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T CELL INDEPENDENT THY-1 ALLO-ANTIBODY RESPONSE WITH THE USE OF TRANSGENIC MICE[1]

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We have introduced a mouse Thy-1.1 gene into the germline of Thy-1.2 mice. The introduced gene was shown to be expressed at very high levels in thymocytes when compared with the endogenous gene. Transgenic thymocytes were shown to evoke a higher than normal primary anti-Thy-1.1 antibody response in plaque-forming cell (PFC) assays. This result suggests that a direct quantitative interaction of the Thy-1 antigen activates the B cell response.

The Thy-1 antigen is a single domain glyocprotein that is homologous to the basic structural domain of the immunoglobulin (Ig) superfamily of genes (1). Thy-1 has been most extensively studied on the cell surface of lymphocytes, although it is expressed in several different tissues during development (2), most notably at high levels in nerve tissue of all vertebrates (3, 4). The expression of Thy-1 on the cell surface varies between different species; for example, mouse and rat thymocytes have abundant Thy-1, but only a small percentage of human thymocytes are positive (5). In contrast, the human thymus contains Thy-1 positive epithelial cells that induce thymocyte differentiation (6). Such patterns of distribution and the homology with the Ig superfamily clearly suggest that Thy-1 is involved in specific cell recognition even though its specific function is not known. In mice, the Thy-1 molecule contains 112 amino acids after processing with three branches of N-linked carbohydrates (3). It has two allelic forms; Thy-1.1 and Thy-1.2, which differ by a single amino acid at position 89: Arg (Thy-1.1) to Gln (Thy-1.2) (2, 3). This difference is sufficient to generate a strong allo-antibody response (7) and provides a good model to study allo-antibody response against cell surface antigens. To facilitate such studies, strains of mice have been bred that are congenic at the Thy-1 locus. These strains are identical to all known genetic loci except the Thy-1 locus, although it is very difficult to exclude other differences at unknown loci. A different approach to produce congenic strains has become available since the development of successful methods to introduce foreign genes into the germline of mice (8, 9). To study the regulated expression of the Thy-1 gene and the anti-Thy-1 allo-antibody response at the molecular level, we describe in this report the introduction of the murine Thy-1.1 gene into fertilized eggs of Thy-1.2 bearing mice. We show that the introduced gene is expressed in the transgenic mice, and the transgenic Thy-1.1/Thy-1.2 thymocytes evoke a strong anti-Thy-1.1 antibody response in Thy-1.2 mice.

MATERIALS AND METHODS

Animals. C57BL/10 (B10), CBA/J (CBA), AKR/J (AKR), (CBA/J × C57BL/10)F_1, and (CBA × B10)F_1 mice were bred in the animal Breeding Laboratory, National Institute for Medical Research, Mill Hill or were supplied by the Imperial Cancer Research Fund, London.

Construction of hybrid gene. The mouse genomic Thy-1.1 gene and the human Thy-1 gene, which were both cloned in the vector PBSV (10, 11) were each restricted with PvuI × ApaI restriction enzymes and were ligated by T4 DNA ligase. After transformation of HB101, plasmids, which contained the 5′ end of the mouse genomic Thy-1.1 gene and the 3′ end of the human Thy-1 gene, were selected (Fig. 1).

Transgenic mice. The mouse/human Thy-1 hybrid gene (H-M-Thy-1) was removed from the vector by an EcoRI restriction digest. The 9 kb M-H-Thy fragment was purified and was microinjected preferably into the male pronucleus of fertilized eggs of (CBA × B10)F_1 mice. The injected eggs were implanted into the oviducts of pseudopregnant (CBA × B10)F_1 mice (9). After birth and weaning, the pups were analyzed for the presence of the injected DNA by Southern blot analyses (14) of tail DNA. Transgenic mice were bred to expand the population. The mice used in this analysis were 4-wk-old F_2 mice.

Southern blotting. Mouse tissue DNA was digested to completion by restriction enzymes and was electrophoresed on a 0.8% agarose gel. After denaturation and neutralization, the DNA was transferred to a nitrocellulose filter by Southern blotting (12). The filter was hybridized with a ^{32}P-labeled SacI-NcoI fragment, which was cut from the 3′ end of the human Thy-1 gene (Fig. 2) and was processed as described (10).

Monoclonal antibody, indirect immunofluorescence, and radioimmune assays. Mouse monoclonal anti-Thy-1.1 antibody HO-22.1 and anti-Thy 1.2 antibody HO-13.4 (13) were obtained from hybridoma culture supernatants. Target cells (1×10^6) were incubated with 50 μl of 1/10 dilution (minimal essential medium (MEM) containing 0.1% NaAz + 5% fetal calf serum; FCS) of monoclonal antibody for 30 min at 4°C. After three washings by MEM with 5% FCS and 0.1% NaAz, cells were resuspended in 50 μl of 1/20 dilution of fluorescein isothiocyanate (FITC)-rabbit anti-mouse Ig (Nordic Immunology, Tilburg, The Netherlands). After three washings, the stained cells were examined under a fluorescence microscope. For radioimmune assays, ^{125}I-sheep anti-mouse Ig was used as the second antibody (Nordic Immunology). Absorption assays were performed as described in Figure 4.

In vitro plaque-forming cell (PFC) response. Suspensions of thymocytes, brain and spleen cells from AKR or transgenic mice were suspended in Eagle's MEM, and were frozen and thawed three times. Thy-1.1 antigens were cultured with spleen cells of Thy-1.2-derived mice by a modified Mishell and Dutton technique (14) by using a flat 24-well plate.

The Thy-1.1 spleens (10^7 viable cells) were suspended in 1 ml of RPMI 1640 medium containing 10% FCS, 10 mM HEPES, and 5 ×

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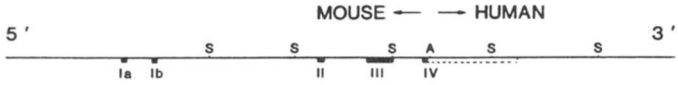

Figure 1. Structure of the mouse-human Thy-1.1 gene. The exons and introns are indicated. The gene contains two alternative first exons (10, 20). The translational start codon is in exon II, which codes for the signal peptide. Exon III codes for the extracellular Ig-like domain. Exon IV contains part of a transmembrane sequence and the translational stop codon. The 3′ end of the mouse gene was replaced with the 3′ end of the human gene at the ApaI (*A*) site, which leaves the Thy-1.1 protein structure unchanged. *S* indicates SacI sites.

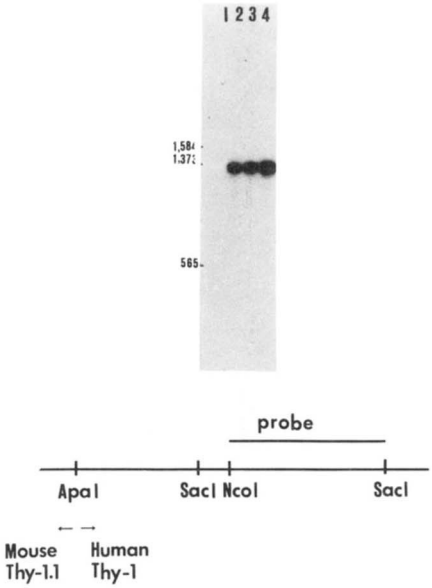

Figure 2. Southern blot analysis of SacI digested DNA from transgenic mouse T12. DNA (10 μg) from different tissues was electrophoresed on agarose gels, was blotted, and was hybridized with a radioactivity labeled NcoI × SacI restriction fragment corresponding to the 3′ end of the human gene. *lane 1*, (CBA × B10)F_1 thymus DNA; *lane 2*, T12 thymus DNA; *lane 3*, T12 brain DNA; lane 4, T12 heart DNA. The *numbers* indicate the position of a λxHinIII/EcoRI marker DNA.

10^{-5} M 2-mercaptoethanol, and were incubated at 37°C in 5% CO_2-air. After 4 days, direct (IgM) anti-Thy-1 PFC in the cultures were determined as described by Fuji et al. (7), with the use of AKR mouse thymocytes as a target (15, 16).

RESULTS

Introduction of the Thy-1.1 gene in (Thy-1.2) mice. To be able to discriminate the injected Thy-1.1 gene from the endogenous Thy-1.2, we first modified the Thy-1.1 gene. The non-coding last exon and 3′ flanking sequences were replaced with the corresponding sequences of the human Thy-1 gene at a common ApaI site, which occurs at identical positions in both genes (10, 17). This leaves all of the Thy-1.1 antigen coding sequences unchanged. The hybrid gene (M-H Thy-1) was isolated without vector sequences as a 9 kb EcoRI restriction fragment and was injected into fertilized eggs of (CBA × B10)F_1 mice. Of the 13 mice born, four were found to contain the M-H Thy-1 gene (mice T6, T9, T11, and T12) by Southern blot analysis of tail DNA. Two of the mice (T6, T12) expressed the foreign Thy-1 gene at very high levels, and the others expressed it at lower levels. Mouse T12 was used for the results described below because mouse T6 died before mating. Although tested less extensively, it should be pointed out that T6 show essentially the same results as mouse T12.

Southern blots (Fig. 2) show a single band when the DNA from transgenic mouse T12 and its offspring are hybridized with a probe that is isolated from the human 3′ end of the introduced gene. Overexposures of the autoradiograph also show the endogenous mouse Thy-1 gene. Together with a comparison of hybridization intensities of the introduced and endogenous gene with 5′ end mouse Thy-1 probes (not shown), we conclude that at least 40 copies of the injected gene have integrated. When several different restriction enzymes are used that cleave the Thy-1 gene fragment only once, a single major band is observed, which indicates a head to tail configuration of 40 Thy-1 genes in a single unique integration site.

Expression of Thy-1.1 antigen in different tissues. To analyze the expression of the exogenous Thy-1.1 and the endogenous Thy-1.2 on the cell surface in different tissues of the transgenic mice, cell suspensions of several tissue were examined by indirect immunofluorescence by using anti-Thy-1.1 (HO 22.1) or anti-Thy-1.2 (HO 13.4) monoclonal antibodies as the first antibody, followed by staining with an FITC-rabbit anti-mouse Ig. Thymocytes from the transgenic animal stained brightly after binding both anti-Thy-1.1 and anti-Thy-1.2, whereas the controls AKR J (Thy-1.1) or F_1 CBA (Thy-1.2) thymocytes are only stained after binding anti-Thy-1.1 or anti-Thy-1.2 antibody, respectively (Fig. 3). The same pattern was obtained for brain tissues, whereas liver, kidney, and heart showed no detectable fluorescence in any of the animals (data not shown). To quantitate the amount of Thy-1.1 antigen expressed on the transgenic tissues, absorption assays were carried out. Increasing numbers of cells from different transgenic tissues and control AKR (Thy-1.1) thymocytes were used to absorb anti-Thy-1.1 antibody (1/10 HO 22.1). The cells were pelleted, and the remaining antibody in the supernatant was plotted as a function of increasing cell numbers (Fig. 4). The result shows that the transgenic thymocytes and brain cells (50% absorption at 3 to 5 × 10^5 cells) expressed a high amount of Thy-1.1 antigen on the cell surface, 6 to 10 times higher than control AKR thymocytes (50% absorption at 3 × 10^6 cells). Transgenic liver, kidney, spleen, and heart tissue were also tested for absorption. Liver and spleen showed some absorption (50% absorption at 3 to 7 × 10^7 cells),

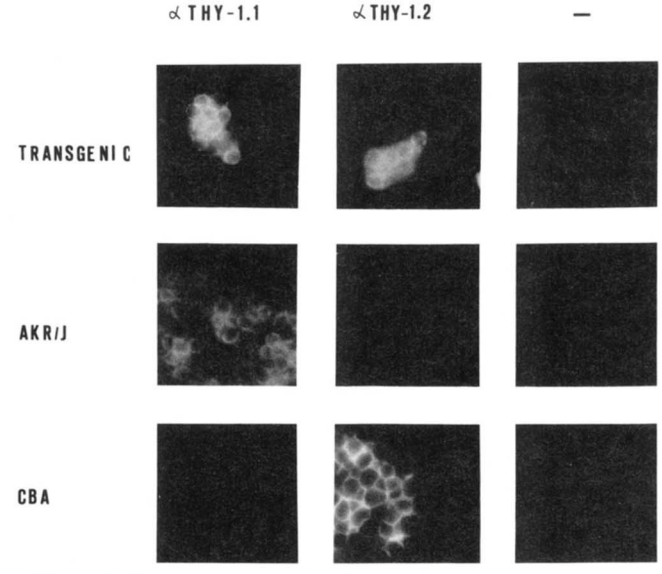

Figure 3. Fluorescence antibody binding by transgenic thymocytes. Transgenic, AKR/J, and CBA thymocytes were assayed with α-Thy-1.1 and α-Thy-1.2 as described in *Materials and Methods*. No fluorescence is observed in a control staining in the absence of anti Thy-1 antibody.

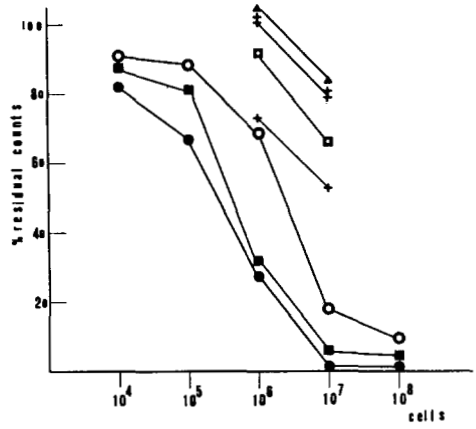

Figure 4. Anti Thy-1 antibody absorption assay with the use of various transgenic mice tissues. Monoclonal anti-mouse Thy-1.1 antibody (100 μl diluted 1/10) (HO 22.1) was absorbed with various amounts of thymocytes (●) or brain (■), liver (□), spleen (+), heart (⊕), kidney (▲) of transgenic mice and thymocytes (○) of AKR mice. After centrifugation, 50 μl of the supernatant were assayed in duplicate for residual antibody activity by an incubation with 5×10^6 AKR thymocytes as target. This was followed by a second incubation with ^{125}I-sheep anti-mouse Ig. The results are the mean of duplicate experiments and are plotted as the counts bound by residual antibody in the supernatant vs the number of cells in the binding.

which is probably caused by the presence of peripheral T cells in these tissues. Kidney and heart were negative. The same tissues were analyzed by S1 nuclease protection assays to assess the amounts of Thy-1.1 and Thy-1.2 RNA. The results show that the amount of Thy-1 antigen on the cell surface closely follows the levels of RNA transcription (Kollias and Spanopoulou). From these results, we conclude that the introduced Thy-1.1 gene is expressed in a correct tissue-specific manner and at least in mouse T12 at 6 to 10 times higher than normal levels due to increased amounts of tissue specific transcription.

Thymocytes of the transgenic mice evoke a strong primary anti-Thy-1.1 antibody response in vitro. Thymocytes, brain and spleen cells from the transgenic mice were used to induce a primary anti-Thy-1.1 antibody response in a PFC in vitro. As might be expected from the quantitation assays, the thymocytes from the Thy-1.1 transgenic mice evoked a very strong primary anti-Thy-1.1 PFC response in CBA mice (Table I). This response was even stronger than the high response obtained with normal AKR/J (Thy-1.1) thymocytes in CBA mice. A clear anti-Thy-1.1 PFC response was obtained with only 100 transgenic thymocytes and peaked at 10^5 cells per well. More than 2000 PFC per 10^7 CBA spleen cells were obtained, which is 5 to 10 times the PFC obtained for 10^5 AKR/J thymocytes per 10^7 CBA spleen cells. Thymocytes from (CBA × B10)F_1 mice or C57BL/10 mice did not develop any primary anti-Thy-1.1 PFC response in CBA mice. In agreement with previous studies (16), brain cells from the transgenic mice induced a much weaker response, although higher than that induced by AKR/J cells. Finally, spleen cells also evoked a weak response, presumably because there is only a low level of Thy-1.1 antigen (see Fig. 3). Interestingly, large numbers of brain cells or spleen cells inhibited the anti-Thy-1.1 PFC response in vitro for reasons that we do not understand. Although these results indicate that the primary PFC response evoked by the transgenic thymocytes is Thy-1.1 specific, they do not completely rule out a contribution from other antigens.

TABLE I

Primary anti-Thy-1 antibody responses induced by Thy-1.1 transgenic mice[a]

Source of Antigen	Dose	Donor mice/PFC per Well (SE)			
		Transgenic mice	AKR	CBA × B10	B10
Thymocytes	10^2	24 (4)	0		
	10^3	224 (16)	0		
	10^4	548 (32)	0		
	10^5	2552 (58)	326 (16)	0	0
	10^6	2172 (165)	656 (73)	0	0
Brain	10^4	188 (16)	0		
	10^6	452 (150)	73 (8)		
	10^7	0	20 (2)		
Spleen	10^3	2 (2)			
	10^5	88 (4)			
	10^6	44 (2)			

[a] Spleen cells (1×10^7 cells/well) from CBA mice were cultured with frozen and thawed thymocytes, brain and spleen cells from Thy-1.1 transgenic mice, AKR/J mice, CBA × B10 mice, or B10 mice as described (18). After 4 days in culture, direct (IgM) anti Thy-1.1 PFC responses were determined with the use of AKR/J mouse thymocytes as targets. PFC values are the mean value of four wells of one experiment. Additional experiments gave essentially the same result.

TABLE II

Genetic control of primary anti Thy-1.1 PFC responses evoked by Thy-1.1 transgenic mice[a]

Immunogen	Dose	Recipient Strain/PFC per Well (SE)		
		CBA	CBA × B10	B10
Thymocytes (transgenic)	10^3	120 (4)	138 (12)	4 (1)
	10^5	1005 (33)	1113 (45)	44 (4)
Brain (transgenic)	10^3	94 (8)	98 (4)	0
	10^5	250 (5)	202 (14)	0
Thymocytes (AKR/J)	10^3	44 (3)	54 (3)	0
	10^5	146 (36)	116 (12)	0

	Dose	CBA	BALB/c	DBA/2	B10	B10BR	BDF1
Thymocytes	10^5	1,380 (72)	56 (4)	72 (7)	172 (13)	0	556 (29)

[a] Spleen cells were taken from individual mice (age and sex matched). For each strain more than 15 mice were used as responders in separate assays. The PFC shown here are representative of one mouse from each strain (mean of four wells). The other 14 PFC assays show similar values. In the lower part of the table, spleen cells were collected from three mice of each strain.

Genetic control of the anti-Thy-1.1 antibody response. Because the Thy-1.1 transgenic mice were made from (CBA × B10)F_1 mice for technical reasons (11), the thymocytes of the transgenic mice will have a combination of cell surface antigens from CB10 and CBA. Some of these could possibly give a response in the PFC assay with CBA spleen cells. To exclude this possibility and to confirm that the response is Thy-1.1 specific, but dependent on other genetic loci, we measured the PFC response to the transgenic thymocytes by using 15 individual CB10, CBA, or (CBA × B10)F_1 mice. As shown in Table II, CBA or (CBA × B10)F_1 mice developed more than 1000 PFC per well in every case. In contrast, B10 mice responded poorly, indicating that certain IgH type strains are poor responders. This was confirmed by using several other mouse strains as responders (18), both B10 and DBA/2 mice responded poorly, whereas BDF1 mice responded well to transgenic thymocytes (Table II), in agreement with Zalesky et al. (19) who used conventional thymocytes. This result confirms that the PFC response is anti-Thy-1.1 specific and is not influenced by variation of the other (CBA × B10) cell surface markers.

DISCUSSION

The results from this study show that the Thy-1 gene can be introduced into the germ line of mice and can give rise to animals that express the exogenous Thy-1 gene in a tissue specific manner. This shows that the regulatory sequences in cis on the 9 kb EcoRI fragment are sufficient to allow specific and efficient expression of the gene, although the levels varied between different transgenic mice. In the mice used in this study (T12), the (introduced) Thy-1.1 antigen is expressed at 6 to 10 times the level of the Thy-1.2 antigen (Fig. 3). This is probably caused by the large number of integrated Thy-1.1 genes, although we do not know whether all of the genes, or to what extent individual genes, are expressed. The results show that the high level of Thy-1.1 expression results in a very strong specific anti-Thy-1.1 primary antibody response by either CBA or (CBA × C57BL/10)F_1 mice. This response is much stronger than any previously observed primary anti-Thy-1.1 antibody responses, such as that obtained by AKR/J thymocytes and CBA spleen cells (18). Because the high level of the response is the same in all of the tested (CBA × C57BL/10)F_1 mice, it suggests that the Thy-1 antigen itself is directly responsible for the primary B cell activation. Whether the strong response is caused exclusively by the very high quantity of Thy-1 on the cell surface, or secondry structural changes that might occur as a result of the high expression, is presently not known. Other T cell surface antigens of the Ig superfamily that occur at a much lower level on the thymocyte cell surface fail to give the primary response.

The possibility to introduce genes into the germline of mice and to express different amounts of any given cell surface marker therefore allows the study of particular cell-cell interactions at the molecular level. In the case of the Thy-1 antigen, both the level of Thy-1 expression can be changed without changing other surface markers, and more interestingly, the Thy-1 protein structure on the cell surface can be modified by gene manipulation. The latter possibility would allow a study of the function of the Thy-1 antigen in vivo and its function in the primary B cell response in particular.

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