gifts from Drs. J. Kappler and H. Hengartner, respectively). To distinguish cortical epithelial and medullary epithelial cells we used the mAb ER-TR4 and ER-TR5 (17).

Mice. Inbred C57B1/6J mice (B6) were obtained from Iffa-Credo (Les Oncins, France). SJL/J, C57B1/10J and A/J mice were obtained

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[3] Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; HGAL, Poly (His-Glu) Poly-Ala: Poly-Lys.

from Olac (Bicester, UK). C57B1/10J × A/J (B × A) and SJL/J × A/J (S × A) F_1 hybrids were bred in our facility.

Transgenic mouse strains were derived by independently injecting A^k_α or A^k_β cloned genomic DNA into fertilized eggs. The injected DNAs were ClaI fragments from plasmids pCA6 and pCA9 (18) (kind gifts from Dr. B. Malissen). These fragments comprise the entire A^k_α and A^k_β genes from AKR mice. Flanking regions extend approximately 5 and 3 kb 5′ and 3′, respectively, of A_α (see Fig. 2 of Ref. 18). DNA was injected as previously described (19) into fertilized B6 or (B6 × SJL) F_2 eggs and the eggs reimplanted into foster mothers. Tails from the resulting offspring were analyzed by Southern blotting for integration of the injected DNA. Permanent transgene heterozygous strains were derived and are maintained by backcrossing against B6 or SJL. Southern blots are performed at each generation, and positive animals mated to B6 or SJL mice to yield the next generation. To obtain "double-positive" transgenic mice, matings are set up between two mice, each positive (heterozygous) for one transgene. The offspring are typed and used in various experiments; animals carrying none or only one of the transgenes serve as experimental controls.

RNA isolation and S1 analysis. Expression of the transgenes was never evaluated in the founder animals, which often have a mosaic distribution of the transgene in their tissues, but instead in transgene-positive second generation progeny. RNA was isolated from several tissues according to Auffray and Rougeon (20) and tested by S1 nuclease analysis. Mouse organs to be analyzed (spleen, heart, kidney, brain, lung, thymus, pancreas, liver) were excised, rinsed in PBS and rapidly frozen in liquid nitrogen. Immediately after adding 1 ml of LiCl/urea-solution (3 M LiCl, 6 M urea, 10 mM NaAc, pH 5) and 100 μl 10% SDS, the frozen tissues were homogenized with a tissue homogenizer (Polytron, Kinematica, Luzern) at highest speed for 20 s. Pancreas tissue was homogenized in LiCl/urea/SDS solution containing 0.2% DEPC (J. Bohme, personal communication). After incubation for 10 h at 4°C, precipitated RNA was pelleted by centrifugation for 10 min at 10,000 rpm in an HB4 rotor, the supernatant discarded, and the pellet resuspended in 2 ml LiCl/urea solution. After 30 min at 4°C, samples were centrifuged as before. Pellets were resuspended in proteinase K solution (200 μg/ml proteinase K, 10 mM Tris, 200 mM NaCl, 2 mM EDTA, 0.5% SDS) and incubated at 37°C for 30 min, followed by phenol/chloroform and chloroform extractions. RNA was ethanol-precipitated, pelleted, dried, and redissolved in H_2O at a concentration of 1 to 2 mg/ml.

Peritoneal macrophages were elicited as described elsewhere (21) by an i.p. injection of thioglycolate (DIFCO). They were harvested 3 days later by washing the peritoneal cavity with RPMI, and cultured at 37°C in RPMI supplemented with 10% FCS and 50 μM 2-ME. The medium was changed after 6 h to remove nonadherent cells, and mouse γ-IFN was added to a final concentration of 40 U/ml. The cells were harvested after 60 h and RNA was prepared exactly as described above.

Quantitative S1 nuclease analysis was carried out with 10 μg total RNA essentially as previously described (22). In order to distinguish transgene-encoded message (A^k_α or A^k_β) from endogenous gene-encoded message ($A^{b\,or\,s}_\alpha$ or $A^{b\,or\,s}_\beta$), we prepared ^{32}P-end-labeled probes that could base-pair perfectly with κ-haplotype mRNA, but lacked complementarity to b- or s-haplotype message at their two most 5′ nucleotides (Fig. 1). Hybridization of these haplotype-specific probes with total RNA and subsequent incubation with S1-nuclease resulted in cleavage of mismatched transcript-probe hybrids and removal of the 5′-end label from probes hybridized to endogenously encoded transcripts (Fig. 1). Thus only probe paired with transgene-encoded message could give rise to detectable signals. The optimal concentration of S1-nuclease necessary to discriminate transgene from endogenous message was determined in pilot experiments using splenic RNA of B10.A (k-haplotype) and (B6 × SJL)-F_1 mice (b/s-haplotype) as test substrates.

5′-End-labeled probes were synthesized by primer extension (23). Single-stranded A_α and A_β DNA templates were obtained by cloning A_α and A_β cDNA into M13 and preparing recombinant ssDNA from phage supernatants according to standard protocols: 10 pmol of primer-oligo-nucleotide (5′-AGCAAACTCAGGAAGC-3′ for A^k_α; 5′-GAAGGGCTGGAACTGG-3′ for A^k_β) were end-labeled for 30 min at 37°C with 10 μl γ-[^{32}P]ATP, 3 μl 5× kinase buffer (100 mM Tris (pH 7.75; 50 mM $MgCl_2$; 50 mM 2-MEI, 250 μg/ml BSA) and 5 U T4-kinase in a total volume of 15 μl. After end-labeling, T4-kinase was inactivated at 65°C for 10 min. One microliter of annealing buffer (as kinase buffer made 1 M in NaCl) and 5 μg single-stranded template-DNA were added, the sample was heated again for 10 min at 65°C and then incubated for 30 min at 22°C to allow annealing of end-labeled primer. Primers were elongated for 30 min at 22°C in a total volume of 40 μl by adding 5 μl of deoxynucleotide triphosphate solution (5 mM each), 2 μl 5 × kinase-buffer, and 4 U Klenow-polymerase. Klenow-polymerase was inactivated at 65°C (410 min) and the template/probe hybrid digested with 20 U *PstI* (any other restriction endonuclease cutting close to the 5′ end of the cDNA insert within the M13 sequence is suitable). Digestion was carried out at 37°C in a total volume of 100 μl adding the appropriate concentrated restriction enzyme buffer, enzyme, and water. After incubation for 90 min the reaction was stopped with an equal volume (100 μl) of sequencing gel loading buffer, the sample was heated for 1 min at 90°C and single-stranded probe was separated on a denaturing 6% acrylamide/urea gel. The gel was exposed for 10 min, the identified probe excised, electroeluted, ethanol-precipitated, and redissolved in distilled water.

Cytofluorimetry. Cytofluorimetric analysis was performed largely as described (24). mAbs were used as tissue culture supernatants or diluted ascites, followed by FITC-conjugated goat anti-mouse IgG(Fc) or anti-rat Ig (Jackson Immunoresearch and Southern Biotechnology Associates), previously absorbed on mouse spleen cells. For two-color analysis of thymus subpopulations, biotinylated H129-19 (anti-CD4) (14) was followed by phycoerythrin-avidin or phycocyanin-avidin, along with directly FITC-conjugated anti-LyT2 (anti-CD8; Becton-Dickinson). Stained cells were analyzed on an ODAM ATC3000, with logarithmic amplifiers. The various subpopulations were quantitated by curvilinear multiparameter gating on scatter, volume, and green and red fluorescences.

Histology and immunohistology. We searched for gross pathologic abnormalities by dissection and examination of the lymphoid organs and of the gut, liver, pancreas, lung, heart, and kidney of transgenic animals. Tissues were also fixed in Bouin's solution, embedded in paraffin, and analyzed by hematoxylin and eosin staining of sections.

Immunohistology was performed as reported previously (24). Essentially, frozen sections of thymus, mesenteric lymph node and spleen were incubated with various mAb, followed by the appropriate second stage horseradish peroxidase conjugates. Binding was visualized by incubating the sections in di-amino-benzidene. Photographs were recorded with a Zeiss photomicroscope equipped with Plan-Apo objectives and a contrasting filter (Schoot; IL490).

T cell hybridoma stimulation. The T cell hybridoma SKK 45.10 (15) (a kind gift from Dr. P. Marrack) recognizes KLH in the context of A^k molecules. Ag presentation was tested in 96-well plates with 5.10^4 hybridoma cells/well. In some experiments, a fixed amount of Ag (20 μg/ml) was added along with varying numbers of irradiated spleen cells as APC (0.75 to 50 × 10^4); alternatively, varying concentrations of antigen (0.5 to 15 μg/ml) were added along with a fixed (25 × 10^4) number of irradiated spleen cells. 50 μl of supernatant were removed after 24 h and tested for IL-2, by using the IL-2-dependent CTLL line, as described (24).

Autoantibody production. Antinuclear and anti-DNA antibodies were tested by indirect immunofluorescence on tissue sections and on *Crithidia lucillae*, respectively, with an FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch) (25). Antibodies to RNP and Sm Ag were assayed by haemaglutination as described (26).

In vivo responses. Mice were injected s.c. with Ag (usually 25 μg) emulsified in CFA, and were bled 12 to 14 days later. For secondary responses, animals were rested for at least 21 days after injection, then challenged with 5 μg of Ag in PBS, injected i.p. They were bled 7 days later.

Specific antibody production was measured by an ELISA assay, with Ag-coated plates. The developping reagent was a polyvalent antibody (goat anti-mouse IgG + IgM (H + L) coupled to alkaline phosphatase (Jackson Immunoresearch)). Tests usually involved three dilutions of the sera (1/150, 1/1500, and 1/15000); the results are expressed as OD units, either directly or extrapolated from nonsaturating time points.

RESULTS

A^k_α and A^k_β expression in transgenic lines. Large fragments of A^k_α or A^k_β genomic DNA were injected into (B6 × SJL) F_2 or into B6 eggs to create the transgenic lines listed in Table I. The reportedly elevated injection efficiency into hybrid embryos (27) prompted us in the initial set of experiments to choose fertilized eggs from B6 × SJL crosses as recipients. Three independent lines—A_α10, A_α26, and A_β62—resulted from these injections. For A_α26 and A_β62, parallel backcrosses against both parental strains were initiated and have now proceeded for eight generations (Table I). This provides us the opportunity to evaluate the influence of two very different genetic backgrounds (B6 and SJL) on transgene function, a potential

TABLE I

A_α^k and A_β^k transgenic mouse lines

Transgenic Line	Injected DNA	Copy Number (Approximate)	Recipient Strain	Backcross (Generation)
A_α10	A_α^k	50	B6 × SJL	B6 (VIII)
A_α26	A_α^k	20	B6 × SJL	B6 (VIII)
A_α26S				SJL (VIII)
A_α46	A_α^k	4	B6	B6
A_β62	A_β^k	10	B6 × SJL	B6 (XI)
A_β62S				SJL (IX)
A_β42	A_β^k	15	B6	B6

that has already been exploited in assessing the effect of allelic variation on A_β/A_α pairing (28). Subsequent sets of injections were performed directly into genetically homogeneous B6 eggs. Although significantly less efficient, as expected (27), these injections yielded lines—A_α46 and A_β42—which do not require further backcrossing and are devoid of complications resulting from a mixed genetic background. In this paper, we concentrate on the analysis of lines that overexpress A^k molecules: A_α10, A_α26, and A_β62.

Integration of the injected DNA is expected to be random in these lines (27), and we observed no obvious linkage to MHC or coat color genes; the integration sites have not been analyzed further. In all cases, transmission of the transgene from one generation to the next is Mendelian (autosomal). Integration of the foreign DNA does not have noticeable ill effects on the health or breeding potential of the mice in any of these lines, at least when it is in the heterozygous state (no attempts have yet been made to obtain homozygous animals). In general, the transgenes do not noticeably distort the anatomy or cellular composition of lymphoid organs. An exception is the A_β62 transgene, which occasionally leads to somewhat reduced B cell numbers. This phenomenon, which is not influenced by the additional presence of an A_α transgene, may be due to toxicity of high levels of free A_β in B cells (S. Gilfillan and H. McDevitt, personal communication, and our unpublished data).

Before undertaking immunological experiments, it was important to establish that the transgenes are transcribed efficiently and accurately. S1 nuclease analysis was performed on total RNA extracted from various mouse organs and from peritoneal macrophages cultured in the presence or absence of IFN-γ. Since all mouse lines express their endogenous A genes, we used haplotype-specific probes that allowed specific detection of transgene-encoded message. The extreme 5′ end of these probes was designed so that it lacks complementarity to endogenous gene-encoded message but hybridizes perfectly to transgene-encoded RNA. Thus, endogenous message can not protect the labelled 5′ end of these probes against S1-nuclease digestion and only probe hybridized to transgene transcripts can give rise to a signal (Fig. 1).

Figure 2, *A* to *C*, presents the results of representative S1-mapping experiments on RNA from transgenic lines A_β62, A_α10, and A_α26. Clearly, in all three lines, transgene expression closely parallels the pattern normally found for MHC class II genes: high level expression in spleen, lung and thymus, low levels of mRNA in kidney and heart and no detectable message in brain and pancreas. This point is most readily made by comparing transcript patterns from the A_β^k transgene and the A_α endogenous gene in the *upper* and *lower panels* of Figure 2*A*.

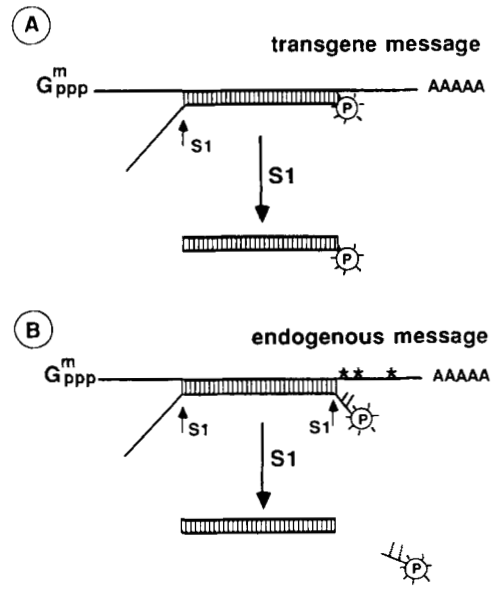

Figure 1. Specific detection of transgene-encoded mRNA in the presence of endogenous gene-encoded transcripts of a different haplotype. *A*, The ^{32}P-end-labeled probe specific for the k-haplotype pairs perfectly along a specified stretch with transgene-encoded mRNA. S1-nuclease digestion gives rise to a labeled fragment which can be separated on a denaturing polyacrylamide gel and detected by autoradiography. G^mppp represents the 5′ cap of the mRNA; AAAAA signifies the poly A tail at the 3′ end of the message. *B*, The very same probe can not pair at its 5′-end with endogenous message (b- or s-haplotype) due to haplotype-specific nucleotides (*) at this position. Although the probe hybridizes well to a specified stretch of the rest of the message, it can not give rise to a signal because the unpaired 5′-end label is highly susceptible to single-strand specific nucleases and thus is removed during S1-nuclease digestion.

The levels of transgene expression vary considerably between the different lines. They are essentially normal in A_α46 (not shown) but elevated in lines A_α10, A_α26, and A_β62 (Fig. 2), when compared with levels in B × A F_1 hybrid controls. This may well be due to the high transgene copy numbers in these lines. Interestingly, transgene overexpression in A_α10 and A_α26 is proportionately most important in lung and thymus, suggesting a major contribution of macrophages to the high expression phenotype.

In order to determine whether the injected transgenes can respond to IFN-γ, a potent activator of class II gene transcription in many antigen presenting cells, we isolated peritoneal macrophages from transgenic mice and cultured them with or without IFN-γ. As shown in Figure 2*D*, IFN-γ treatment led to a strong induction of transgene expression, similar to that of naturally occurring MHC class II genes.

The transgenes present in the various lines thus seem to respond to the transcriptional controls that normally operate on MHC class II genes. To further substantiate this finding, we examined the cell-type distribution of transgene-encoded A^k molecules by cytofluorimetry and immunohistology. Two problems hindered immunodetection of A_α^k or A_β^k: a lack of reagents capable of detecting the individual A chains, and an inefficient formation of mixed-haplotype heterodimers between the host's A_α or A_β chains (b or s alleles) and the transgene products. This block is apparently more drastic for A_α^k than for A_β^k, as

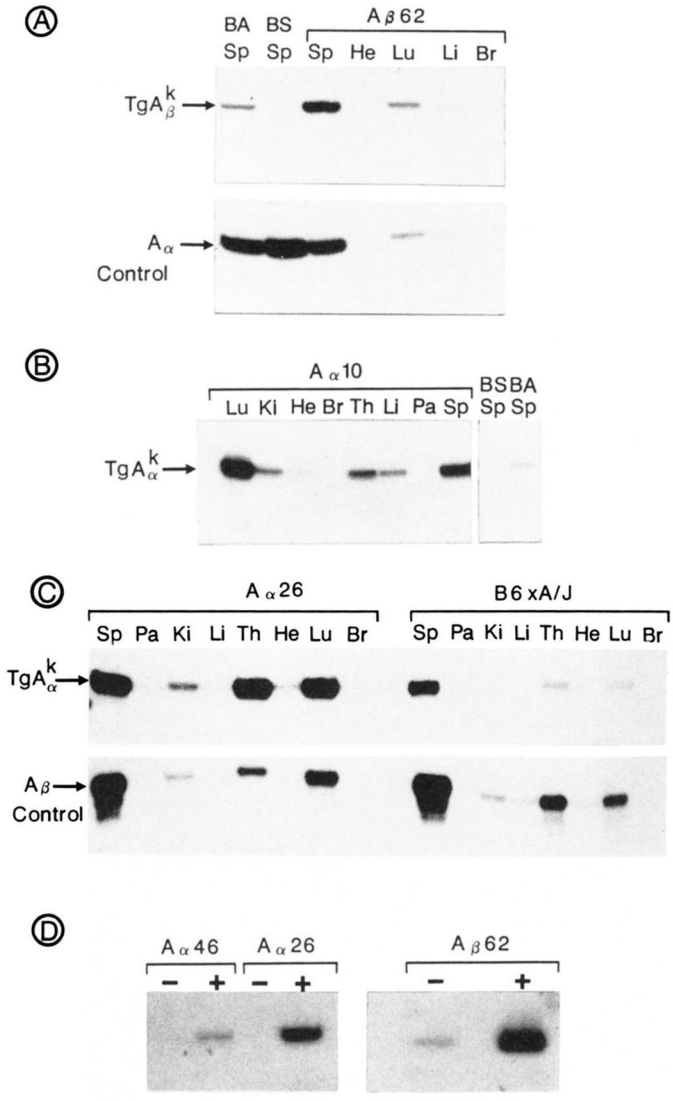

Figure 2. Faithful expression of the A^k transgenes in several mouse lines. *A*, $A_\beta 62$. Quantitative S1-nuclease analysis was performed with an A^k_β-specific probe (*upper panel*) on total RNA extracted from spleen (Sp), heart (He), lung (Lu), liver (Li), and brain (Br). Specificity controls were brought by spleen RNA from B10 × A/J (BA; H-$2^{b,k}$) or B6 × SJL (BS; H-$2^{b,s}$) F_1 hybrids: no signal is detected with the BS sample, whereas the A^k_β mRNA in the BA sample protects a fragment of the expected size. Hybridization of RNA from the same tissues to an A_α probe which does not discriminate between haplotypes (*lower panel*) provides a standard for specific expression of class II genes. *B*, Aα10. An A^k_α-specific probe was used in conjunction with RNA from the tissues listed in A, as well as thymus (Th), kidney (Ki), and pancreas (Pa). *C*, Aα26. The same A^k_α specific probe was used with RNA from tissues of Aα26 or of control B6 × A/J mice. In the lower panel, the same RNAs were probe with an Aβ probe which does not discriminate between haplotypes. *D*, Macrophages from various lines. A^k_α or A^k_β-specific probes were used to monitor the induction of transgene-encoded mRNAs in response to gamma-interferon: thioglycolate-induced peritoneal macrophages were cultured without (− lanes) or with (+ lanes) IFN-γ, and the RNA extracted from the cultured cells was analyzed by quantitative S1-mapping as above.

discussed elsewhere (28). The implication for this paper is that, although the A^k_β chain can be demonstrated in $A_\beta 62$ or $A_\beta 62S$ mice (in association with the host's $A^{b\text{ or }s}_\alpha$ chain), detection of A^k_α will only occur in offspring resulting from a cross with an A^k_β transgenic animal. Consequently, the data presented below were mostly obtained from transgene "double-positive" animals.

Flow cytometric analysis was performed on splenocytes from double-positive animals, using A^k_α- or A^k_β-specific mAbs; B × A F_1 hybrids (H-$2^{b\times k}$) were also included as positive controls. The fluorescence histograms (Fig. 3) indicate that the transgenes do give rise to surface A^k protein, which can be detected with anti-A^k_α or anti-A^k_β reagents. Two-color cytofluorimetric studies with an anti-μ counterstain showed that these A^k molecules are found on virtually all B cells, as are endogenous gene-encoded class II molecules (not shown). Aside from the antibodies used to obtain the curves in Figure 3, a number of other mAbs recognized transgene-encoded A^k molecules on the surface of spleen cells (data not shown; the mAbs are listed in *Materials and Methods*). Since these reagents interact with distinct determinants on A^k_α or A^k_β (29, 30), we have some confidence that we are indeed detecting bona fide A^k molecules. This point is strengthened by results of the functional analyses reported below.

Also evident from the profiles shown in Figure 3 is that surface A^k levels vary markedly between the transgenic F_1 lines. Although the $A_\alpha 46 \times A_\beta 62$ combination shows fluorescence intensities that are not very different from those of normal B × A heterozygotes, splenocytes from $A_\alpha 26 \times A_\beta 62$ transgene double-positives are significantly brighter. In the experiment shown in Figure 3*A* the increase over B × A levels is quite dramatic, approximately 10-fold. Analysis of a large number of animals from $A_\alpha 26 \times A_\beta 62$ crosses has consistently shown this result, with fluorescence intensities ranging from 3- to 10-fold (usually about 5-fold) over intensities from B × A splenocytes. This A^k overexpression does not significantly influence the number of class II molecules on the cell surface, as stainings with anti-A^b mAb give similar fluorescence intensities for $A_\alpha 26 \times A_\beta 62$ double-positives and transgene-negative littermate controls (not shown). Staining with nondiscriminating reagents which detect transgenic as well as host A molecules thus give 2- to 5-fold higher staining intensities with double-positive transgenics than with control littermates. These observations hold true whether the transgenes are on the B6 or the SJL genetic background ($A_\alpha 26 \times A_\beta 62$ or $A_\alpha 26S \times A_\beta 26S$).

To investigate expression on other Ia-positive cell types, we examined cryostat sections of thymus, lymph node and spleen after immunofluorescent or immunoperoxidase staining. Again, the mice were double-positive transgenics, resulting from crosses of the $A_\alpha 26$ and $A_\beta 62$ lines. The patterns observed after A^k_α or A^k_β specific staining (mAb H116-32 and 39E) imply a normal distribution of class II molecules in these organs, and were superimposable on the staining pattern of the host MHC (mAb 2A2). Further, the overexpression of A^k molecules originally detected on splenic B cells was also observed in epithelial cells, macrophages and dendritic cells. A representative section is shown in Figure 4. The anti-A^k_β staining is clearly much more intense on the thymus section from the $A_\alpha 26 \times A_\beta 62$ mouse than from the B × A control mouse. This is true of the cortical epithelial cells as well as of the medullary epithelial or interdigitating cells. It should be noted that the two thymus sections were stained, developed and photographed under the same conditions. Ia overexpression is also evidenced in thymic sections stained with the public anti-I-A mAb 2A2 (Fig. 5, *a* and *b*).

Thus, the A^k_α and A^k_β transgenes seem to be expressed, at the RNA and protein levels, with full tissue and cell-type specificity. In some transgenic strain combinations, such as $A_\alpha 46 \times A_\beta 62$ and $A_\alpha 46 \times A_\beta 42$, the levels of complex are roughly similar to those of analogous natu-

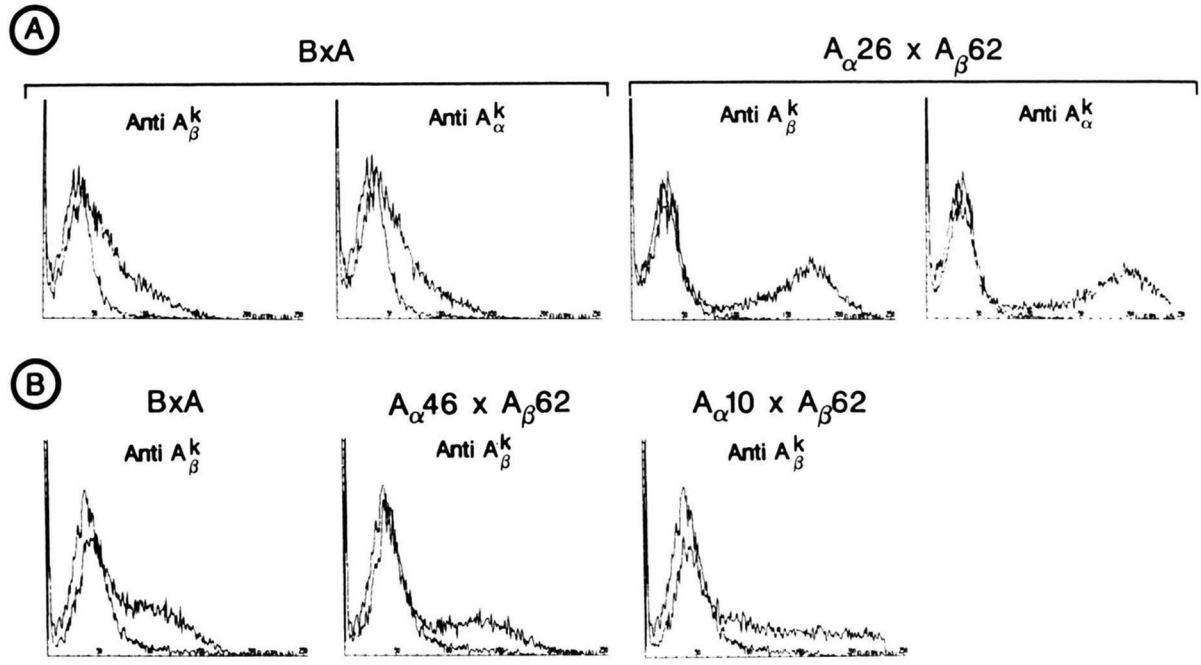

Figure 3. Cell-surface expression of transgene-encoded A^k molecules. Two representative experiments are shown in *panels A* and *B*. Splenocytes were tested by flow cytometry after staining with anti-A^k_β (39E) or anti-A^k_α (H-116-32) mAb. The spleen cell suspensions were prepared from B10 × A/J F_1 hybrid mice (B × A) as positive controls, or from hybrids between the Aα10, Aα26, Aα46, and Aβ62 transgenic mouse lines, as indicated. The axis represents fluorescence intensity on a logarithmic scale; the ordinate shows cell number.

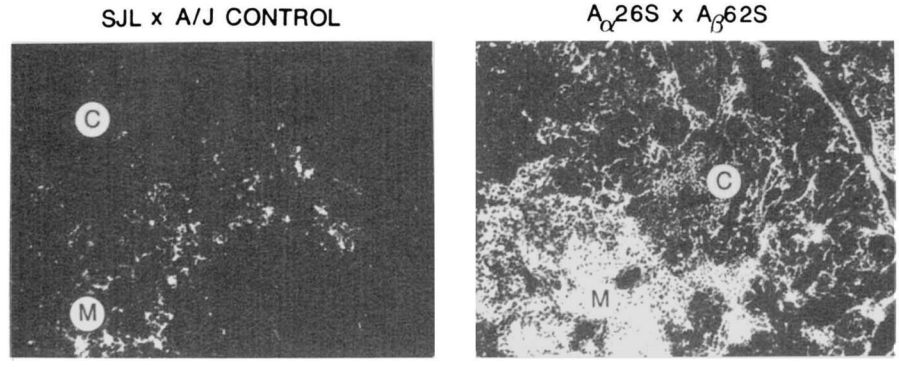

Figure 4. Ia molecule overexpression in the thymus. Cryostat sections of thymic tissue was prepared from 8-wk-old animals, either a hybrid between the $A_\alpha 26S$ and the $A_\beta 62S$ transgenic mouse lines, or from a control SJL × A/J F_1 hybrid. The sections were stained in parallel with the anti-A^k_α reagent H116-32 and an FITC-labeled goat anti-mouse IgG (Fc) antibody. Fluorescence micrographs were taken under the same conditions. M, medulla; C, cortex.

rally occurring mice. In other combinations, such as $A_\alpha 10 \times A_\beta 62$ and $A_\alpha 26 \times A_\beta 62$, surface A complex levels are significantly elevated (about 5-fold on average; these animals will be referred to as 5× mice, for short). The remainder of this report will focus on the consequences of this overexpression for immune system organization and function.

Ia molecule overexpression: consequences on immune system organization. We first sought to determine whether elevated levels of Ia Ag modify the architecture of the immune system or the distribution of cells within various lymphocyte subpopulations.

Examination of hematoxylin-eosin stained sections of paraffin-embedded tissues (spleen, lymph node and kidney) did not reveal any gross histological differences between the 5× transgenic mice and controls (data not shown). Neither did finer immunohistologic analyses. For example, thymic stromal components appear indistinguishable: staining with ER-TR4, which detects cortical epithelial cells, shows a comparable fine network in the thymus of both animals (Fig. 5, *c* and *d*) and the medullary epithelial stroma also appear the same upon staining with ER-TR5 (Fig. 5, *e* and *f*). The architecture of the peripheral lymphoid organs also seems unaltered by Ia molecule overexpression: lymph node paracortical T cell areas (not shown) and B cell follicles (revealed with an anti-B220 reagent—Fig. 5, *g* and *h*) both appear normal.

It remained possible that within this apparently normal architecture, lymphocyte subpopulations were disturbed by Ia overexpression. The most likely organ to show such an effect would probably be the thymus, where T cells are selected to be restricted by but tolerant of the self MHC molecules (see Refs. 31 and 32 for review and references). Kruisbeek and co-workers (33) have reported that mAb blocking of Ia Ag in the thymus prevents the maturation of class II-restricted $CD4^+$ T cells. Thus, one might expect thymuses from 5× mice to contain more mature $CD4^+CD8^-$ T cells. Nonetheless, immunohistologic studies with mAb directed against Thy-1, CD4, CD8, CD5, and MEL14, showed a normal distribution of thymocyte subsets (not shown). Two-color flow cytometric analysis was also performed on transgene double-positive 5× mice and on control littermates (carrying only one or none of the transgenes), by using anti-CD4 and anti-CD8 reagents. Three representative experiments are reported in Table II. A clear influence of genetic background on the relative proportions of the various subpopulations is observed: substantially more mature thymocytes occur

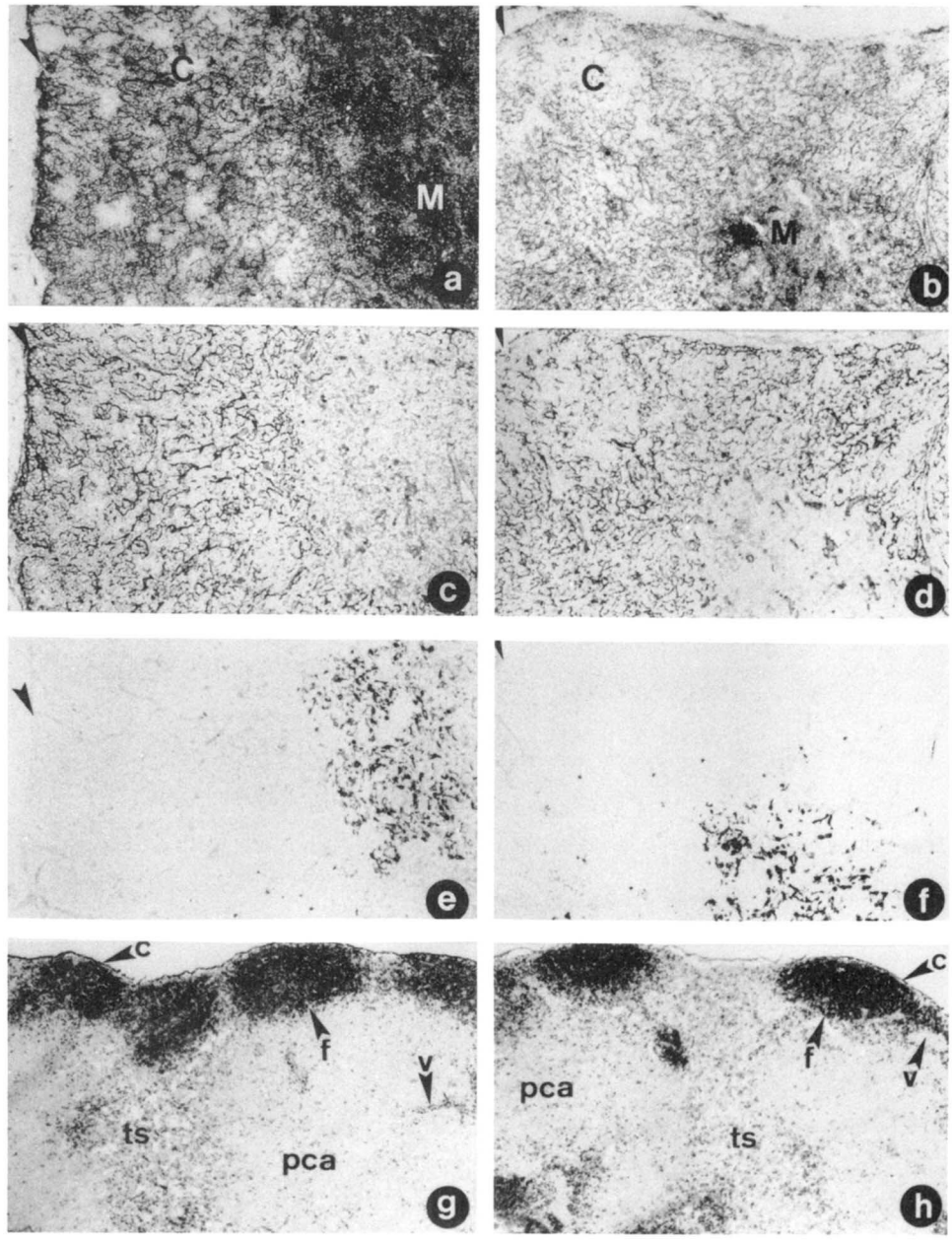

Figure 5. Adjacent frozen sections of the thymus of I-A overexpressing mice (*a*, *c*, and *e*) and littermate control mice (*b*, *d*, and *f*). Sections *a* and *b* are labeled with 2A2 (anti A_α); sections *c* and *d* are labeled with ER-TR4; sections *e* and *f* are labeled with ER-TR5. C = cortex; M = medulla. The *arrowheads* in *a* to *f* indicate (for orientation) the thymic capsule. The mesenteric lymph node of an I-A-overexpressing mouse is shown in *g*; *h* represents the mesenteric node in littermate controls. These sections are labeled with RA2 6B2 (anti-B220). c = capsule; f = lymphoid follicle; pca = paracortical area; ts = trabecular sinus; v = high endothelial venule.

on the SJL than on the B6 background. There is, however, little influence of the transgenes on subpopulation percentages: thymuses of 5× mice do not have more mature, class II-restricted $CD4^+CD8^-$ T cells; if anything, they have marginally less (although this decreased percentage remained, in most cases, within the normal range). We did repeatedly observe a shift in staining intensity with the anti-CD4 antibody; cortical thymocytes ($CD4^+CD8^+$) appear duller in 5× mice than in control littermates (refer to Table II as well as the two-color plots shown in Fig. 6). This reduction in the number of CD4 molecules varies somewhat with individual 5× animals, ranging from 15 to 60%. It seems to affect the immature $CD4^+CD8^+$ population more than the mature $CD4^+CD8^-$ cells (Table II). The anti-CD8 fluorescence intensities are not similarly affected.

Lymphocyte populations were also investigated in peripheral lymphoid organs. As can be seen in the representative experiments reported in Table III, 5× mice are not much different from control littermates. B cell numbers, as well as fluorescence intensity with anti-Ig reagents, are not altered, at least no more than they are by single transgenes alone. T cell subpopulations are also normal. The staining intensity with anti-CD4 is identical in all samples, regardless of transgenes and Ia levels.

To summarize this section, overexpression of Ia molecules does not seriously alter the organization of the immune system: no anatomical anomalies were detected, nor were any severe perturbations of lymphocyte subpopulations.

Ia molecule overexpression: consequences on immune system function. Next, we sought to determine whether the overexpression of Ia molecules affected various immune system functions. Considering previously published results on variants or on transfected cells (6–10), we first tested antigen presentation potential. The T cell hybridoma SKK45.10, specific for KLH in the context of A^k molecules (15), was used to assay the APC potential of splenocytes from 5× mice versus control animals. Under conditions of limiting antigen concentration or

TABLE II

Thymocyte subpopulations in "5×" transgenic mice

Experiment	$CD4^-CD8^-$[a]	$CD4^+CD8^+$	$CD4^-CD8^+$	$CD4^+CD8^-$
Experiment I: SJL Background				
Transgenic mice[b]				
Control (negative)	4.1	57.7 (145)	14.4	23.7 (162)
Control (A_α26S)	4.9	57.5 (135)	15.5	22.3 (161)
Control (A_β62S)	4.4	61.6 (153)	12.1	21.8 (157)
5× (A_α26S/A_β62S)	4.9	61.3 (119)	17.6	16.1 (139)
Experiment II: B6 Background				
Transgenic mice				
Control (negative)	4.7	81.1 (120)	5.2	9.0 (142)
Control (A_α26)	4.3	80.8 (121)	3.1	11.8 (155)
Control (A_β62)	6.6	75.5 (115)	4.0	13.9 (154)
5× (A_α26 + A_β62)	5.0	80.5 (106)	6.0	8.3 (146)
Experiment III: SJL Background				
Control (A_α26)	3.2	66.7 (161)	8.2	21.8 (191)
5× (A_α26 + A_β62)	3.8	69.3 (121)	15.8	11.0 (151)

[a] Thymocytes were stained with anti-L3T4 (CD4) and anti-LyT2 (CD8) mAb, and analyzed by two-color flow cytometry, as described in *Materials and Methods*. The four T cell subpopulations were quantitated by curvilinear gating on two-dimensional cytograms of anti-CD4 vs anti-CD8 fluorescence intensities. Values indicate the percentage of cells in each subpopulation for individual mice. Values in parentheses are the mean fluorescence intensities of anti-CD4 staining (as channel number on a 1-256 log scale; a 30-channel shift correspond to a two-fold difference in fluorescence intensity). These numbers should only be compared within experiments, as laser or ATC settings varied between experiments.

[b] Matings were set up between animals carrying the A_α26 and A_β62 transgenes on the SJL (Expts. I and III) or B6 (Expt. II) backgrounds. Offspring were analyzed by Southern blotting of tail DNA to determine which transgenes they carried. The genotypes of the individual mice are listed in this column.

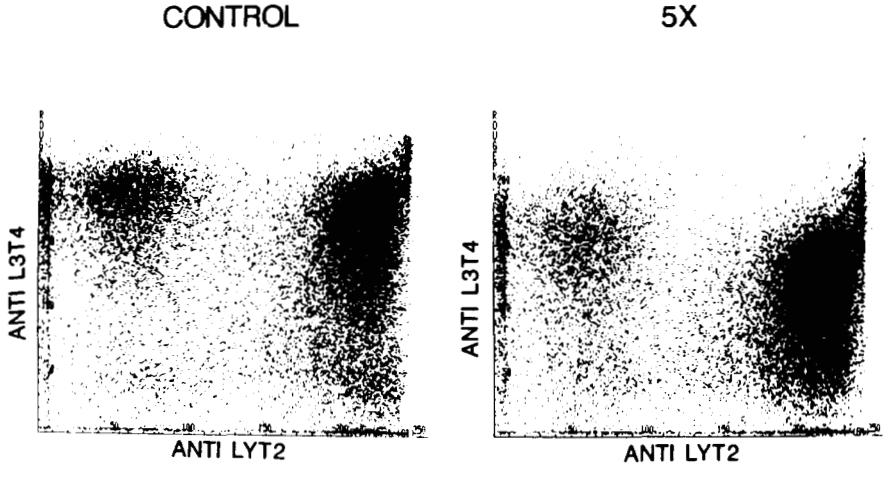

Figure 6. Reduction in CD4 staining intensity on thymocytes from Ia-overexpressing transgenic mice. Thymocytes from a 5× mouse or a control littermate were stained with reagents directed against the CD4 (anti-L3T4) or CD8 (anti-LyT2) differentiation markers. The plots derive from two-color flow cytometric analysis.

TABLE III

Peripheral lymphocyte populations in 5× mice

Experiment	Ig^+	$CD4^+$	$CD8^+$
Experiment I (Lymph nodes)			
Control (Negative)	51	23 (150)	12
Control (A_α26S)	31	29 (146)	17
Control (A_β62S)	54	21 (158)	11
5× (A_α26S × A_β62S)	40	25 (151)	11
Experiment II (Spleens)			
Control (negative)	42	33	15
Control (A_α26S)	36	35	10
Control (A_β62S)	34	37	14
5× (A_α26S + A_β62S)	48	31	11
Experiment III (Lymph nodes)			
Control (negative)	43	19	
Control (A_α26)	47	16	
Control (A_β62)	70	17	
5 × (A_α26 + A_β62)	60	13	

APC number (Fig. 7), presentation by splenocytes from 5× mice was clearly more effective than presentation by control B × A spleen cells. The dose/response curves for 5× mouse splenocytes are shifted by a factor of 5 to 10 relative to the control, which correlates well with the increase in surface A^k levels in these spleens. Incidentally, this experiment also confirms results obtained by Lechler and co-workers (34) showing that both A_α and A_β need to be of the k haplotype for presentation to SKK45.10: we detect no T cell activation with spleen cells from A_α26 or A_β62 mice—only from the double positive animals.

Having, as anticipated, observed a heightened presentation potential in 5× mice, we asked whether immune responses in general are more efficient. We first performed an in vivo experiment parallel to the in vitro assay described above: limiting doses of KLH were injected into 5× and control mice, and specific antibody response measured in an ELISA assay. KLH was the Ag of choice because it is an effective immunogen, not under restrictive Ir gene control: preliminary experiments with B6 and B10.A mice had found no influence of the MHC on responses to low doses of KLH. The results plotted in Figure 8*A* show that the primary anti-KLH responses in 5× mice are not much different from those of control mice: very low doses (15 and 20 ng/mouse) do not elicit strong responses in any of the animals, whereas a dose of 150 ng is the first effective dose, whether in 5× mice or in control littermates. Primary responses to a nonsaturating dose of BSA are also indistinguishable (Fig. 8*B*). Finally, neither the time nor the magnitude of the secondary response to KLH differ greatly with 5× vs control mice (Fig. 8*C*).

We also tested the secondary antibody response to the synthetic copolymer HGAL, which is thought to be con-

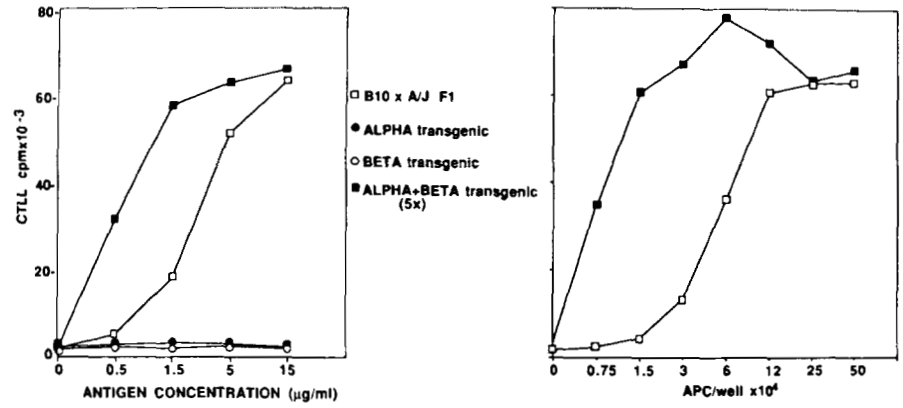

Figure 7. Ia molecule overexpression enhances in vitro Ag presentation. The response of the T cell hybridoma SKK45-10 (KLH-specific, A^k-restricted; 15) was measured using spleen cells from several mice as antigen presentors. These mice included a control B10 × A/J F_1 hybrid (open squares), a 5× (Ia-overexpressing) double-transgenic mouse (Aα26 × Aβ62; *dark squares*) and Aα26 or Aβ62 single transgenic mice (*dark* and *open circles*, respectively). IL-2 production was quantitated by the CTLL assay, and represented by [^{3}H]thymidine incorporation by the CTLL cells. Maximum incorporation by the CTLL cells in these experiments was between 70 and 80 cpm × 10^{-3}, in response to saturating levels of IL-2.

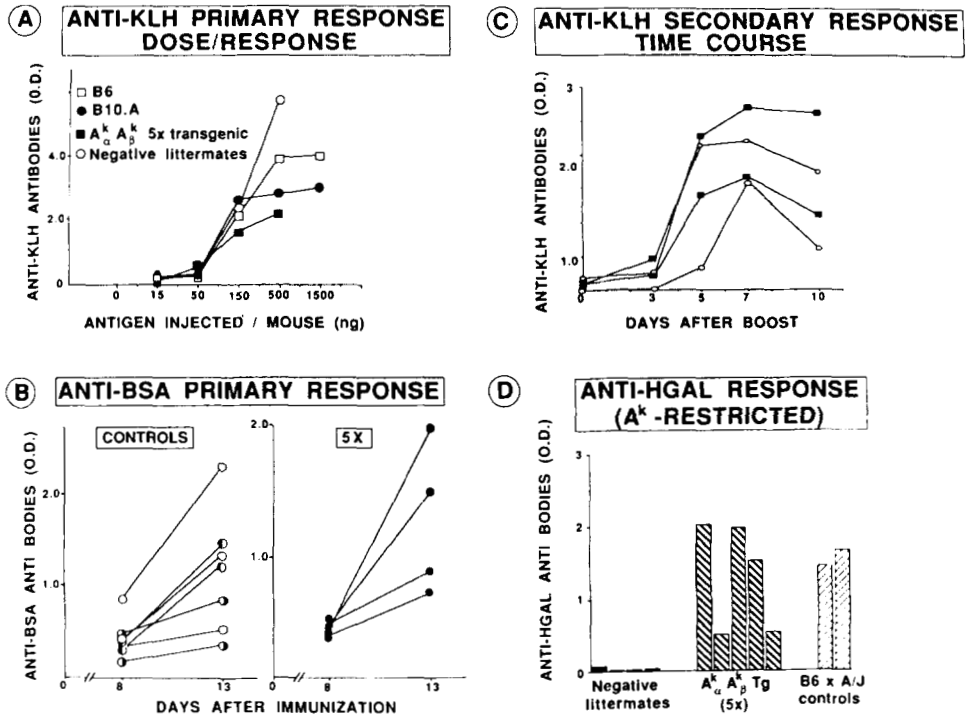

Figure 8. Ia molecule overexpression does not significantly elevate in vivo antibody responses. *A*, KLH primary responses. KLH-specific antibodies were detected by an ELISA assay after immunization with limiting doses of KLH. Immunization was performed by injecting into the footpad the indicated dose of Ag, emulsified in CFA. Antibody responses were measured 12 days later. Each point represents a single animal. The values shown here represent the optical density reading at a 1/1500 dilution of the sera, either uncorrected (values below 2.0), or extrapolated from readings at shorter phosphatase reaction times. *B*, BSA primary responses. BSA-specific antibodies were measured in response to primary immunization with 20 μg of BSA, as above. *Right panel*: Ia-overexpressing Aα26 × Aβ62 double transgenic mice. *Left panel*: control littermates, either nontransgenic (○) or A_α26 and A_β62 single transgenic mice (○ and ●, respectively). *C*, KLH secondary responses. Animals (5× or control littermates) were boosted with 10 μg of KLH in phosphate-buffered saline, 6 wk after a primary immunization with 500 ng of KLH in CFA. The mice were bled at several times and anti-KLH antibody titred in an ELISA assay. *D*, HGAL secondary responses. Negative littermates, 5× mice or normal B6 × A/J controls were immunized with 25 μg of HGAL in CFA, i.p. They were boosted 3 wk later with 5 μg of HGAL in PBS, and bled 8 days later. HGAL-specific antibodies were determined by an ELISA assay.

trolled by the A^k molecule (35, 36). As shown in Figure 8*D*, the combination of A^k_α and A^k_β transgenes lead to a strong anti-HGAL response, absent in the control littermates. These data formally establishes that the response to HGAL is indeed linked to the A molecule. Both chains are necessary for the full response, although a partial response can be obtained with the A^k_β transgene alone (not shown). The data also indicate that the response seen in mice with the overexpressed restricting element is of the same order as that in normal A^k-positive mice (B × A).

One potential consequence of an overexpression of Ia antigens could be a breakdown in Ir gene control: it is conceivable that responses not normally observed with A^k mice might surface with 5× animals, a greater density of A^k molecules compensating for their poor intrinsic presentation capability. Hence, we challenged transgene double-positive mice from A_α26S × A_β62S crosses with GLØ or beef insulin, antigens to which A^k E^- or A^s E^- mice do not respond. 5× mice did not respond to either of the antigens (not shown).

One could envisage that Ia molecule overexpression might lead to an overstimulation of the immune system, spontaneously or in conjunction with environmental stimuli; two abnormal conditions might ensue: lymphoproliferation or autoimmune reactivity. No signs of excessive lymphocyte proliferation were noticed in 5× animals, at ages ranging from 3 wk to 10 mo. Thymus, spleen and lymph node size and cell content were never significantly different from those of control littermates. As noted above, lymphoid organs from 5× mice had normal anatomical and histologic structure.

To test for autoimmune reactivity, we assayed anti-organ and anti-nuclear antibodies in the sera of several 5× mice ranging in age from 1 to 10 mo. Some of the animals were evaluated for autoantibodies before and after immune stimulation (immunization with CFA). We were unable to find any anti-organ auto-antibodies on cryostat sections of various organs (data not shown). Table IV presents results from the antinuclear antibody

TABLE IV

Antinuclear and anti-DNA antibodies in transgenic mice

Mice	Number of Positive Mice/Number of Mice Tested (Titer of Individual Positive Sera)			
	Antinuclear antibodies	Anti-DNA antibodies	Anti-RNP antibodies	Anti-Sm antibodies
Controls				
Transgene-negative littermates	1/12 (1/160e)	0/12	0/12	0/12
A_α^k or A_β^k only	2/15 (1/80e; 1/640e)	2/15 (1/10e; 1/40e)	0/15	0/15
Double-positives				
A_α^k/A_β^k	1/8 (1/40e)	0/8	0/8	0/8

tests. The frequency of individuals exhibiting general antinuclear and anti-DNA antibodies was not significantly different with 5× mice vs control animals. Antinuclear antibodies occurred occasionally, the highest titers being in a transgene-negative mouse (1/160e) and in a single-positive $A\beta$ transgenic mouse (1/640e). All animals were negative for anti-RNP and anti-Sm antibodies. Furthermore, immune stimulation by injection of CFA did not elicit autoantibodies.

DISCUSSION

Several transgenic mouse lines carrying an A_α^k or A_β^k gene on an H-2^b or H-2^s background are described in this report. The transgenes appear to respond normally to all tissue and cell-type regulatory controls: tissue distribution and lymphokine inducibility of the transgene-encoded mRNAs are normal, and these mRNA do not appear in class II-negative organs such as the brain, even in lines with the highest transgene copy numbers (Fig. 2). Furthermore, immunohistology has established that A^k molecules are found on all expected cell types—and only on these cell types—whether they reside in the thymus or in peripheral organs.

Although the transgenes operate properly in a qualitative sense, they do show large quantitative variations in transcriptional effectiveness—variations that correlate well with transgene copy number. Some lines, such as $A_\alpha 10$, $A_\alpha 26$ or $A_\beta 62$, clearly have exaggerated levels of A^k mRNA in the spleen and lung, and this overexpression results in elevated levels of A^k protein on the cell surface in the appropriate crosses. For example, cytofluorimetric analysis showed that $A_\alpha 26 \times A_\beta 62$ matings produce mice with an Ia molecule density roughly 3- to 5-fold greater than normal. Immunohistologic studies further established that all classes of Ia-positive cells show a similar enhancement of Ia molecule expression. One implication of these findings is that Ia surface density is directly linked to steady-state mRNA amounts, protein transport or modification steps not being rate limiting (at least at the densities studied here).

These "5×" mice have allowed us to study the biologic consequences of generalized Ia overexpression, a subject which has been the topic of some speculation (see Ref. 11 and references therein). We find that the overall organization of the immune system is hardly affected by transgene-induced overexpression. The shape, size and architecture of lymphoid organs are normal, and there appear to be only slight changes in the distribution of lymphocyte subpopulations. While these effects are minor and somewhat variable in extent, they have been observed reproducibly in numerous experiments; it is interesting to consider them in light of current ideas on the selection of the T cell repertoire (31, 32). In the thymus, we frequently observe a decrease in the frequency of mature, class II-restricted, $CD4^+CD8^-$ cells (Table II). This decrease is presumably due to more efficient negative selection: an increased density of A molecules may lower the affinity requirements for the deletion of a particular clonotype. Lower surface levels of CD4 protein are also routinely found on thymocytes of 5× mice (Table II, Fig. 6), consistent with the idea that thymic selection operates concomittantly on the TCR and on CD4 and CD8 accessory molecules (37, 38). One envisages the TCR, Ia and CD4 molecules as participating in a three-way interaction. Our results complement those of McCarthy et al., which demonstrate that treatment of mice with anti-CD4 antibodies leads to (is compensated by ?) higher levels of surface TCR. It is not clear, though, whether the decrease in the density of CD4 on thymocytes as a consequence of increased Ia density on thymic stroma reflects a direct effect on double-positive thymocytes, less stringent requirements in a positive selection step, or a more efficient negative selection step.

The effects on $CD4^+$ thymocytes are different from those observed with $CD4^+$ T cells from the peripheral lymphoid organs of 5× mice. In these locations, we find similar frequencies of $CD4^+$ cells in all mice, and the levels of CD4 protein are indistinguishable (Table III). The peripheral T cell population has thus been "corrected," relative to the mature cells imported from the thymus. Such post-thymic modifications of the T cell repertoire have been described previously (39).

Just as the organization of the immune system is not greatly perturbed by A^k overexpression, immune function also appears essentially unaffected, at least by the assays we have so far employed. We did detect, as expected from several previous studies (6–10), an increased antigen presentation capacity by spleen cells from 5× mice (Fig. 7). There were, however, no apparent changes in the kinetic course or magnitude of in vivo immune responses, as assayed under non-saturating conditions (Fig. 8). One must assume that Ag presentation is not the rate-limiting step in such immune responses. Similarly, Ia molecule overexpression does not, in and of itself, provoke lymphoproliferative or autoimmune disorders. It would be interesting to perform the same studies in the context of an autoimmune-prone mouse, it being conceivable that Ia overexpression could accelerate disease on such a genetic background, but not affect resistant B6 or SJL mice.

In short, we find Ia molecule overexpression to be of remarkably little consequence to the murine immune system. Provided expression remains above a critical threshold (10), quantitative variations in Ia Ag must not play a central role in immune regulation. Of course, this contention can only refer to variations in the 5-fold range, as we have described in this report. Yet, fluctuations of this magnitude are known in at least one case to turn a "responder" mouse into a "nonresponder" (5).

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