Functional expression of a microinjected E^d_α gene in C57BL/6 transgenic mice

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The class II major histocompatibility antigens, I-A and I-E, have been detected on the surface of certain immunocompetent cells, including B lymphocytes and monocytes[1–3]. These molecules are involved in cell–cell interactions in the immune responses[4,5]. Each class II antigen consists of two subunits, α and β chains, and the genes encoding these subunits have been well characterized at the molecular level[6–9]. To analyse the regulatory mechanism of E_α gene expression and the role of the I-E antigen in the regulation of the immune responses, we have produced transgenic mice by microinjecting cloned E^d_α genes into fertilized eggs of C57BL/6 mice of *b* haplotype. This strain of mouse carries a deletion in the upstream (5′) region of the E_α gene covering the transcriptional promoter and, therefore, does not express this gene[10]. Interestingly, this genetic defect of the E_α gene is accompanied by the inability of the host mouse to respond to a certain set of antigens, phenomena generally termed *Ir* gene control[11,12]. We report here that the E^d_α genes are expressed in these transgenic mice to form the I-$E^d_\alpha E^b_\beta$ antigen on the surface of B lymphocytes and monocytes and that these I-E antigens are functional in terms of the induction of a mixed lymphocyte reaction and the restoration of immune responsiveness to poly(L-glutamic acid–L-lysine–L-phenylalanine) (GL-Phe).

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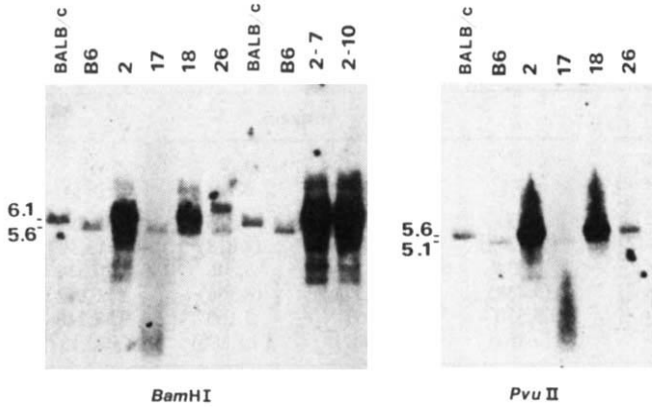

Fig. 1 Southern blot analysis of the E_α gene in transgenic mice[32]. DNAs (5 μg) from liver of BALB/c, C57BL/6 or transgenic mice were digested with *Bam*HI or *Pvu*II, electrophoresed on a 0.5% agarose gel, transferred to a nitrocellulose filter and hybridized with the ^{32}P-labelled 2.6-kb *Sal*I fragment which includes the first exon of the E_α gene. The host C57BL/6 mice carry a deletion in this region[32]. Mice 2, 17, 18 and 26 were born from ova injected with E^d_α DNA. Mouse 17 did not contain injected genes. The offspring (nos 2-7 and 2-10) of mouse 2 were produced by mating mouse 2 with a female C57BL/6 mouse. DNAs from mice 2-7 and 2-10 were also analysed by *Bam*HI digestion. Sizes of DNA fragments are in kilobases.

Approximately 200 copies of the 14-kilobase (kb) *Sac*II/*Xho*I fragment containing the entire E^d_α gene sequence were microinjected into each fertilized egg of the C57BL/6 mouse. In total, 30 mice were born. These mice were partially hepatectomized and the DNA isolated from the livers was analysed by Southern blotting to determine whether the injected E^d_α gene is retained in the host DNA. Because of the aforementioned deletion, BALB/c DNA yielded a DNA fragment 0.5 kb larger than that of C57BL/6 DNA when these DNAs were digested either with *Bam*HI or *Pvu*II and the digests analysed with a probe covering the 5′ end of the E_α gene (see Fig. 1). This allows us to distinguish the injected E^d_α gene from the endogenous defective E^b_α gene present in the host C57BL/6 mouse. As expected, DNA from transgenic mice gave the 5.6-kb *Bam*HI or 5.1-kb *Pvu*II band characteristic of the C57BL/6-derived, defective E^b_α gene (Fig. 1). In addition, DNA from transgenic mice 2 and 18 gave the 6.1-kb *Bam*HI band and 5.6-kb *Pvu*II band diagnostic of the E^d_α gene, indicating that these mice have retained the injected BALB/c E^d_α gene. DNA from the third transgenic mouse, no.

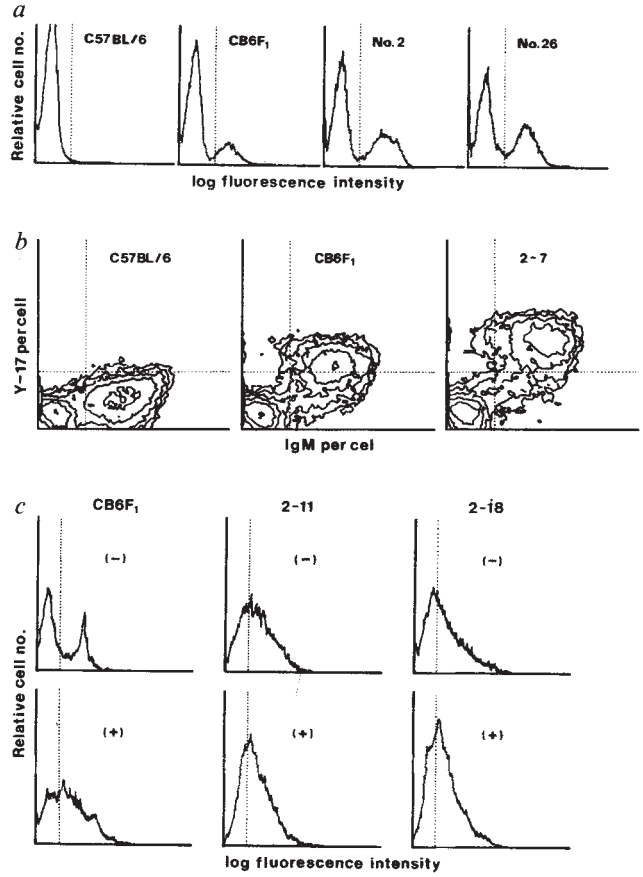

Fig. 2 Expression of the I-$E^{d/b}$ antigens on lymphocytes and peritoneal monocytes of the transgenic mice. *a*, Mononuclear cells were prepared from peripheral blood of $CB6F_1$ and C57BL/6 mice and of mice 2 and 26 on a Ficoll–Hypaque gradient. Cells were incubated with the biotinated Y17 antibody, followed by fluoresceinated avidin and analysed with a fluorescence-activated cell sorter, FACS-440 (Becton-Dickinson). *b*, Spleen lymphocytes from $CB6F_1$, C57BL/6 mice and an offspring (no. 2-7) of mouse 2 were incubated with the Y17 antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse μ chain, followed by phycoerythrin-conjugated avidin and analysed with a cell sorter. *c*, Peritoneal exudate cells were prepared from $CB6F_1$ mice and from the offspring of mouse 2 which had been injected intraperitoneally with 1 ml 3% proteose peptone 3 days earlier. Cells (1×10^6 in 1 ml) were incubated in a 24-well plastic plate at 37 °C and non-adherent cells were removed by washing with Hank's balanced salt solution. Adherent cells were cultured with or without 50% (v/v) supernatant of Con A-stimulated spleen cells for 2 days. This supernatant contains, respectively, 100 U ml^{-1} and 650 U ml^{-1} γ-interferon and interleukin-2 activity. The cells were then stained with the Y17 antibody and analysed with a cell sorter.

26, also displayed the 5.6-kb *Pvu*II band but the size of the corresponding *Bam*HI fragment was somewhat greater than 6.1 kb. This discrepancy may result from loss of the 5′ *Bam*HI site. From an approximate calculation based on the intensities of the E_α^d bands, mice 2 and 18 seem to have at least 10 copies of the E_α^d gene. These E_α^d genes were transmitted to a fraction of offspring, two examples of which (nos 2-7 and 2-10) are shown in Fig. 1. In the following studies, both the original transgenic mice and their offspring were used.

Expression of the E_α gene in the transgenic mice was examined by the surface staining of peripheral blood mononuclear cells and splenic lymphocytes using a monoclonal anti-I-E antibody, Y17 (ref. 13). Because this antibody can recognize combinations of E_α and E_β molecules of various haplotypes, including $E_\alpha^d E_\beta^b$, we could examine whether the product of the microinjected E_α^d gene can pair with the endogenous E_β^b chain and be displayed on the cell surface as the hybrid $E_\alpha^d E_\beta^b$ molecule. As shown in Fig. 2*a*, about one-third of the peripheral blood lymphocytes from mice 2 and 26 could be stained with Y17, indicating that the lymphocytes of the transgenic mice expressed the hybrid I-$E^{d/b}$ antigen. The fluorescence intensity of the Y17-positive cells of the transgenic mice seems to be significantly higher than that of a $CB6F_1$ (C57BL/6 × BALB/c F_1) mouse. As expected, the I-$E^{d/b}$ antigen was mainly expressed on B lymphocytes bearing immunoglobulin M (IgM) molecules on the cell surface (Fig. 2*b*). We also analysed E_α^d gene expression on the monocytes of the transgenic mice. Peritoneal monocytes of the transgenic and $CB6F_1$ mice, as the control, were cultured for 48 h with or without the culture supernatant of concanavalin A (Con A)-stimulated murine spleen cells containing 50 U ml^{-1} of γ-interferon and stained with the Y17 antibody. As expected, the I-$E^{d/b}$ molecules were detected on a very small fraction of the unstimulated cells and on most of the stimulated cells of the $CB6F_1$ mouse. In contrast, most of the peritoneal monocytes from the transgenic mice expressed the I-$E^{d/b}$ antigen in the absence of the Con A supernatant and the addition of the supernatant to the culture media did not increase the level of I-$E^{d/b}$ expression. The reason for this constitutive expression is unknown, but it may be related to the fact that the transgenic mice examined (nos 2-11 and 2-18) carry a large copy number (>20) of E_α^d genes. In any case, it is interesting that not only the injected E_α^d genes but also the endogeneous E_β^b gene seem to be constitutively expressed in these mice.

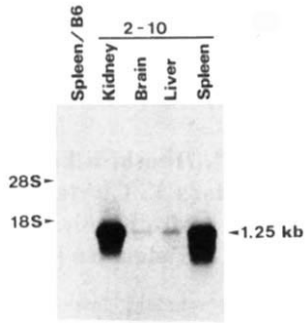

Fig. 3 Northern blot analysis of E_α mRNA from various tissues of a transgenic mouse. Total RNA was phenol-extracted from spleen, liver, kidney and brain of a transgenic mouse and adsorbed to oligo(dT)-cellulose; 2 μg poly(A)$^+$ RNA from spleen and 5 μg poly(A)$^+$ RNA from liver, kidney and brain were denatured, electrophoresed on a 1% formaldehyde-containing agarose gel, transferred to a nitrocellulose filter and hybridized with a ^{32}P-labelled 3.5-kb *Sal*I fragment containing exons 2, 3 and 4 of the E_α gene as described previously[33]. As a control, 2 μg mRNA from C57BL/6 spleen was also analysed. Sizes of RNA molecules are shown in kilobases.

To examine the transcription of the microinjected E_α^d gene, we performed a Northern blot analysis of the E_α transcript in various tissues from a transgenic mouse, no. 2-10. Figure 3 shows that detectable amounts of E_α messenger RNA of 1.25 kb were observed in poly(A)$^+$ RNA from all analysed tissues of the transgenic mouse. The microinjected E_α^d gene seemed to be transcribed at a low level in liver and brain cells. Although kidney cells of a transgenic mouse contained a relatively large amount of E_α mRNA, similar amounts of E_α transcripts were also observed in BALB/c kidneys (data not shown). The class II antigens are known to be expressed on B lymphocytes and macrophages in lymphoid organs[1-3,14], liver Kupffer cells[15,16], brain astrocytes[16], kidney pelvis cells[15] and blood vessel endothelial cells[17]. The observed transcripts of the E_α gene in tissues of the transgenic mouse may be due to these cells. Recently, a rabbit β-globin gene was reported to be expressed in skeletal muscle and testis but not in erythroid cells in transgenic mice[16]. Immunoglobulin μ heavy-chain genes were also expressed in heart as well as in lymphocytes[19]. We cannot

Table 1 I-$E^{d/b}$ antigen in a transgenic mouse is functional in terms of induction of a mixed lymphocyte reaction and restoration of the response to GL-Phe

	Stimulator cells				Antigens			
	(−)	C57BL/6 (A^bE^-)	Transgenic ($A^bE^{b/d}$)	$CB6F_1$ ($A^{b/d}E^{b/d}$)	(−)	GL-Phe	KLH	PPD
C57BL/6	5,767 (±999)	7,233 (±1,149)	25,622 (±1,459)	90,557 (±15,417)	6,445 (±1,268)	6,326 (±837)	6,373 (±818)	47,296 (±13,307)
Transgenic	—	—	—	—	4,434 (±438)	15,228 (±4,612)	5,846 (±306)	20,314 (±5,061)
$CB6F_1$	—	—	—	—	5,530 (±865)	25,363 (±1,067)	5,160 (±1,580)	58,340 (±22,137)

2.5×10^5 responder lymph node cells from normal C57BL/6 mice were stimulated with 1×10^6 mitomycin C (MMC)-treated cells from C57BL/6, $CB6F_1$ or transgenic mice in 0.2 ml complete culture medium in a microtitre plate (Falcon 3072, Becton-Dickinson) for 4 days. Complete culture medium consisted of RPMI 1640 culture medium, 10% heat inactivated fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 100 mM HEPES, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM glutamine. MMC treatment of stimulator cells: 5×10^6 spleen cells were incubated in 1 ml complete culture medium in the presence of 50 μg MMC at 37 °C in 5% CO_2 in air; after 30 min, cells were washed three times with Hank's balanced salt solution. Cells were pulsed with 1 μCi ^{3}H-TdR for the final 16 h of culture. Cells were collected on a filter paper with a semi-automatic cell harvester (Labo Mash Science, Tokyo) and the uptake of ^{3}H-TdR was measured by standard scintillation counting. The proliferative responses of responder cells are expressed as the uptake of ^{3}H-thymidine (mean ± s.d. of triplicate cultures). The antigen-specific T-cell proliferative response was assayed according to the methods of Corradin *et al.*[31]. C57BL/6 mice (three in one group), $CB6F_1$ mice (three in one group) and a transgenic mouse were immunized with 50 μg GL-Phe (given by Dr R. Schwartz, NIH) and emulsified in the complete Freund's adjuvant. After 1 week, draining lymph nodes were removed and single-cell suspensions made. Cells were cultured in complete culture medium containing 10% heat-inactivated horse serum instead of FCS in the presence of 200 μg ml^{-1} GL-Phe, 100 μg ml^{-1} KLH or 50 μg ml^{-1} purified protein derivative (PPD) from *Mycobacterium tuberculosis* (Mitui Pharmaceuticals, Tokyo). Cells were cultured for 4 days and the proliferative response assayed as described above.

exclude the possibility that the microinjected E_α^d genes are expressed in inappropriate cells of the transgenic mouse. To assess this possibility more precisely, further studies, including *in situ* RNA hybridization using tissue samples, are required.

Finally, we examined whether the newly expressed I-$E^{d/b}$ antigen has immunological functions. Spleen cells of the transgenic mice could induce a significant proliferation of C57BL/6 lymphocytes (Table 1), suggesting that the I-$E^{d/b}$ antigens of the transgenic mice could be successfully recognized as allo-antigens by the T lymphocytes from C57BL/6 mice. It is known that mouse strains of *H-2b, s, f* and *q* haplotypes do not respond to certain antigens such as GL-Phe, GL-Leu and pigeon cytochrome *c* (*Ir* gene phenomena)[20-22]; this unresponsiveness is correlated with the defect in I-E antigen expression in these strains[23]. We therefore used responsiveness to GL-Phe as another criterion for the functional expression of the I-$E^{d/b}$ antigens in the transgenic mice, and attempted to demonstrate directly that the *I-E* gene defect is the cause of the unresponsiveness. For this purpose, lymph node cells from mice primed with GL-Phe in complete Freund's adjuvant were assayed for a proliferative response to GL-Phe and, as a control, to keyhole limpet haemocyanin (KLH). We found that GL-Phe could induce significant proliferation of the lymphocytes from $CB6F_1$ mice (positive control) and from the transgenic mouse but not of lymphocytes from C57BL/6 mice (negative control) (Table 1). The proliferation was specific to the priming antigen (GL-Phe) because no proliferation above the background level was observed in the presence of KLH. These results suggest strongly that the product of the microinjected E_α gene is expressed on the surface of the antigen-presenting cells of the host mouse (C57BL/6) in such a way as to allow the restoration of responsiveness to the antigen GL-Phe.

In the past, several groups have successfully introduced cloned genes for major histocompatibility antigens into cultured cell lines[24-26], and these transformants have been used to study antigen recognition by T lymphocytes[27-30]. However, this approach is not suitable for *in vivo* analysis of the roles of the major histocompatibility antigens in the generation of the diversity of T-lymphocyte repertoires. To date, this latter type of analysis has been carried out by using H-2 congeneic mice, chimaeric mice and H-2 mutant mice. In the present study, we produced transgenic mice which express functional I-E antigens by microinjecting the cloned E_α^d DNA into fertilized eggs of C57BL/6 mice. These transgenic mice have several advantages over the aforementioned mice: (1) the transferred, cloned gene is well characterized; (2) a cloned gene can be modified by *in vitro* mutagenesis or exon shuffling; (3) it is possible to establish new strains carrying the E_α^d gene by crossing these mice with other B6 or B10 congeneic mice. Thus, these transgenic mice should be a powerful tool for the analysis of the effect of well-defined class II genes on immune responses.

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Neutralization of human T-lymphotropic virus type III by sera of AIDS and AIDS-risk patients

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Human T-lymphotropic virus type III (LAV, HTLV-III)[1,2] is aetiologically linked to acquired immune deficiency syndrome (AIDS) and persistent general lymphadenopathy (PGL)[2-7]. Specific radioimmunoassays (RIA), enzyme-linked assays, immunofluorescence assays (IFA) and immunoblotting techniques are being used widely to detect serum antibodies to HTLV-III in infected patients and in those at risk of infection[4-7]. However, these assays do not functionally identify those antibodies that neutralize the infectivity of the virus. We have used three methods of titrating serum neutralizing factors: inhibition of syncytium induction, neutralization of envelope pseudotypes of vesicular stomatitis virus (VSV) and reduction of infectivity of HTLV-III for a cell line permissive to virus replication. We report here that sera from subjects in various disease categories possess only low-level neutralizing activity, even when antibodies to viral membrane antigens are present in high titre. Envelope pseudotypes prepared from four HTLV-III isolates made in three different countries are equally sensitive to neutralization by positive sera, including sera from patients yielding two of the virus isolates.

To examine neutralizing properties of sera containing anti-HTLV-III, we initially determined whether such sera would inhibit the induction of syncytia or would neutralize VSV(HTLV-III) pseudotypes. When uninfected cells bearing HTLV-III receptors are mixed in excess with virus-producing cells, large multinucleated syncytia form within 6 h and are an indicator of viral envelope glycoprotein activity[8]. From previous studies[9-12] of HTLV-I and HTLV-II, we would expect antibodies binding to the functional epitopes of the viral envelope antigen both to inhibit syncytium formation and to neutralize the infectivity of pseudotypes.

Table 1 shows the mean titres of sera from patients naturally infected with one of the three known HTLV strains. As expected, sera containing antibodies to HTLV-I and HTLV-II specifically inhibited syncytium formation induced by these viruses and also