Regulation of Expression of B7 by Murine Langerhans Cells: A Direct Relationship Between B7 mRNA Levels and the Level of Surface Expression of B7 by Langerhans Cells

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Cultured BALB/c epidermal Langerhans cells express high levels of the costimulatory molecule B7 on their surfaces relative to levels expressed on fresh Langerhans cells. Quantitation of relative amounts of B7 mRNA in fresh epidermal cells and cultured epidermal cells following amplification of mRNA signals via reverse transcriptase–polymerase chain reaction, hybridization of PCR products with radiolabeled internal oligonucleotide probes, resolution of hybrids in non-denaturing polyacrylamide gels, and detection by autoradiography revealed dramatically (approximately one thousandfold) higher levels of B7 mRNA in cultured epidermal cells (10–40% I-A$^+$) as compared with fresh epidermal cells (1–4% I-A$^+$). Levels of B7 mRNA in cultured epidermal cells were also substantially greater than those detected in a reference B lymphoma cell line (CH-1). Analysis of B7 mRNA expression in subpopulations of cultured epidermal cells demonstrated that essentially all of the B7 mRNA was present in Langerhans cells; cells bearing I-A and CD45 antigens. Cultured keratinocytes did not contain appreciable amounts of B7 mRNA. These results are consistent with previous data regarding surface expression of B7 by cLC and also demonstrate that fLC are essentially devoid of B7 mRNA and surface protein. Key words: CD28/CTLA-4/costimulatory molecules/accessory cells. *J Invest Dermatol 101:883–886, 1993*

Previous studies demonstrated that Langerhans cells (LC) become potent accessory cells after culture in GM-CSF containing media for brief periods [1,2]. The increased accessory cell activity of cultured LC (cLC) relative to fresh LC (fLC) probably reflects increased expression of major histcompatibility complex antigens [3,4] and costimulatory molecules [5], and may mimic changes that occur in LC as they migrate from skin to regional lymph nodes after epicutaneous antigen exposure *in vivo* [6]. Several potentially important costimulatory and/or adhesion molecules including intercellular adhesion molecule 1 (ICAM-1) [5], lymphocyte function–associated antigen 3 (LFA-3) [7], and B7/BB1 [8] have been identified on the cell surfaces of LC and have been implicated as important determinants of the potent accessory cell activity of cLC and other dendritic cells [9]. Recent studies have emphasized the central role that B7 (expressed on the surfaces of accessory cells) plays in the activation of naive CD4$^+$ T cells [10], and in the avoidance of antigen-specific clonal anergy [11,12] via engagement of the T-cell co-receptors CD28 [13,14] and CTLA-4 [15].

Although several novel approaches to treatment or prevention of allograft rejection [16,17] and experimental neoplasia [18,19] have already evolved from studies of B7 and its role in T-cell activation, regulatory mechanisms that control expression of B7 by accessory cells have not been extensively studied. Since we began our studies regarding B7 expression by murine LC, it has been demonstrated that 1) murine cLC stain more intensely with the B7-binding fusion protein CTLA4Ig than fLC [8], and 2) CTLA4Ig inhibits the accessory cell activity of cLC [8]. Symington *et al* reported similar results in a study of B7 expression by human LC, and also suggested that B7 mRNA was present in both fLC and cLC [20]. The studies detailed here employ a sensitive polymerase chain reaction (PCR)-based technique to quantitate mRNA levels and demonstrate that although B7 mRNA is abundant in cLC, fresh murine EC (LC and keratinocytes) lack appreciable B7 mRNA. Our results indicate that significant amounts of B7 mRNA and protein are not expressed in normal uninflamed murine epidermis, and suggest that the level of expression of B7 by LC is determined by B7 mRNA levels.

MATERIALS AND METHODS

Antibodies, Fusion Proteins, and Flow Cytometry CTLA4Ig [15] and the irrelevant control L6 (a murine-human fusion IgG_1 [15]) were provided by Peter Linsley (Bristol-Myers Squibb, Seattle, WA). Mouse monoclonal antibody (MoAb) MK-D6 (anti-I-A^d) and an irrelevant IgG_{2a} control were purchased from Becton Dickinson (Mountain View, CA). Rat MoAb 53-6.72 (anti-CD8, IgG_{2a}) YN/1.7.4 (anti-ICAM-1 [CD54], IgG_{2a}), M5/114.5.2 (anti-I-A^d, anti-I-E^d, IgG_{2b}), and M1/89.18.7.HK (anti-CD45, IgG_{2b}) were obtained from the American Type Culture Collection (Rockville, MD). Biotin-conjugated goat anti-human IgG [$(Fab')_2$], biotin-conjugated goat anti-rat IgG [$(Fab')_2$], and R-phycoerythrin streptavidin were purchased from TAGO (Burlington, CA). Fluorescein isothiocyanate–monoclonal rat anti-mouse Ig (κ chain specific) was obtained from Zymed (San Francisco, CA). EC were stained for simultaneous expression of I-A antigens and ICAM-1 as described [5]. Expression of B7 on

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MGL and TAB made equal contributions to the studies reported in this paper.

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Abbreviations: cEC, cultured epidermal cell; cLC, cultured Langerhans cell; fEC, fresh epidermal cell; fLC, fresh Langerhans cell; RT, reverse transcriptase.

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LC (I-A$^+$ cells) and KC (I-A$^-$ cells) was similarly evaluated substituting CTLA4Ig (or the irrevelant fusion protein L6) and biotin-conjugated goat anti-human IgG for YN/1.7.4 and biotin-goat anti-rat IgG. CTLA4Ig and L6 were used at 5 μg/ml.

Preparation of Murine Epidermal Cells and Epidermal Cell Subpopulations Female BALB/c (H-2^d) mice were obtained from Harlan Sprague Dawley (Frederick, MD) and used at 8–12 weeks of age. Fresh and 72-h cultured EC were prepared from BALB/c ear skin by limited trypsinization as previously described [21]. In some experiments, cEC were depleted of, and enriched for, cLC using rat MoAb and sheep anti-rat IgG-coated magnetic beads (Dynabeads M-450, Dynal, Oslo, Norway). cEC were incubated with saturating amounts of the indicated rat MoAb for 1 h at 4°C, washed, and incubated with Dynabeads (approximately 20 beads per cLC) for 30 min at 4°C. Cells and beads were mixed continuously using a tube rotator. Bead-adherent and nonadherent cells were separated from each other with a magnet (Dynal) and RNA was prepared as described below.

Quantitation of mRNA Using Reverse Transcriptase (RT)-PCR Relative amounts of B7, I-Aα chain, and β-actin mRNA present in epidermal cells were quantitated using a modification of a previously described method [22]. Briefly, total RNA was isolated from epidermal cells using acid guanidinium isothiocyanate/phenol (RNAzol B) (Biotex Laboratories, Houston, TX) and chloroform, and quantitated spectrophotometrically. RNA was reverse transcribed and cDNA was amplified using a GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) in accordance with the manufacturer's instructions except that primer concentrations (3′ for reverse transcription and 3′ plus 5′ for PCR) were adjusted to 1 μM. After reverse transcription, cDNAs were amplified during 20–25 cycles (20 cycles for β-actin and 25 cycles for B7 and I-Aα chain) of PCR in a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer Cetus) with denaturation at 95°C for 1 min, annealing at 55°C for 1.25 min and polymerization at 72°C for 2 min. Reagent grade primers were obtained from Synthecell (Rockville, MD). Primer and probe sequences for β-actin and I-Aα chain were as previously reported [22]. B7 primers were designed based on the published sequence for murine B7 [23] (5′ B7 primer, 5′CCCGAGTATAAGAACCGGAC;3′ B7 primer, 5′CACAAGTGTGTTCTTGCTATC). These primers result in a 483-bp PCR product corresponding to nucleotides 517-999 of the 1720-bp murine B7 cDNA sequence [23].

PCR products were quantitated as previously described [22]. Briefly, PCR products were hybridized to radiolabeled internal oligonucleotide probes, resolved in nondenaturing polyacryamide gels, and detected by autoradiography. Densitometry was performed using an optical scanner (Microtek 300FS, Torrence, CA) and Image 1.36 software (NIH, Bethesda, MD). The probe for B7 mRNA was designed based on the published B7 sequence [23] and corresponds to nucleotides 775-809 (B7 probe, 5′CGCTTCTCTTGGTTGGAAAATGGAAGAGAATTACC).

RESULTS

Expression of ICAM-1 and B7 by Fresh and Cultured LC Previous studies demonstrated that murine cLC express much higher levels of ICAM-1 [5] and B7 [8] than fLC. We stained BALB/c fEC and cEC for simultaneous expression of I-A antigens and ICAM-1 (or B7) using the rat MoAb YN1/1.7.4 (or the fusion protein CTLA4Ig). Cultured LC expressed levels of ICAM-1 and B7 > fiftyfold higher than levels of the corresponding antigens on fLC; little if any B7 was identified on fLC, or fresh or cultured keratinocytes (T. A. Borkowski and M. C. Udey, unpublished observations).

Relative mB7 mRNA Levels in Fresh and Cultured EC To assess the relationship of B7 surface expression to mRNA levels in cLC we employed quantitative RT-PCR [22]. B7 specific sense and anti-sense primers and an internal oligonucleotide probe were designed based on the published sequence of murine B7 [23]. Conditions allowing the amplification and detection of an appropriately sized PCR product complementary to the internal B7 probe in CH-1 cells, a B-lymphoma cell line known to contain B7 mRNA [23], were identified. Varying amounts of RNA from CH-1 cells, fEC, 72-h cEC, PAM212 cells (transformed murine keratinocytes), and L cells (transformed murine fibroblasts) were subjected to RT-PCR using B7 and β-actin primers. PCR products were hybridized to excess amounts of radiolabeled internal probes, resolved in nondenaturing gels, and detected by autoradiography.

Data presented in Fig 1 demonstrate that cEC contain readily detectable B7 mRNA. The intensity of the B7 signal derived from 20 ng/ml cEC RNA was greater than that derived from reactions initiated with concentrations of CH-1 RNA a thousandfold higher. Little if any B7 signal was identified in RNA prepared from fEC, transformed keratinocytes, or fibroblasts, although β-actin signals were readily identified in each of these samples. Note that at the highest concentrations of cEC RNA tested, the intensity of the band corresponding to the appropriately sized B7 PCR product decreased and irrelevant bands appeared.

Quantitation of mB7, I-Aα, and β-Actin mRNA Levels in Cultured EC Cultured BALB/c EC are comprised of 10–40% cLC; the vast majority of the remaining cells are keratinocytes. To interpret the results of cell-depletion studies designed to identify the cell of origin of the B7 mRNA signal, it was necessary to first define conditions that allowed relative quantitation of I-Aα chain mRNA (chosen as a marker for LC) and β-actin mRNA (found in both LC and keratinocytes). We defined conditions that resulted in direct (log-linear) relationships between the amounts of total cEC RNA added into the RT-PCR reaction tubes and the intensities of the mRNA-derived signals using primers and probes specific for B7, I-Aα and β-actin (M. G. Lee and M. C. Udey, unpublished observations). Conditions expected to result in signal intensities that fell on the linear portion of each curve were chosen for subsequent experiments.

LC Contain All of the B7 mRNA in Cultured EC Depletion of cells bearing class II major histocompatibility complex and CD45 antigens from cEC with appropriate rat MoAb and sheep anti-rat Ig coupled to magnetic beads resulted in the coincident loss of mRNA signals for I-Aα chain and B7 (Fig 2); depletion of CD8$^+$ cells had no effect on either signal. RNA isolated from class II major histocompatibility complex and CD45 antigen–enriched preparations in the same experiment were enriched in mRNA encoding I-Aα chain and B7 (compare with B7 mRNA-derived signals observed with 1250 ng/ml cEC RNA [Fig 1]). Cultured keratinocytes (depleted of contaminating LC by extensive washing) also lacked B7 and I-Aα chain mRNA (Fig 2). These results demonstrate that cLC

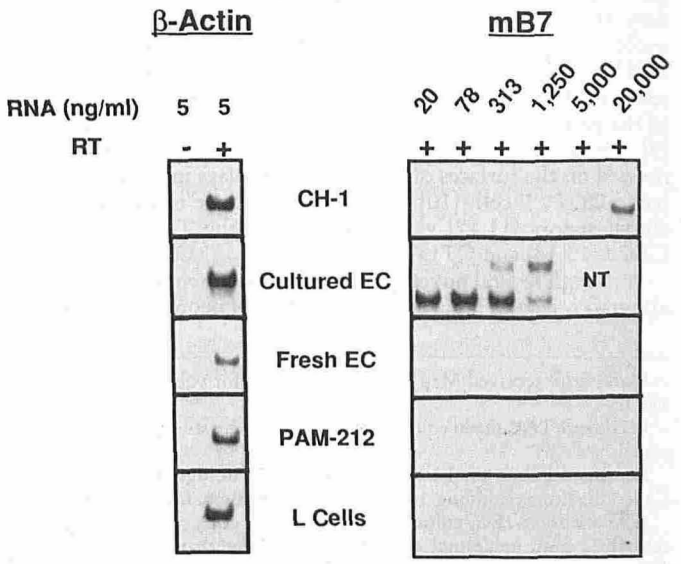

Figure 1. Cultured epidermal cells contain abundant mB7 mRNA. Varying amounts of total RNA from the cells indicated were subjected to PCR (with and without RT) using primers specific for mB7 and β-actin. PCR products were hybridized to radiolabeled internal oligonucleotide probes, resolved in non-denaturing gels and detected by autoradiography. Note that the amount of specific mB7 PCR product decreases when high levels of total RNA from cEC are added into the reaction tubes. Fresh EC were comprised of 1.9% I-A$^+$ cells; cEC contained 34.9% I-A$^+$ cells.

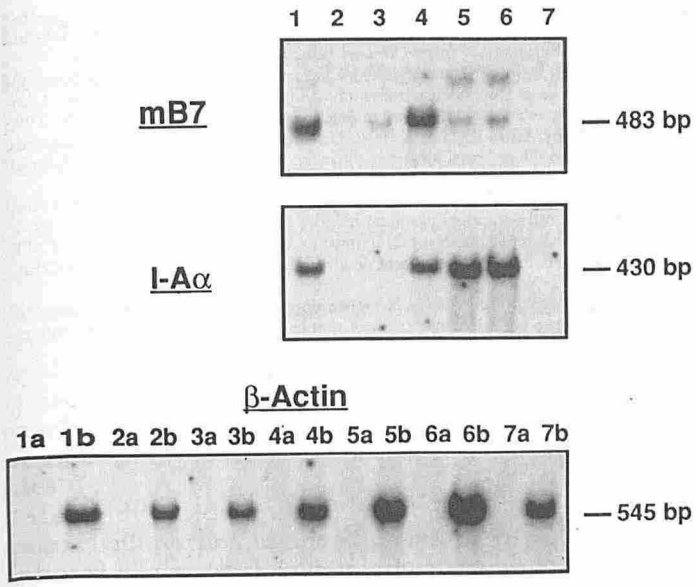

Figure 2. Langerhans cells contain all of the mB7 mRNA present in cultured epidermal cells. RNA was isolated from cEC *(lane 1)*, I-A$^-$ cEC (≤ 0.3% I-A$^+$; *lane 2*), CD45$^-$ cEC (≤ 0.5% I-A$^+$; *lane 3*), CD8$^-$ cEC (10.2% I-A$^+$; *lane 4*), cEC enriched for I-A$^+$ cells *(lane 5)*, cEC enriched for CD45$^+$ cells *(lane 6)*, and cKC (≤ 0.1% I-A$^+$; *lane 7*) and relative amounts of mRNAs encoding mB7, I-Aα, and β-actin in EC subpopulations were determined as described in *Materials and Methods*. Total RNA concentrations in the PCR reaction tubes were 125 ng/ml, 2.5 ng/ml, and 0.16 ng/ml for B7, I-Aα, and β-actin, respectively. β-Actin signals derived from samples subjected to PCR in the absence *(a)* and in the presence of *(b)* of RT are shown.

contain essentially all of the B7 and I-Aα chain mRNA present in cEC.

DISCUSSION

The central role that engagement of CTLA-4 and CD28 on T cells by B7 expressed on accessory cells plays in T-cell activation is well documented. The potent accessory cell activity of cLC and their phenotypic and functional relationship to other dendritic cells has also been extensively studied. Likely determinants of LC and dendritic cell accessory cell activity include levels of class I and class II major histocompatibility complex antigens and costimulatory and/or adhesion molecules such as ICAM-1, LFA-3, and B7. Recent studies suggest that B7 is perhaps the most important of these costimulatory molecules. This view is consistent with the observation that LC are the only epidermis-derived cells that effectively present nominal antigens to normal T cells [24]. Murine keratinocytes, cells that have the capacity to express class II major histocompatibility complex antigens [25] and ICAM-1 [26] but apparently lack the ability to synthesize B7 mRNA or protein, fail to present antigens to T cells but instead induce antigen specific unresponsiveness (clonal anergy) [27]. We hypothesize that exposure of LC to ultraviolet radiation or chemical fixatives may render them unable to synthesize B7 mRNA and protein, and thereby convert LC from potent accessory cells into cells that (in conjunction with antigen) induce tolerance [28]. Our results do not exclude the possibility that murine keratinocytes (like human keratinocytes [29–31]) express functionally important costimulatory molecules whose nucleotide sequences are distinct from that of B7.

The present studies indicate that the surface level of B7 on murine fLC and cLC is directly proportional to the level of B7 mRNA present, a result that presumably reflects increased B7 mRNA synthesis or enhanced B7 mRNA stability in cLC. It will be of interest to identify cytokines or other microenvironmental influences that regulate B7 mRNA levels in LC and to determine if mRNAs encoding other costimulatory molecules expressed by cLC (including ICAM-1 and LFA-3) are coordinately controlled. Attempts to study ICAM-1 mRNA levels in LC using the techniques described in the present study were inconclusive because ICAM-1 mRNA was readily detected in keratinocytes, and it was not possible to completely deplete keratinocytes from LC-enriched preparations (based on residual keratin 1 mRNA content). Recently developed methods that permit propagation of Langerhans cell-like cells from mouse [32] and human peripheral blood [33] and from mouse bone marrow [34] may allow these questions to be addressed more directly in future experiments.

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