The activation of T cells requires two distinct signals. One signal involves interaction of the antigen-specific T-cell receptor (TCR) with class II major histocompatibility complex (MHC) molecules and antigenic peptides; whereas the second signal involves antigen-nonspecific "co-stimulation," generally delivered by antigen-presenting cells (APCs) of hematopoietic origin.

Several molecules have been proposed to provide this co-stimulatory signal, the best characterized of which is the CD28 molecule expressed on T cells. Several related CD28 ligands, B7-1 and B7-2, have been described, and are found on APCs. Our recent demonstration that the majority of T cells in psoriatic lesions express CD28 was not surprising, because 60% to 80% of peripheral blood (PB) T cells express CD28. It is also known that during T-cell activation CD28 expression is increased. When psoriatic lesions were further investigated using several different monoclonal antibodies (MoAbs), it was observed that keratinocytes stained with anti-BB-1 MoAbs (now known to bind to B7-3/BB-1), but not with anti-B7-1 MoAbs.

As a follow-up to these studies, we recently immunostained skin samples using a commercially available MoAb against B7-1. We were surprised to observe that this MoAb stained the vast majority of T cells in both epidermal and dermal compartments in psoriatic lesions, as well as in cutaneous lesions obtained from patients with cutaneous T-cell lymphoma (mycosis fungoides). Immunostaining of the majority of skin-seeking T cells was unexpected because B7-1 has been reported not to be expressed by circulating PB T cells; although it can be upregulated on blood-derived T cells after activation in vitro. When this MoAb was pre-absorbed by incubating with Chinese hamster ovary (CHO) cells transfected with a full-length B7-1 cDNA, all staining was abolished, thus indicating that this result was not caused by a second reagent contaminating the MoAb preparation, but that the anti-B7-1 MoAb was also binding to the T cells. These data indicate that T-cell expression of B7-1 can occur during T-cell activation in vivo, at sites of cutaneous inflammation or neoplasia.

MATERIALS AND METHODS

Clinical samples. Punch biopsies (3 mm) were obtained from five untreated psoriatic plaques and four untreated plaques of mycosis fungoides. Each biopsy sample was derived from a different patient after obtaining informed consent. PB samples were obtained by venipuncture and collected in EDTA-containing syringes from normal healthy adult volunteers. Mononuclear cells were isolated from the Ficoll-Hypaque interface after centrifugation. Highly purified resting T cells were further enriched from these interface cells by vigorous negative selection using a cocktail of MoAbs against B cells, monocytes, natural killer cells, and activated (ie, HLA-DR+) T cells, together with magnetic beads as previously described. These T cells were greater than 98% CD8+, lacked HLA-DR, and did not proliferate in response to phytohemagglutinin A (PHA, 10 μg/mL). Dermal mononuclear cells were also obtained from psoriatic lesions by keratome sampling of untreated lesions and isolating lymphocytes and dendritic cells as previously described. Briefly, the epidermis is separated from dermis using disperse, and the dermal fragments minced and placed in culture medium (RPMI 1640 + 10% fetal calf serum [FCS]; Gibco, Grand Island, NY) for 2 days. After this incubation period, mononuclear cells are isolated from the loosely attached dermal fragments and surrounding suspension by centrifugation. After resuspending the pellet in RPMI + 10% FCS, these cells include primarily T lymphocytes and dermal dendritic cells which can be distinguished by their forward- and side-scatter properties. These cells were then stained using a commercially available MoAb against BB-1. We were surprised to observe that this MoAb stained the vast majority of T cells in both epidermal and dermal compartments in psoriatic lesions, as well as in cutaneous lesions obtained from patients with cutaneous T-cell lymphoma (mycosis fungoides). Immunostaining of the majority of skin-seeking T cells was unexpected because B7-1 has been reported not to be expressed by circulating PB T cells; although it can be upregulated on blood-derived T cells after activation in vitro. When this MoAb was pre-absorbed by incubating with Chinese hamster ovary (CHO) cells transfected with a full-length B7-1 cDNA, all staining was abolished, thus indicating that this result was not caused by a second reagent contaminating the MoAb preparation, but that the anti-B7-1 MoAb was also binding to the T cells. These data indicate that T-cell expression of B7-1 can occur during T-cell activation in vivo, at sites of cutaneous inflammation or neoplasia.
T CELLS COEXPRESS CD28 AND B7-1 IN SKIN

properties using a flow cytometer (see below). All cells were maintained in a humidified incubator with 5% CO2 at 37°C.

**MoAbs.** The primary antibodies used included MoAbs against B7-1 (clone L307.4 Becton Dickinson Advanced Cellular Biology, San Jose, CA), B7-3/BB-1 (anti-BB-1, obtained as a gift from Peter Linsley, Bristol Myers Squibb Pharmaceutical Corp, Seattle, WA), and CD28 (Becton Dickinson). Anti-IFLA-DR MoAb (L243) was obtained through the America Type Culture Center (Rockville, MD). Isotype control antibodies included preparations of either IgG or IgM antibodies (Coulter Corp, Hialeah, FL). All antibodies were used at a final concentration of 10 μg/mL.

**Immunoadsorption studies.** To verify that the MoAb against B7-1, L307.4, recognized the B7-1 antigen, CHO cells transfected with a full-length B7-1 cDNA (gift of P. Linsley) were stained. The B7-1 transfectants, but not vector-only transfected cells were strongly positive using the MoAb L307.4. Moreover, serial absorption of the anti-B7 MoAb by the CHO-B7-1 transfected cells showed proportional decreases after repetitive cycles of incubation, until there was essentially no significant immunoreactivity of the anti-B7 MoAb using fresh CHO-B7-1 transfected cells (see Fig 1). The immunoadsorption was performed by sequentially mixing 1 mL of the anti-B7-1 (10 μg/mL) with 107 CHO-B7-1 transfected cells, or vector-only transfected CHO cells, for 1 hour. After this incubation, the cells were pelleted and the MoAb in the supernatant reexposed to a fresh batch of transfected cells, and the process repeated a total of four times.

**Flow cytometry analysis.** Between 5 × 10^5 to 5 × 10^6 T cells were stained with a primary MoAb, and these primary MoAbs detected using fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse IgG (Tago Inc, Burlingame, CA). For the transfected CHO-B7-1 cells, they were used at 10^6 cells/mL and stained in a two-step procedure in the same manner as the T lymphocytes. For double-labeling experiments, directly conjugated (phycoerythrin [PE]-labeled) primary MoAb against CD3 was used together with the appropriate blocking steps in connection with the indirect immunofluorescence staining as above for B7-1. Flow cytometric analysis was performed using a FACScan equipped with Lysis 2 software (Becton Dickinson, Mountainview, CA).

**Immunostaining procedure.** Five-micron thick cryostat sections of skin were immunostained using a sensitive avidin-biotin immunoperoxidase technique (Vectastain Kit, Vector Labs, Burlingame, CA), with 3-amino-4-ethyl carbazole as the chromogen producing a positive-red-reactive product as previously described. For double-labeling experiments a directly conjugated (PE-labeled) primary MoAb against CD28 was used together with appropriate blocking steps in conjunction with anti-B7-1 MoAb and FITC-conjugated goat-antimouse IgG as described above. An Olympus BH-2 fluorescence microscope equipped with both red and green filters was used to visualize the positively stained cell types.

**T-cell activation.** Polyclonal proliferation of resting PB T cells was induced using immobilized anti-CD3 MoAb (G19-4, gift of J. Ledbetter, Bristol Myers Squibb Pharmaceutical Corp) and IL-2 (100 Units, Boehringer-Manheim, Indianapolis, IN) for 12 days in RPMI 1640 plus 10% FCS as previously described.

**RESULTS**

**Immunostaining of cryostat sections of skin lesions for B7-1, CD28, and B7-3/BB-1.** Because of the unexpected immunoreactivity of the lymphocytes with the B7 MoAb L307.4 as described below, we carefully tested the antigenic recognition capability of this MoAb. The object of this testing was to verify that the commercially obtained MoAb, L307.4, bound to the B7-1 molecule, and that the preparation was not contaminated with a second antibody that was reacting with the T cells. The anti-B7 MoAb strongly reacted with CHO-B7-1 transfected cells, but not control-transfected, B7-1-negative CHO cells (Fig 1). Additionally, upon serial testing of the MoAb after sequential exposure to CHO-B7-1^+ cells, flow cytometry showed that by four absorption cycles, the staining intensity of fresh CHO-B7-1^+ positive cells was reduced to below isotype control, background levels (Fig 1). Similar staining was observed for both lots of MoAb, L307.4, and the staining intensity diminished as the MoAb concentration was reduced from 10 μg/mL to 1 μg/mL; and was nonexistent at concentrations below 1 μg/mL. Thus, we concluded that the MoAb was indeed recognizing an epitope exposed on the B7-1 molecule.

Immunostaining cryostat skin sections using this anti-B7-1 MoAb showed that virtually all of the round lymphoid cells in both the epidermis and dermis of psoriatic and mycosis fungoides lesions were positive (Fig 2). This staining pattern was observed for all biopsy specimens, and a representative example is shown in Figure 2A, which is a psoriatic plaque stained with anti-B7-1. Note the strong and diffuse staining of the round lymphoid cells in both epidermal and dermal compartments (Fig 2B). The determination that these indicated cells are actually lymphocytes is based on their staining in serial sections for CD3 and either CD4 or CD8 (data not shown). The prominent accumulation of CD3^+ T cells, but not B lymphocytes, in psoriatic plaques has been previously documented by several groups.

Staining of serial sections also showed that greater than 95% of the CD3^+ cells in the psoriatic plaque were also CD28^+ as previously described.
Fig 3. (A) Mycosis fungoides plaque with diffuse and intense positive B7-1 immunoreactivity on enlarged round lymphocytes in both dermis and epidermis. Note the large positively stained Pautrier microabcess. (B) Mycosis fungoides plaque with diffuse and intense positive CD28 immunoreactivity on lymphocytes in dermis and epidermis including the Pautrier microabcess. (C) Mycosis fungoides plaque stained for BB-1 shows strong and diffuse staining of epidermal keratinocytes, but no positive staining of lymphocytes.

Fig 2. (page 2582) (A) Psoriatic plaque with diffuse and intense positive B7-1 immunoreactivity on round lymphoid cells. (B) Within a psoriatic plaque, B7-1 positive lymphoid cells are present in both epidermal and dermal compartments. (C) Psoriatic plaque stained with anti–B7-1 plus FITC-conjugated goat-antimouse antibody, followed by phycocerythrin-conjugated anti-CD28 antibody. Upper panel viewed through a fluorescein filter shows numerous positive round lymphoid cells (curved arrow), and a dendritic epidermal Langerhans cell (straight arrow). The latter was identified by characteristic dendritic morphology best observed when focusing up and down on the field. The lower panel is the same field examined using the rhodamine filter and shows that all the B7-1+ lymphoid cells are also CD28+ (curved arrow). (D) Psoriatic plaque stained for BB-1 shows strong and diffuse staining of keratinocytes in both the basal and suprabasal portion of the epidermis. No staining of the lymphoid cells in either epidermis or dermis were observed.
Pautrier microabscess. Staining of serial sections showed was B7-1+, as previous described. Staining the psoriatic lesions for B7-3BB-1 with anti-BB-1 MoAb showed positive staining of basal and suprabasilar keratinocytes, but no staining of lymphoid cells (data not shown). The ability of B7-1-absorption to abrogate T-cell immunoreactivity shows that the same antibody is binding both to recombinant B7-1 and to the T cell.

The keratinocytes, endothelial cells, and dermal dendritic cells were B7-1+. An occasional epidermal Langerhans cell was B7-1+, as previous described. Staining the psoriatic lesions for B7-3/BB-1 with anti-BB-1 MoAb showed positive staining of basal and suprabasilar keratinocytes, but no staining of lymphoid cells (Fig 2D).

Figure 3A shows the B7-1 staining pattern of a representative mycosis fungoides lesion. Note the strong and diffuse staining of the lymphoid cells (identified as T cells by staining of serial sections with CD3, CD4, and CD8) in both the dermis and epidermis including a large Pautrier microabscess. Staining of serial sections showed that the infiltrating lymphocytes diffusely expressed CD28 in both dermal and epidermal compartments, including the large Pautrier microabscess present in the epidermis (Fig 3B). When the CHO-B7 absorbed MoAb preparation was used, all immunoreactivity on the lymphocytes was abolished (data not shown). Staining mycosis fungoides lesions for B7-3/BB-1 showed strong positivity in the epidermis (particularly basal layer keratinocytes), but no staining of lymphocytes was observed (Fig 3C).

Flow cytometric analysis of psoriatic lesional T cells. To verify that the immunoreactivity for B7-1 on the T cells in these skin biopsies was not caused by a fixation artifact, lesional T cells were directly obtained from fresh unfixed keratome samples, and subjected to two-color immunofluorescence analysis by flow cytometry. The T cells could be separated from dermal dendritic cells by their forward and side-scatter properties (Fig 4A). The exact nature of the round lymphoid cells was confirmed by their CD3 expression (Fig 4C). These CD3+ T cells were also positive for B7-1 using the MoAb L307.4 (Fig 4B). Using the CHO-B7-1 absorbed antibody, this B7-1 immunoreactivity was abolished (Fig 4D). For the representative experiment shown in Fig 4, the mean fluorescent intensity on lesional T cells for the control MoAb was 2.4, and for B7-1 it was 26.7, yielding a B7-1:control ratio of 11:1. Thus, specific B7-1 staining on lesional T cells was greater than 1 log above background.

Flow cytometric analysis of PB T cells. Resting PB T cells were HLA-DR+ (data not shown), but expressed a low level of B7-1 that was consistently seen as only a slight shift of the fluorescence intensity over isotype control staining (Fig 5A). For example in Fig 5A, the mean fluorescent intensity on lesional T cells for the control MoAb was 19.6, and for B7-1 it was 61.6, yielding a B7-1:control ratio of only 3:1. Thus the amount of specific B7-1 staining on PB T cells was significantly less than the values of 10 times above background seen for T cells isolated from psoriatic lesions (Fig 4). After CHO-B7-1 absorption, this B7-1 immunoreactivity was diminished to near background levels. There was no increase in B7-1 expression observed when T cells were stimulated for 12 days on tissue-culture plates coated with immobilized anti-CD3 MoAb (Fig 5B) — mean fluorescent intensities: control MoAb = 18.0, B7-1 = 44.2, ratio = 2.5:1, despite induction of HLA-DR expression on these cells and visible activation with blastogenesis and cluster formation as seen by phase-contrast microscopy (data not shown).
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Fluorescence Intensity

0 10^1 10^2 10^3

Cell Number

Fluorescence Intensity

0 10^1 10^2 10^3

Cell Number

Fig 5. Staining of PB T cells with anti-87-1 MoAb. (A) Highly purified resting T cells were isolated from PB and stained with anti-87-1 MoAb (--), with isotype-matched control MoAb (-----), or with anti-87-1 MoAb that had been serially absorbed (4 cycles) on B7-1 CHO cells before staining as outlined in Materials and Methods (---). (B) Highly purified resting T cells were stimulated with immobilized anti-CD3 MoAb (G19-4) for 12 days, removed, and stained with anti-B7-1 MoAb or with isotype-matched control MoAb.

DISCUSSION

This report documents that virtually all lymphocytes in the dermis and epidermis of a benign (ie, psoriasis) and malignant (ie, mycosis fungoides) T-cell-mediated skin disease express B7-1, a CD28 ligand. These T cells also coexpress CD28 raising the interesting possibility of autocrine costimulation via B7-1:CD28 interaction. The antibody we used to identify B7-1, L307.4, was raised against B7-1- transfected cells (L. Lanier, personal communication, November 1993), and is specific for B7-1, although the exact epitope on the B7-1 molecule that it recognizes has not been precisely identified. Nonetheless, we believe the MoAb is indeed recognizing a specific site on B7-1 based on the considerations above and on our own results absorbing the MoAb against CHO-B7-1 cells.

Compared with PB T cells that only weakly express B7-1, the psoriatic and mycosis fungoides dermal T cells were more strongly B7-1+. Based on our current results, it appears that once the PB T cells enter the skin, there is accumulation of a subset that is expressing B7-1. At least two possible explanations for these results can be suggested: (1) local activation of a B7-1+ T-cell population of a population that, initially, only weakly expresses B7-1, or (2) selective recruitment from the blood of an extremely low frequency (<1% of total T cells), and currently undetectable, subset of strongly B7-1+ T cells. With respect to the first possibility, because our in vitro stimulation experiments did not upregulate B7-1 on PB T cells, there may be other activation pathways for induction of B7-1 operative in vivo in these two skin diseases that are not being simulated by our in vitro protocol. Thus, based on the available data, we cannot definitely confirm either of the two aforementioned possibilities.

It should be mentioned that using this MoAb against B7-1, our results dealing with PB T cells have some similarities as well as differences with previous reports using different MoAbs against B7-1. In contrast to these previous reports, we observed a low level of B7-1 expression on resting PB T cells. However, after 12 days of stimulation no further increase was observed, which is in agreement with Sansom and Hall, but in disagreement with Azuma et al. Sansom and Hall required more than 30 days of stimulation before detection of B7-1 on PB T cells. We followed the stimulation protocol exactly as described by Azuma et al., and our only explanation for these differences is that the MoAbs used to detect B7-1 were different amongst each study. The finding of B7-1 on T cells in these two persistent dermatoses in vivo may reflect the fact that such lesional T cells have undergone chronic stimulation over several months.

Another important finding relates to the discordant expression of B7-1 versus B7-3/BB-1 on the T cells and keratinocytes. In the past, B7-1 and B7-3/BB-1 were thought to be identical antigens (termed B7/BB-1). However, we first described a discordant pattern of expression on activated keratinocytes in vitro and in vivo, and the finding that these are distinct epitopes has since been confirmed by others. These current findings further confirm and extend the conclusion that B7 and BB-1 are not identical antigens because the T cells express B7-1, but not B7-3/BB-1; whereas the keratinocytes express B7-3/BB-1, but not B7-1 immunoreactivity. It should be noted that the anti-B7-3/BB-1 MoAb has cross-reactivity with B7-1 under certain circumstances, such as expression of the B7-1 gene in CHO cells. However, in our tissue sections (figures 2D and 3C) and in flow cytometry (data not shown), anti-B7-3/BB-1 MoAb stains keratinocytes but not T cells. It may not be possible to determine the cause of this phenomenon until the B7-3/BB-1 gene is further identified and/or cloned. However, it does suggest that reactivity with the anti-B7-3/BB-1 MoAb might recognize a posttranslational modification of a protein product,
one which might not be made in T cells. Immunoprecipitation-based studies as well as efforts to clone the B7-3/BB-1 gene are underway to definitively establish the identity of B7-3/BB-1 and to permit direct comparison with the B7-1 gene product.

In conclusion, these results indicate that B7-1 (but not B7-3/BB-1) is expressed by lymphocytes in two common and chronic skin disorders; one benign (psoriasis) and one malignant (mycosis fungoides). These lymphocytes also express CD28, and such cell-surface expression would theoretically permit a T cell to be able to undergo self-costimulation via interaction with B7-1. Such participation of costimulatory molecules may contribute to the ongoing T-cell proliferation that occurs in the skin of patients afflicted by these disorders.

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T lymphocytes in skin lesions of psoriasis and mycosis fungoides express B7-1: a ligand for CD28

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