Regulation of Transgenic Class II Major Histocompatibility Genes in Murine Langerhans Cells

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I-E is a class II major histocompatibility complex molecule normally expressed by Langerhans cells. A series of transgenic mice were developed previously that carry E_α^d gene constructs with promoter-region deletions that cause expression of I-E by different cell types when maintained on a B6 (I-E[−]) genetic background. To study cis-acting gene sequences that regulate expression of class II proteins by Langerhans cells, we identified transgenic I-E expression by tissue immunoperoxidase staining and by epidermal cell suspension immunofluorescence cytometry. Mice with a transgene containing 1.4 kilobase pairs (kb) of flanking sequence 5′ to the E_α initiation site expressed barely detectable levels of I-E on a tiny percentage of Langerhans cells, indicating that sequences promoting Langerhans cell expression of E_α exist between 2.0 and 1.4 kb 5′ of the E_α initiation site. Removal of an additional 170 bp of 5′ flanking sequence caused near-normal levels of expression by approximately one third of epidermal Langerhans cells, which contrasts with studies that showed minimal transgene expression by splenic dendritic cells in these animals. Thus, sequences between 1.4 and 1.23 kb 5′ of the E_α initiation site decrease expression of I-E by epidermal Langerhans cells, but enable I-E expression by splenic dendritic cells. These studies identify Langerhans cell–specific regulatory sequences and genetic regions controlling major histocompatibility complex class II gene expression in Langerhans cells and splenic dendritic cells. The genetic regions identified may be particularly important because differential regulation of class II major histocompatibility complex protein synthesis by Langerhans cells and dendritic cells may be crucial to immune functions of intact animals. *Key words: dendritic cells/antigen presentation. J Invest Dermatol 104:329–334, 1995*

Specific immune responses are generated and regulated through the expression of genes encoded by the major histocompatibility complex (MHC), the products of which are cell-surface glycoproteins that combine with antigenic peptides to form complexes recognized by T cells [1,2]. Class II MHC molecules, also known in the mouse as immune-response–associated (Ia) antigens, are heterodimers composed of two chains of approximately equal sizes termed α and β [3,4]. Two types of murine Ia antigens, called I-A and I-E, are normally coexpressed on thymic epithelial and interdigitating reticular cells as well as on B lymphocytes, macrophages, and dendritic cells [5–10]. Thymic expression of Ia molecules is critical both for the induction of tolerance to self antigens [10,11] and for selection of self-MHC–restricted T cells [12–14]. For antigen-specific activation outside of the thymus, CD4(+) T cells must interact with Ia molecules complexed with the specific antigen and expressed on the surface of antigen-presenting cells (APCs). Surface expression of these Ia molecules is regulated and represents a pivotal point in control of the diverse immune processes requiring CD4(+) T cells, including cell-mediated immunity and the generation of antibodies by B cells.

Dendritic cells are Ia(+) APCs that are especially active in the induction of primary antigen-specific immune responses [6]. The term dendritic cell may be applied to several populations of cells, the phenotypes and functional characteristics of which are dynamic, depending on their anatomic location and their history of exposure to various stimuli [15,16]. Langerhans cells are a type of dendritic cell found in the epidermis, where they are normally the only cell type that express Ia antigen [6,17]. When freshly isolated from epidermis, Langerhans cells can process antigen, and this function is associated with synthesis of Ia antigen [18–20]. More "mature" dendritic cells, isolated from spleen, retain the ability to present antigen but do not synthesize Ia antigen and are poor APCs [19,20]. Therefore, Ia antigen expression is differentially regulated in Langerhans cells and splenic dendritic cells. This differential regulation may be crucial to directing the immune responses of intact organisms to particular antigens [6,19,20].

The genetic basis for class II gene expression has been studied *in vitro* by transfecting specific gene constructs into various cell types, and *in vivo* by developing transgenic animals [10,21–28]. Strains of

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Abbreviations: APCs, antigen-presenting cells; Ia, immune-response–associated.

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mice exist in which the endogenous genes encoding the E_α chain of the I-E heterodimer are not expressed because of a mutation in the regulatory portion of the E_α gene [4]. E_α transgenes containing at least 2.0 kilobase pairs (kb) of 5′ flanking DNA cause appropriate expression of E_α mRNA and surface protein in these mice [10,28–30]. Transgenes containing less 5′ flanking sequence (E_α^d genes) generally result in normal expression of I-E in the thymus of these animals but may result in lower levels of expression by specific peripheral cell types, partial expression by a given cell type, or expression limited to one or several of the cell types that express I-E in normal animals. Expression of I-E by B cells, macrophages, and splenic dendritic cells has been characterized in these animals, and specific 5′ flanking sequences with cis-regulatory function in these cell types have been identified. However, no such studies have been made of I-E expression by Langerhans cells [10,26,27,31].

In our current study, we examined expression of I-E_α transgenes by Langerhans cells in these animals. We show that I-E_α expressed by Langerhans cells is regulated differently from that expressed by macrophages, B cells, and splenic dendritic cells, and we define specific regions in the 5′ flanking sequences of the E_α gene that differentially control that expression. Because dendritic cells are the main, if not the only, cells capable of priming CD4(+) T cells and initiating certain immune responses [6,7,32], characterization of I-E expression by all dendritic cells in these transgenic animals is critical for precise understanding of the mechanisms of immune function restricted by I-E. Furthermore, identification of these specific regulatory sequences may allow precise targeting of Langerhans cell and dendritic cell gene expression.

MATERIALS AND METHODS

Generation of Transgenic Mice Generation of the experimental animals, including the embryo microinjection, E_α^d gene subcloning, and analysis of DNA, have been described previously [10,26–30]. Two strains of transgenic mice with wild-type phenotype were used; strains 107 and Eα16 [10,26,27].

Preparation of Cell Suspensions After sacrifice, the mice were soaked in 70% ethanol and shaved. Strips of body-wall skin were cut, the dermis was separated from fat, and the strips were placed dermal-side down in 0.3% trypsin in GNK (0.3% trypsin [type XI; Sigma, St. Louis, MO], 0.17% glucose, 150 mM NaCl, 5 mM KCl). Strips were incubated overnight at 4°C, and the epidermis was removed the next morning by gentle scraping. The epidermis was placed in fresh trypsin, GNK, and 0.1% DNAse (deoxyribonuclease I; ICN, Irvine, CA) and incubated in a 37°C water bath with continuous shaking for 10 min. Medium A (Minimal Essential Medium [Gibco, Grand Island, NY] containing 10% fetal bovine serum, 1% Penicillin/streptomycin, and 0.1% DNAse) was added and the tubes were placed on ice. The cell preparation was filtered through nylon mesh to remove stratum corneum and hair, and the cells were washed in medium A and pelleted at 1000 rpm for 10 min at 4°C. Cells were further enriched in Ficoll (density 1.083) at 1200 rpm for 20 min at 4°C. Interface cells were harvested and washed twice in medium A.

Epidermal Cell Culture For some experiments interface cells were cultured for 3 d at 37°C in RPMI 1640 medium supplemented with 5% murine serum and 1 × antibiotic-antimycotic (Gibco).

Epidermal-Sheet Preparation Ears were treated with Neet depilatory for 10 or 12 min, and the hair was wiped off with a Kimwipe. The ears were rinsed in distilled water. The dermis and subcutaneous tissue were separated by blunt dissection from the underlying ear cartilage, and any remaining cartilage and fat were removed by scraping with the edge of a forceps. Ear skin was soaked in 0.5 M NH_4SCN at 37°C for 20 min (no CO_2), and the epidermis was removed and washed three times in phosphate-buffered saline. The epidermal sheets were fixed in acetone (−20°F) for 20 min and then washed and stored in phosphate-buffered saline at 4°C.

Detection of Ia Activity I-A and I-E were detected with monoclonal antibodies 212.A (anti–I-A, IgG2a) [33] and 14.4.4s (anti–I-$E^{k,d}$, IgG2a) [34], respectively. An isotype-matched monoclonal antibody of irrelevant specificity was used as a control in all experiments. Other controls routinely performed to validate our results included staining of nontransgenic littermates (negative control) and of a naturally I-A and I-E(+) strain (5R, positive control). Note that the 5R stain is homozygous for I-E and the experimental animals are heterozygous for the transgene.

Flow Cytometry To minimize binding of mouse monoclonal antibodies to surface Fc receptor, epidermal cells were incubated with 5% mouse serum, and staining was performed in the presence of the same. After incubation with the primary monoclonal antibody (biotinylated 212.A or control antibody), the cells were washed three times and incubated with avidin-phycoerythrin (Becton-Dickinson, Bedford, MA), washed three times, and analyzed by flow cytofluorometry on a FACStar or FACScan (Becton-Dickinson) or EPICS XL-MCL (Coulter Corporation, Hialeah, FL). For double staining, fluorescein isothiocyanate (FITC)-conjugated 14.4.4s antibody was included in the primary incubation solution. To control for the presence of FITC, we used Fab-FITC of irrelevant specificity. All flow cytometry results are presented as dot plots showing fluorescence intensity (logarithmic scales) on the ordinate and abscissa. At least 10,000 events were accumulated for all plots shown.

Immunohistochemistry Epidermal sheets were washed in 100 mM Tris (pH 7.2) and then stained with the Avidin-Biotin-Peroxidase complex technique (ABC, Vector Laboratories, Burlingame, CA) according to standard protocols. Immunohistochemical staining of thymus, lymph nodes, and spleen from selected animals confirmed the patterns of I-E expression reported previously in these animals (our unpublished data).

RESULTS

As expected, animals with transgenes that had previously been reported to express I-E with a normal tissue distribution and at normal levels (wild-type phenotype) showed a 1:1 correspondence between expression of endogenous I-A and expression of I-E on Langerhans cells when analyzed by flow cytometry using two-color immunofluorescence staining or *in situ* by immunoperoxidase **(Fig 1*A,B*)**. However, animals with a transgene containing only 1.4 kb of 5′ flanking sequence and which show restricted expression of I-E [10] showed minimal expression of I-E by epidermal Langerhans cells. Faint expression was identified in only one of five epidermal-sheet preparations, and there was minimal or no change in the fluorescence-activated cell sorter profile of these animals' Langerhans cells when stained for I-A and I-E as compared to staining for I-A alone (**Fig 1*C***, strain 46.2). This contrasts with studies that showed expression of I-E by 100% of dendritic cells isolated from spleens of these animals **(Table I)** [31]. Short-term culture caused these Langerhans cells to express near-normal levels of I-E, like splenic dendritic cells from the same animals **(Fig 2*B*)**. Strikingly, removal of an additional 170 bp of the 5′ flanking sequence resulted in a rightward shift (increase in mean channel fluorescence) in the cytofluorometric profile of almost the entire population of Langerhans cells, indicating expression of transgenic I-E by most epidermal Langerhans cells (with normal levels of I-E expressed by approximately one third) (**Fig 1*D***, strain 244.8). This increased I-E expression was confirmed by the easy detection of a population of I-E(+) epidermal dendritic cells *in situ*. These results indicate that the 170-base pair fragment between −1.4 and −1.23 kb 5′ to the initiation codon of the E_α gene contains sequences suppressing expression of I-E_α by epidermal Langerhans cells. The effects of culturing Langerhans cells from the 244.8 strain were not as clear cut as those seen with the 46.2 stain; after 3 d there was continued I-E expression by most 244.8 Langerhans cells, although some experiments showed a slightly decreased intensity of expression, particularly in the group of Langerhans cells with low baseline levels of expression (**Fig 2*C***, *left upper sector*). For example, the mean channel (x axis) fluorescence was 69.16 for freshly isolated Langerhans cells, *versus* 31.68 for cultured Langerhans cells in one set of experiments. Similar results were seen in two of three experiments. One experiment showed no detectable change. As shown in **Table I**, studies of splenic dendritic cells from these animals showed faint expression, less than 5% of control, in this strain [31]. This discrepancy in expression of I-E by epidermal Langerhans cells and splenic dendritic cells points to specific sequences regulating phenotypic heterogeneity of dendritic cell populations within different anatomic locations.

Removal of an additional 880 bp of 5′ flanking sequence resulted in variable expression in two different strains, which parallel the expression in B cells seen previously in these transgenic lines **(Fig 1*E,F*)**. Near-normal levels of expression were seen in the majority

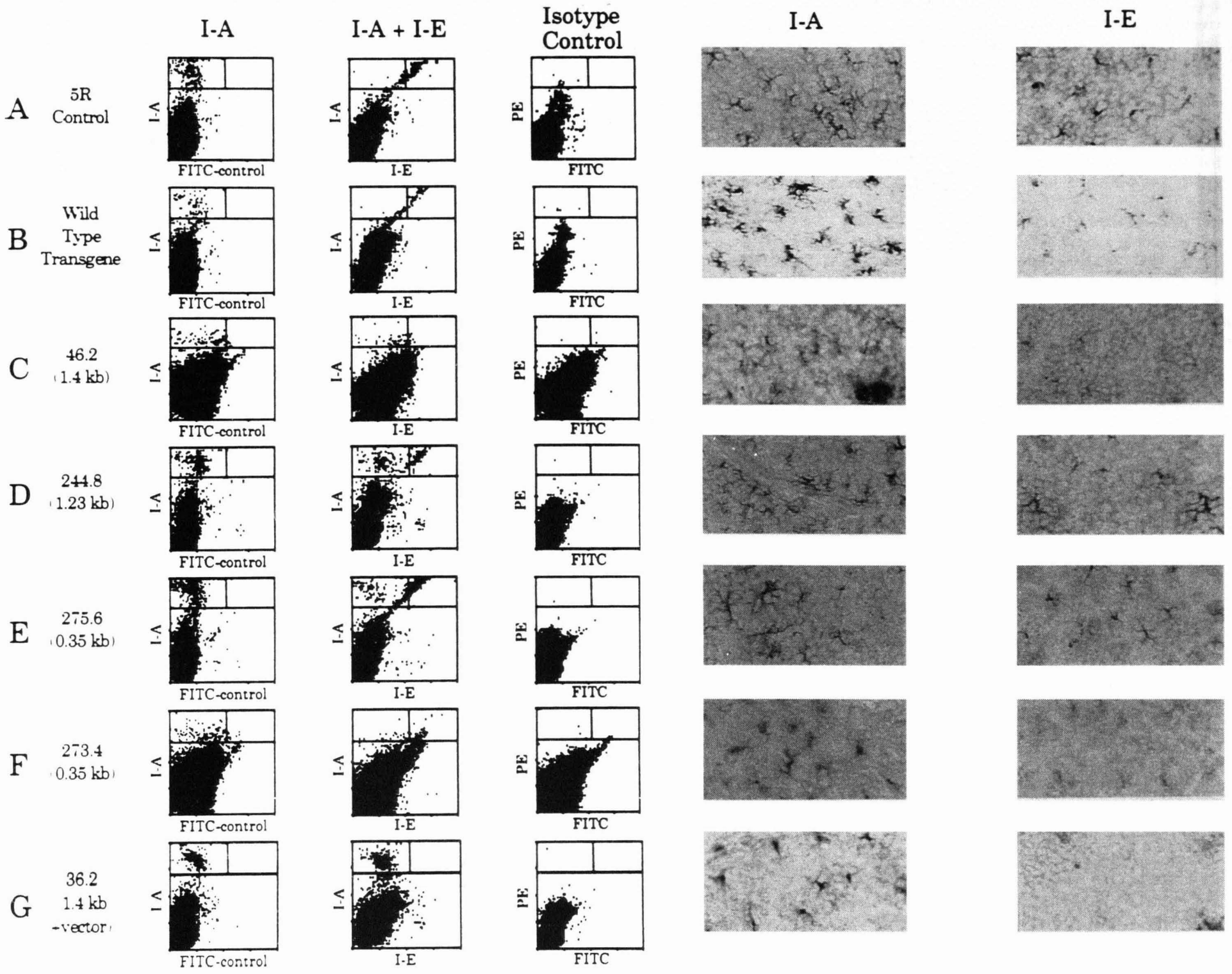

Figure 1. Different genetic regions promote or suppress expression of transgenic MHC class II molecules by murine epidermal Langerhans cells (LC). Figure shows cytometric and *in situ* detection of the MHC class II antigens I-A and I-E in transgenic mice. For flow cytometry, epidermal cells were stained with biotinylated anti–I-A and FITC-anti–I-E (or FITC-labeled antibody of irrelevant specificity as a control), followed by phycoerythrin (PE)-avidin. Data fields are drawn to indicate the population of phycoerythrin (+)/FITC(−) Langerhans cells (I-A[+]/I-E[−], *upper left*) and phycoerythrin (+)/FITC(+) Langerhans cells (I-A[+]/I-E[+], *upper right*). *In situ* studies used biotinylated primary antibodies with an avidin/peroxidase detection system. Photomicrographs were 400× original magnification. *A* and *B*, positive controls. *A*) Results using 5R, a nontransgenic strain that normally expresses I-E; *B*) results using animals with wild-type expression of their transgene. Animals with transgenes containing progressively shorter fragments of 5′ regulatory sequence (strains 46.2, 244.8, 275.6, and 273.4) are displayed (*C,D,E,F*). Animals with I-E expression limited to the thymus (36.2) are shown (*G*) for comparison and serve as perfect specificity controls for the reagents. Note that I-E expression by Langerhans cells in strain 46.2 (*C*) was minimal, and was found *in situ* in a patchy distribution in only one of five animals studied.

Table I. Expression of Membrane I-E on Dendritic Cells (DC) and Langerhans Cells (LC)[a]

	Transgenic Line					
	Eα16, 107-1 (Wild Type)	46.2	244.8	275.6	273.4	36.2
5′ regulatory sequences (kb)	2.0	1.4	1.23	0.35	0.35	1.4 + vector
Percent I-E(+)						
LC	100	<5	>95	>95	>95	0
DC	100	100	<5	100	35	0

[a] The staining and fluorescence-activated cell sorter analysis of Langerhans cells were performed as described in *Materials and Methods*. The data for characterization of I-E expression on dendritic cells are summarized from Levin *et al* [31].

of Langerhans cells in strain 275.6 **(Fig 1*E*)**. This line was shown previously to have an essentially wild-type pattern of I-E expression in the thymus and by peripheral B cells and splenic dendritic cells, but impaired expression by macrophages [31,35]. A second strain containing the same E_α^d construct showed a similar although lower pattern of expression by Langerhans cells (strain 273.4, **Fig 1*F***). This strain also showed a reduction of I-E expression on B cells, macrophages, and splenic dendritic cells, but normal expression in

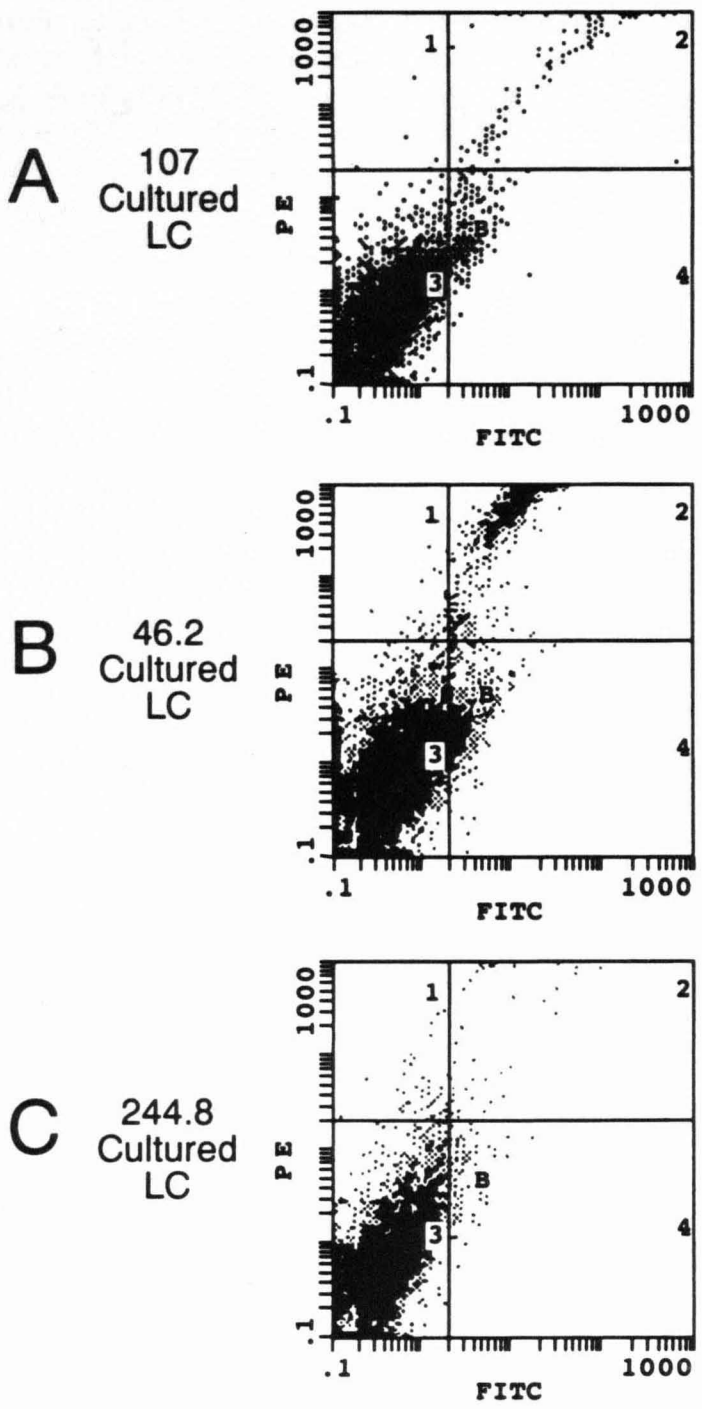

Figure 2. Expression of transgenic MHC class II molecules is altered by Langerhans cell culture. Flow cytometry of Langerhans cells cultured for 3 d and stained for I-A (FITC label, y axis) and I-E (phycoerythrin [PE] label, x axis). *A*) Wild-type cultured Langerhans cells show I-A(+), I-E(+) phenotype similar to that seen in freshly isolated Langerhans cells from the same strain (compare with **Fig 1*B***). *B*) Animals with transgenes containing 1.4 kb of 5′ regulatory sequence (strain 46.2) show a marked increase in I-E expression, with a phenotype similar to that of dendritic cells from these animals (compare with **Fig 1*C***, and see **Table I**) and freshly isolated or cultured wild-type Langerhans cells. *C*) Langerhans cells from animals with transgenes containing 1.23 kb of 5′ regulatory sequence (strain 244.8) show minimal changes after culture.

the thymus [31,35]. This variation between strains containing the same transgene construct is consistent with position effects from different transgene insertion sites and was discussed previously for these two strains [35]. The addition of vector DNA to the transgene suppresses expression by peripheral B cells, macrophages, and dendritic cells, as well as by epidermal Langerhans cells (**Fig 1*G***, strain 36.2).

DISCUSSION

The series of mice that we have described, in which transgenic I-E is expressed by different cells, provides a valuable resource for investigating the role of I-E in immune responses and for identifying regulatory sequences that are specific for different cell types. However, the utility of these animals for *in vivo* studies depends on complete and accurate identification of all potentially I-E(+) cells. Previous studies of these animals focused on expression of I-E by thymic cells, splenic dendritic cells, peripheral macrophages, and B cells [11,26–31]. Expression of transgenic I-E by Langerhans cells has not been characterized previously, so any attempts to analyze I-E–related immune functions using these animals are compromised.

In this report, we characterized the expression of I-E by Langerhans cells in these animals. These studies used multiple founder lines in all cases, and consistent expression of transgenes was obtained except in the lines 275.6 and 273.4. These lines (and one other which shows exactly the same expression pattern as 273.4) [26] were obtained from a construct with approximately 0.3 kb of 5′ flanking DNA. Perhaps this short segment renders this transgene more susceptible to position effects. The lines used in this study, with the exception of 275.6, are characteristic of the transgene used and do not represent an aberrant expression pattern derived from a single founder line with a single integration site.

Of note is the transgenic strain 36.2, in which the transgene contains a portion of vector in addition to 1.4 kb of 5′ flanking sequence. These animals are important because they have normal expression of I-E in the thymic cortex and significant, though reduced, expression in the medulla; this expression permits both positive and at least partial negative selection of T cells capable of recognizing antigen in the context of I-E. As a consequence, these mice are tolerant to I-E in functional immunoassays [10]. Our current study, combined with previous work, demonstrates that these animals lack I-E expression on all known APCs present in the periphery and thus identifies these animals as ideal recipients in adoptive transfer experiments. The use of strain 36.2 as recipients of I-E(+) APCs from otherwise syngeneic animals allows precise, *in vivo* determination of the role of individual cell types in specific immune responses [31].

Additional features of our study are further delineation of specific 5′ regulatory sequences affecting class II molecule expression and identification of sequences that are critical for expression of I-E by Langerhans cells and dendritic cells. In particular, our studies of the 46.2 strain show that cis-acting positive regulatory elements required for expression of I-E by most epidermal Langerhans cells *in vivo* exist between −2 and −1.4 kb 5′ to the I-E_α initiation codon. Comparison of strains 46.2 and 244.8 indicates *in situ* that synthesis of I-E by Langerhans cells can be suppressed almost completely by sequences in the region between −1.4 and −1.23 kb of the I-E gene. However, when the 46.2 Langerhans cells are cultured for 3 d, they express I-E like the dendritic cells from these same animals. This observation is not surprising, given the well-documented tendency for Langerhans cells to resemble dendritic cells after exposure to granulocyte macrophage–colony-stimulating factor produced by keratinocytes during isolation and culture [36,37]. This finding suggests that microenvironmental factors may be the major difference between Langerhans cells and other dendritic cells. It also suggests that the occasional finding of slight *in situ* expression of I-E on a tiny fraction of epidermal Langerhans cells in the 46.2 strain could be a result of changes induced by the preparation of epidermal sheets. Unlike the results with strain 46.2, our experiments were not able to detect significant changes (decreases) in I-E expression by cultured 244.8 Langerhans cells. Interpretation of this finding is problematic because I-E expression normally increases during culture [38] and because the 3-d culture may not be long enough for loss of previously synthesized I-E molecules.

The 170-base pair region highlighted by these studies contains sequences, known as the X′Y′-box [39], that promote I-E expression in other cell types and that are therefore candidates for regulating expression of I-E in Langerhans cells. In contrast to the situation in epidermal Langerhans cells, 100% of splenic dendritic cells and peritoneal macrophages express I-E when these sequences are at the 5′ end of their transgenes [31]. Sequences between −1.23 and 0.35 of the gene decrease I-E expression by all splenic dendritic cells and can completely suppress expression of I-E by most of these cells, but have no effect on a large subset of epidermal Langerhans cells. This segment contains a sequence known as the W-box [40], which is required for expression of I-E in some cell types. Therefore, this area and the area immediately 5′ to it are implicated in the reciprocal regulation of I-E synthesis by epidermal Langerhans cells and splenic dendritic cells. The X′Y′- and W-box sequences, or

intervention directed at them, might be useful in differentially manipulating expression of genes by Langerhans cells and splenic dendritic cells, and are prime targets for further research. More detailed analysis may be possible as techniques for culturing Langerhans cells and dendritic cells become more sophisticated [41–44] and as well-characterized Langerhans and dendritic cell lines become more widely available [45,46].

Langerhans cells are bone-marrow–derived dendritic epidermal cells that are potent presenters of both alloantigen and exogenously acquired antigen in the context of class II MHC molecules [17,36,47,48]. Langerhans cells undergo phenotypic and functional changes *in vitro* when isolated from the epidermis, and *in vivo* when they migrate to regional lymph nodes after antigen sensitization [18–20,36,38]. In particular, after antigen exposure *in vivo,* they acquire the ability to sensitize T cells and stimulate T-cell–dependent, B-lymphocyte antibody responses [18,19]. The ability to process antigen by Langerhans cells correlates with their level of biosynthesis of class II molecules and expression of invariant chain [20]. Cessation of antigen processing and class II molecule synthesis may be crucial for transportation of antigen from the skin, where it is encountered, to the lymphoid organs, where a specific response is generated. This same mechanism may help control presentation of endogenous antigens. Therefore, synthesis of MHC molecules by these cells appears to be a major point of regulation for primary immune responses, and understanding the molecular basis of its control may allow more precise manipulation of the immune system [20]. Our studies implicate the regions of the I-E gene between −1.4 and −1.23 kb, and between −1.23 and −0.35 kb, which contain the elements known as the X′Y′-box and the W-box, respectively, as being crucial in the differential regulation of class II MHC expression by Langerhans cells and dendritic cells. This region and these elements may play major roles in the control of immune responses.

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ANNOUNCEMENT

An international symposium titled "Biological Events in Cutaneous Inflammation" will be held in Kiel, Germany, October 19–21, 1995. The topics will be the role of keratinocyte in cutaneous inflammation, leukocyte adhesion in early inflammation, mechanisms of cellular infiltration, Langerhans cell biology, cell-specific recruitment, proteolysis, fibrosis in cutaneous inflammation, and genodermatoses: recent advances.

The meeting will consist of invited oral presentations. Abstracts will be accepted for oral or poster presentation. Deadline for abstract submission is August 1, 1995. For further information contact Ulrich Mrowietz, M.D., Department of Dermatology, University of Kiel, Schittenhelmstrasse 7, 24105 Kiel, Germany. FAX: (49) 431-5971543.